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 To avoid any influence of food consumption on lipid metabolism, food was withdrawn after the injection.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 m*M* NaCl, 0.1% Triton X-100, and 10 m*M* 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_2O 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

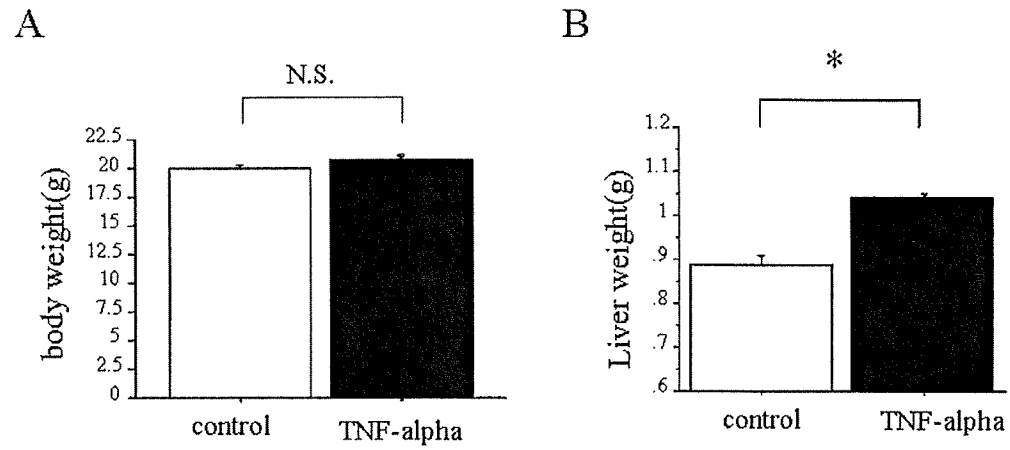

Figure 1. Body weight (A) and liver weight (B) 24 hrs after intraperitoneal injection of TNF-α at a dose of 0.166 mg/kg (TNF-α) or injection with the same volume of saline (control). Values and vertical bars: mean ± SEM ($n = 4$–8 for each). * $P < 0.05$ versus the corresponding controls.

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

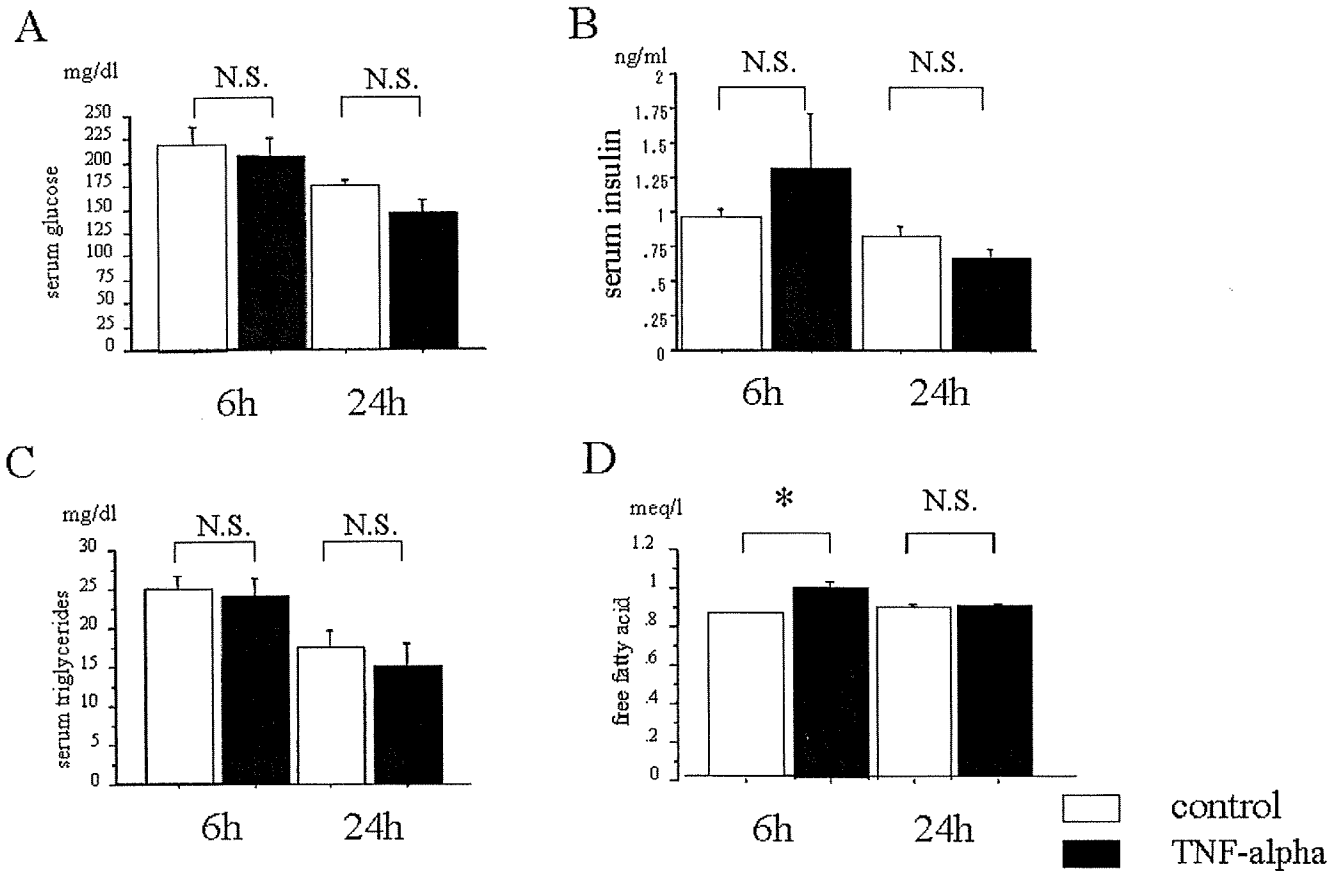

Figure 2. Serum glucose (A), serum insulin (B), serum triglycerides (C), and free fatty acid (D) levels at 6 hrs and 24 hrs after ip injection of TNF-α at a dose of 0.166 mg/kg (TNF-α) or injection with the same volume of saline (control). Values and vertical bars: mean ± SEM ($n = 4$ for each). * $P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

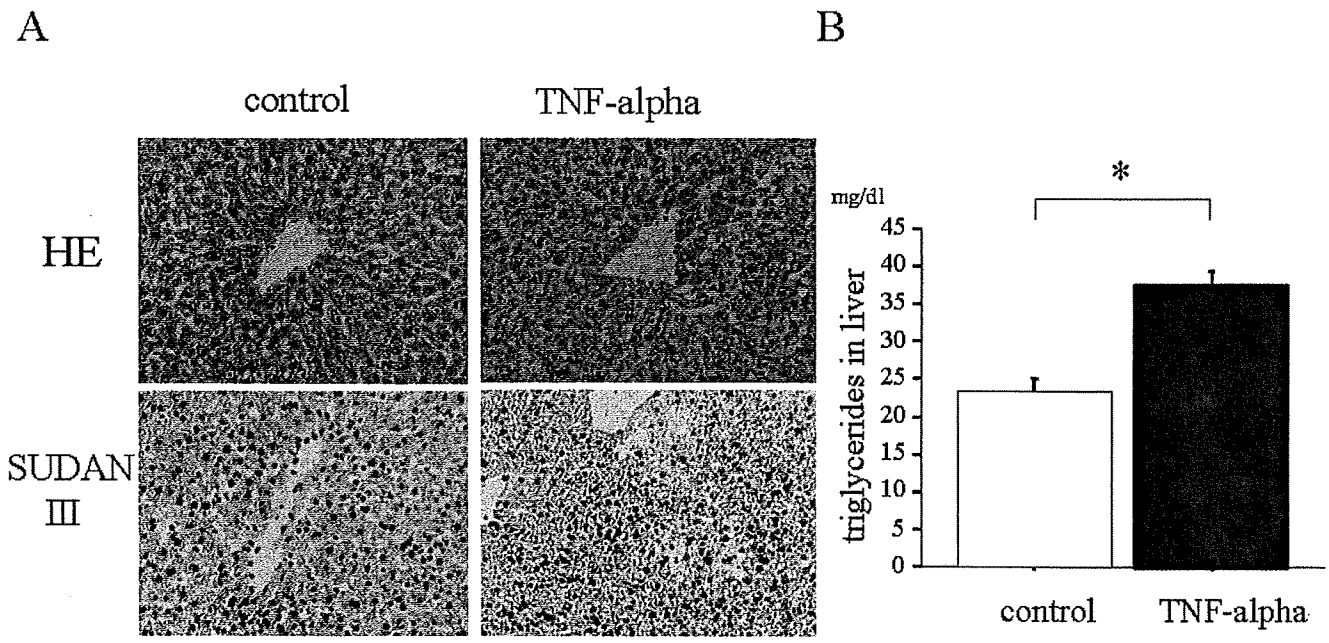

Figure 3. Histological changes (A) assessed by HE and SUDAN III (×200 magnification), and liver triglyceride levels (B) in the liver 24 hrs after ip injection of TNF-α at a dose of 0.166 mg/kg (TNF-α) or injection with the same volume of saline (control). Values and vertical bars: mean ± SEM ($n = 4$ 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) proteins 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

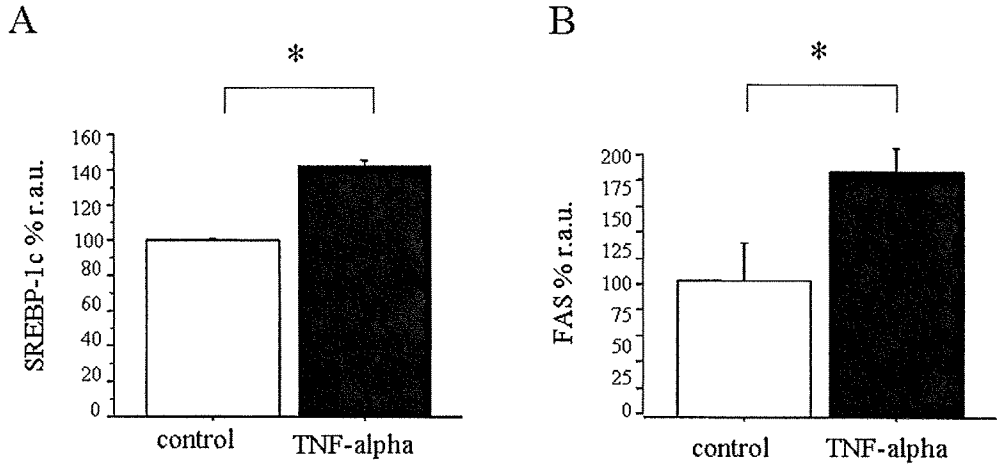

Figure 4. Expression of SREBP-1c mRNA (A) and FAS mRNA (B) in the liver 24 hrs after ip injection of TNF-α at a dose of 0.166 mg/kg (TNF-α) or injection with the same volume of saline (control). Real-time PCR was performed with total RNA from the liver samples. Values and vertical bars: mean ± SEM ($n = 4$ for each). * $P < 0.05$ versus the corresponding controls.

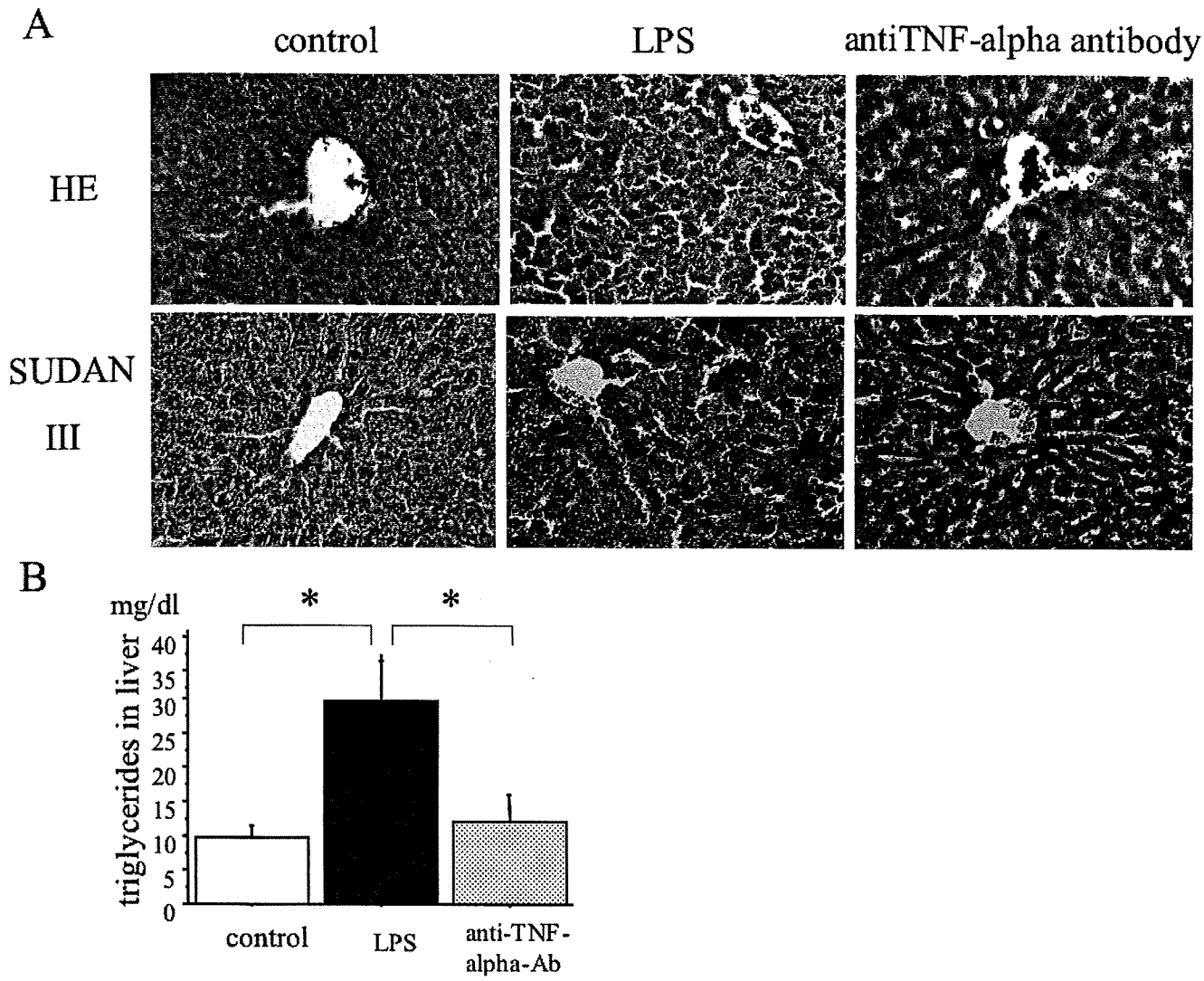

Figure 5. Effect of anti-TNF-α antibody on LPS-induced development of fatty liver 24 hrs after treatment. (A) Histological changes in the liver assessed by HE and SUDAN III staining (×200 magnification). (B) Liver triglyceride levels. Mice were injected with ip anti-TNF-α antibody at a dose of 3.3 mg/kg (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.

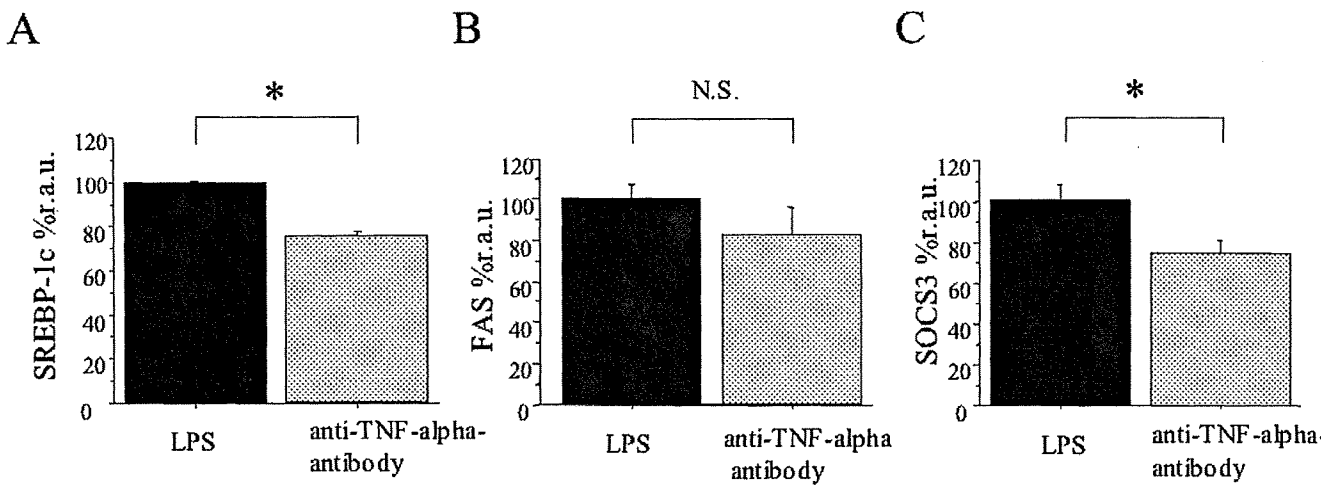

Figure 6. Expression of SREBP-1c mRNA (A), FAS mRNA (B), and SOCS3 (C) in the liver 24 hrs after treatment with anti-TNF-α antibody at a dose of 3.3 mg/kg (anti-TNF-α antibody) or saline (LPS) 30 mins before the administration of LPS at a dose of 3.3 mg/kg. Real-time PCR was performed with total RNA 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.

1. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259:87–91, 1993.
2. Hotamisligil GS, Budavari A, Murray D, Spiegelman BM. Reduced tyrosine kinase activity of the insulin receptor in obesity-diabetes. Central role of tumor necrosis factor-alpha. J Clin Invest 94:1543–1549, 1994.
3. Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM. Tumor necrosis factor alpha inhibits signaling from the insulin receptor. Proc Natl Acad Sci U S A 91:4854–4858, 1994.
4. Stephens JM, Pekala PH. Transcriptional repression of the GLUT4 and C/EBP genes in 3T3-L1 adipocytes by tumor necrosis factor-alpha. J Biol Chem 266:21839–21845, 1991.
5. den Boer M, Voshol PJ, Kuipers F, Havekes LM, Romijn JA. Hepatic steatosis: a mediator of the metabolic syndrome. Lessons from animal models. Arterioscler Thromb Vasc Biol 24:644–649, 2004.
6. Diehl AM. Fatty liver, hypertension, and the metabolic syndrome. Gut 53:923–924, 2004.
7. Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, Natale S, Vanni E, Villanova N, Melchionda N, Rizzetto M. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology 37:917–923, 2003.
8. Feingold KR, Grunfeld C. Tumor necrosis factor-alpha stimulates hepatic lipogenesis in the rat *in vivo*. J Clin Invest 80:184–190, 1987.
9. Feingold KR, Soued M, Serio MK, Adi S, Moser AH, Grunfeld C. The effect of diet on tumor necrosis factor stimulation of hepatic lipogenesis. Metabolism 39:623–632, 1990.
10. Feingold KR, Adi S, Staprans I, Moser AH, Neese R, Verdier JA, Doerrler W, Grunfeld C. Diet affects the mechanisms by which TNF stimulates hepatic triglyceride production. Am J Physiol 259:E177–E184, 1990.
11. Memon RA, Grunfeld C, Moser AH, Feingold KR. Tumor necrosis factor mediates the effects of endotoxin on cholesterol and triglyceride metabolism in mice. Endocrinology 132:2246–2253, 1993.
12. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 109:1125–1131, 2002.
13. Shimomura I, Bashmakov Y, Horton JD. Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. J Biol Chem 274:30028–30032, 1999.
14. Semenkovich CF. Regulation of fatty acid synthase (FAS). Prog Lipid Res 36:43–53, 1997.
15. Bennett MK, Lopez JM, Sanchez HB, Osborne TF. Sterol regulation of fatty acid synthase promoter. Coordinate feedback regulation of two major lipid pathways. J Biol Chem 270:25578–25583, 1995.
16. Shimomura I, Bashmakov Y, Ikemoto S, Horton JD, Brown MS, Goldstein JL. Insulin selectively increases SREBP-1c mRNA in the livers of rats with streptozotocin-induced diabetes. Proc Natl Acad Sci U S A 96:13656–13661, 1999.
17. Boizard M, Le Liepvre X, Lemarchand P, Foufelle F, Ferre P, Dugail I. Obesity-related overexpression of fatty-acid synthase gene in adipose tissue involves sterol regulatory element-binding protein transcription factors. J Biol Chem 273:29164–29171, 1998.
18. Shimomura I, Bashmakov Y, Horton JD. Increased levels of nuclear SREBP-1c associated with fatty livers in two mouse models of diabetes mellitus. J Biol Chem 274:30028–30032, 1999.
19. Ueki K, Kondo T, Tseng YH, Kahn CR. Central role of suppressors of cytokine signaling proteins in hepatic steatosis, insulin resistance, and the metabolic syndrome in the mouse. Proc Natl Acad Sci U S A 101: 10422–10427, 2004.
20. Shi H, Cave B, Inouye K, Bjorbaek C, Flier JS. Overexpression of suppressor of cytokine signaling 3 in adipose tissue causes local but not systemic insulin resistance. Diabetes 55:699–707, 2006.
21. Ginsberg HN. Diabetic dyslipidemia: basic mechanisms underlying the common hypertriglyceridemia and low HDL cholesterol levels. Diabetes 45:S27–S30, 1996.
22. Fungwe TV, Cagen L, Wilcox HG, Heimberg M. Regulation of hepatic secretion of very low density lipoprotein by dietary cholesterol. J Lipid Res 33:179–191, 1992.
23. Wang CS, Fukuda N, Ontko JA. Studies on the mechanism of hypertriglyceridemia in the genetically obese Zucker rat. J Lipid Res 25:571–579, 1984.

24. Feingold KR, Serio MK, Adi S, Moser AH, Grunfeld C. Tumor necrosis factor stimulates hepatic lipid synthesis and secretion. Endocrinology 124:2336–2342, 1989.
25. Feingold KR, Doerrler W, Dinarello CA, Fiers W, Grunfeld C. Stimulation of lipolysis in cultured fat cells by tumor necrosis factor, interleukin-1, and the interferons is blocked by inhibition of prostaglandin synthesis. Endocrinology 130:10–16, 1992.
26. Green A, Dobias SB, Walters DJ, Brasier AR: Tumor necrosis factor increases the rate of lipolysis in primary cultures of adipocytes without altering levels of hormone-sensitive lipase. Endocrinology 134:2581–2588, 1994.
27. Ruan H, Miles PD, Ladd CM, Ross K, Golub TR, Olefsky JM, Lodish HF. Profiling gene transcription in vivo reveals adipose tissue as an immediate target of tumor necrosis factor-alpha: implications for insulin resistance. Diabetes 51:3176–3188, 2002.
28. Crespo J, Cayon A, Fernandez-Gil P, Hernandez-Guerra M, Mayorga M, Dominguez-Diez A, Fernandez-Escalante JC, Pons-Romero F. Gene expression of tumor necrosis factor alpha and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients. Hepatology 34:1158–1163, 2001.
29. Kugelmas M, Hill DB, Vivian B, Marsano L, McClain CJ. Cytokines and NASH: a pilot study of the effects of lifestyle modification and vitamin E. Hepatology 38:413–419, 2003.
30. Brun P, Castagliuolo I, Di Leo V, Buda A, Pinzani M, Palú G, Martines D. Increased intestinal permeability in obese mice: new evidences in the pathogenesis of nonalcoholic steatohepatitis. Am J Physiol Gastrointest Liver Physiol 292:G518–525, 2007.
31. Grunfeld C, Verdier JA, Neese R, Moser AH, Feingold KR. Mechanisms by which tumor necrosis factor stimulates hepatic fatty acid synthesis in vivo. J Lipid Res 29:1327–1335, 1988.
32. Ueki K, Kondo T, Kahn CR. Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. Mol Cell Biol 24:5434–5446, 2004.
33. Ueki K, Kadowaki T, Kahn CR. Role of suppressors of cytokine signaling SOCS-1 and SOCS-3 in hepatic steatosis and the metabolic syndrome. Hepatol Res 33:185–192, 2005.
34. Hessle CC, Andersson B, Wold AE. Gram-positive and Gram-negative bacteria elicit different patterns of pro-inflammatory cytokines in human monocytes. Cytokine 30:311–318, 2005.
35. Adi S, Pollock AS, Shigenaga JK, Moser AH, Feingold KR, Grunfeld C. Role for monokines in the metabolic effects of endotoxin. Interferon-gamma restores responsiveness of C3H/HeJ mice in vivo. J Clin Invest 89:1603–1609, 1992.

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.

Key words : percutaneous radiofrequency ablation, artificial ascites, saline solution

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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，再々々発の腫瘤を治療する機会も多くなり，腫瘤は肝の様々な部位に存在するため，腫瘤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瘤，隣接臓器に近接している腫瘤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瘤に対して，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瘤に加え，腸管近接部の腫瘤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．2 cm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）

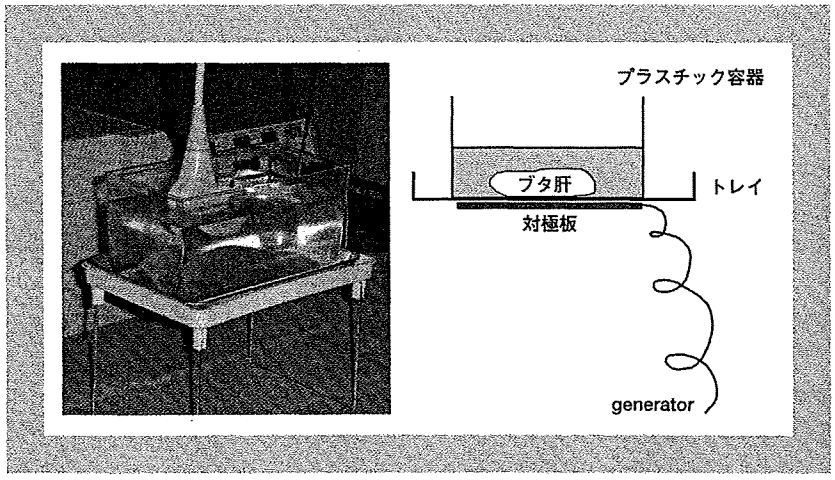

図1　実験装置
生理食塩水，5％ブドウ糖液1,500mLを容器にそれぞれ注入し，人工腹水環境を作製した．

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，横隔膜直下の腫瘤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

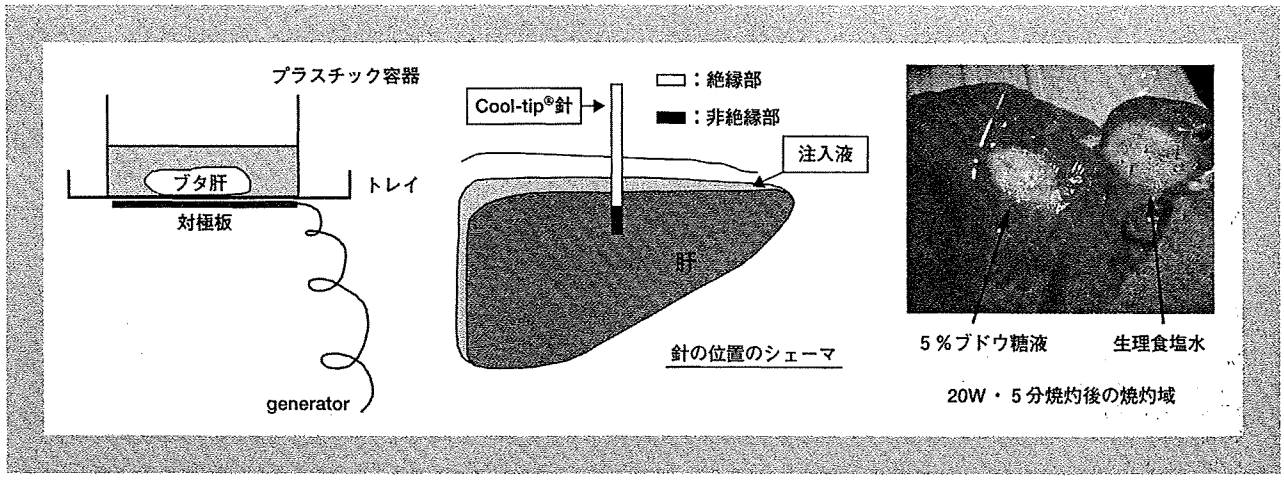

図2　非絶縁部が肝内にある場合

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．

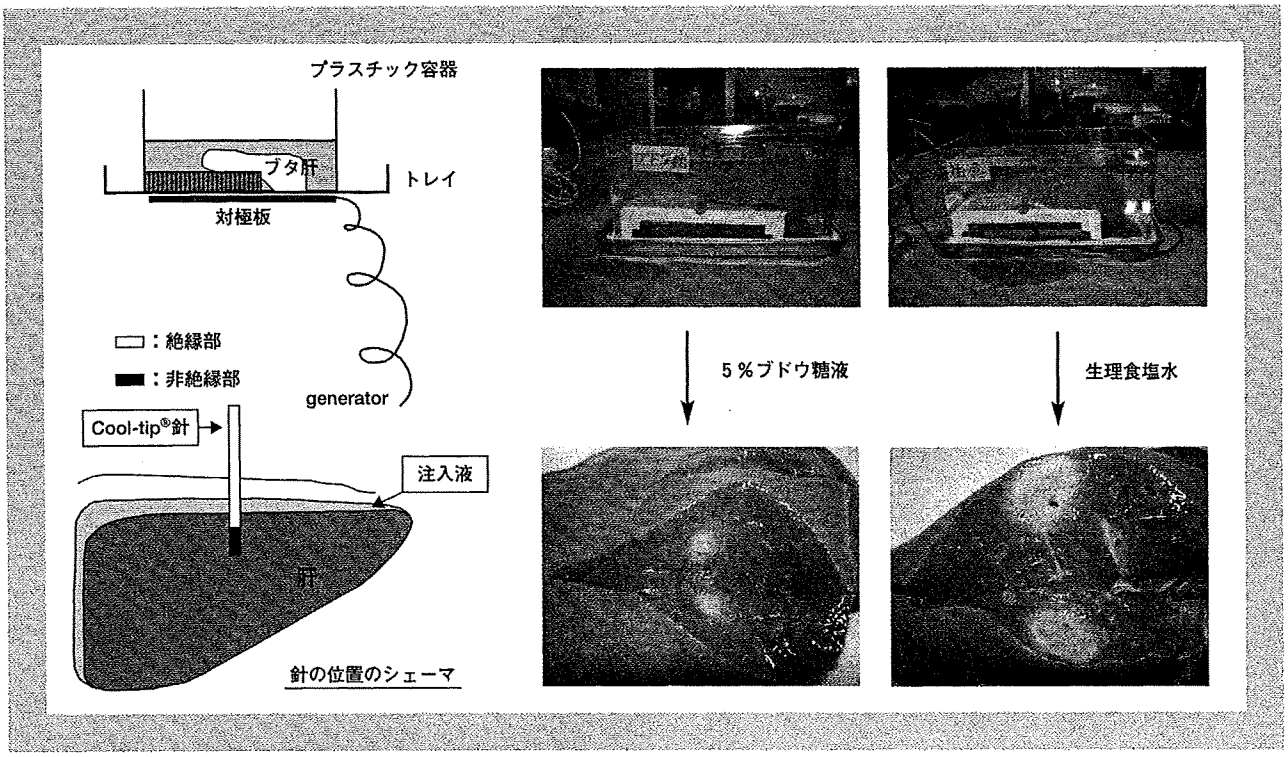

図3　対極板と肝の接触面積を減少させた場合

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S_6，S_2 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．

References

1）Minami Y, Kudo M, Kawasaki T, Chung H, Ogawa C, Inoue T, Sakaguchi Y, Sakamoto H, Shiozaki H : Percutaneous ultrasound-guided radiofrequency ablation with artificial pleural effusion for hepatocellular carcinoma in the hepatic dome. J Gastroenterol **38** : 1066-1070, 2003
2）Minami Y, Kudo M, Kawasaki T, Chung H, Ogawa C, Shiozaki H : Percutaneous radiofrequency ablation guided by contrast-enhanced harmonic sonography with artificial Pleural effusion for hepatocellular carcinoma in the hepatic dome. AJR Am J Roentgenol **182** : 1224-1226, 2004
3）Katayama K, Ooka Y, Uemura A, Shinzaki S, Egawa S, Naito M, Ishibashi K, Kamoi R : Saline injection into the pleural cavity to detect tumors of the hepatic dome with sonography : A new approach for treatment of hepatocellular carcinoma. AJR Am J Roentgenol **179** : 102-104, 2002
4）Kondo Y, Yoshida H, Shiina S, Tateishi R, Teratani T, Omata M : Artificial ascites technique for percutaneous radiofrequency ablation of liver cancer adjacent to the gastrointestinal tract. Br J Surg **93** : 1277-1282, 2006

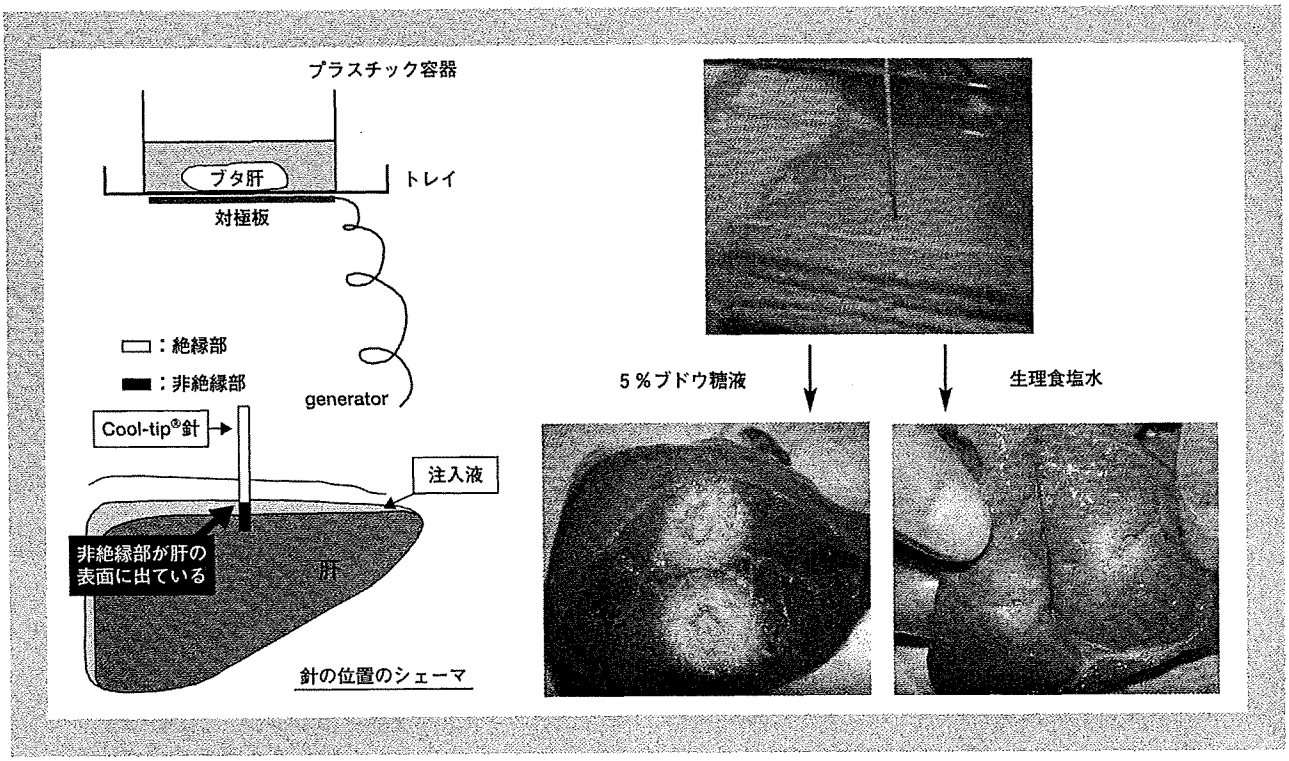

図4　非絶縁部が肝外にある場合

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．

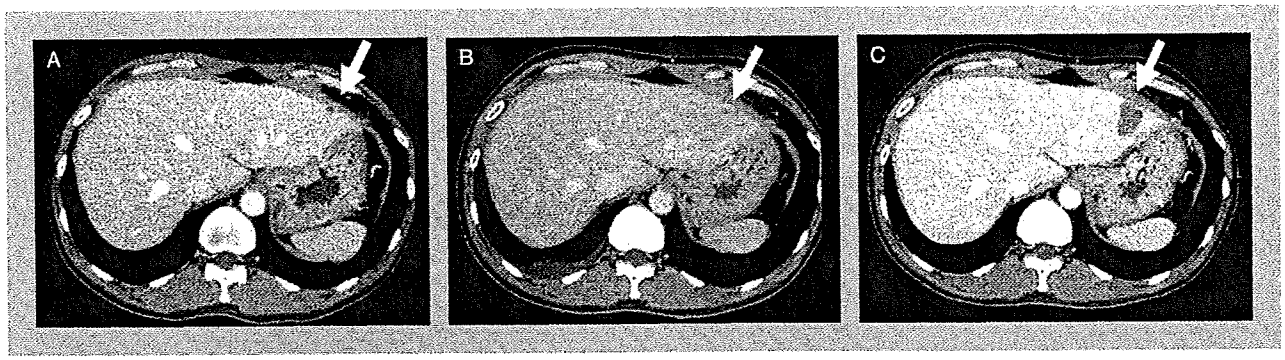

図5　転移性肝癌（45歳，男性）

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．

5）清家正隆：胸水・腹水注入下治療の実際．池田健次 編，肝癌ラジオ波凝固療法―そのノウハウとエビデンス．東京，診断と治療社，154-158，2007
6）Kim YS, Rhim H, Paik SS : Radiofrequency ablation of the liver in a rabbit model : creation of artificial ascites to minimize collateral thermal injury to the diaphragm and stomach. J Vasc Interv Radiol **17** : 541-547, 2006
7）Raman SS, Lu DS, Vodopich DJ, Sayre J, Lassman C : Minimizing diaphragmatic injury during radio-frequency ablation : efficacy of subphrenic peritoneal saline injection in a porcine model. Radiology **222** : 819-823, 2002

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**）．

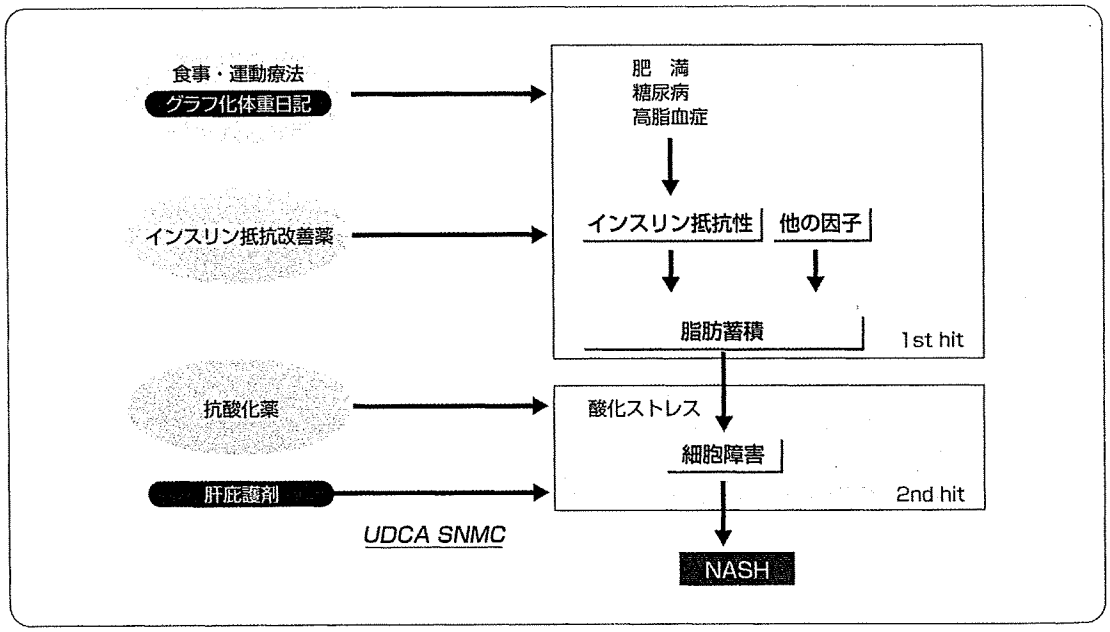

図1　一般的なNAFLDの病態と治療

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

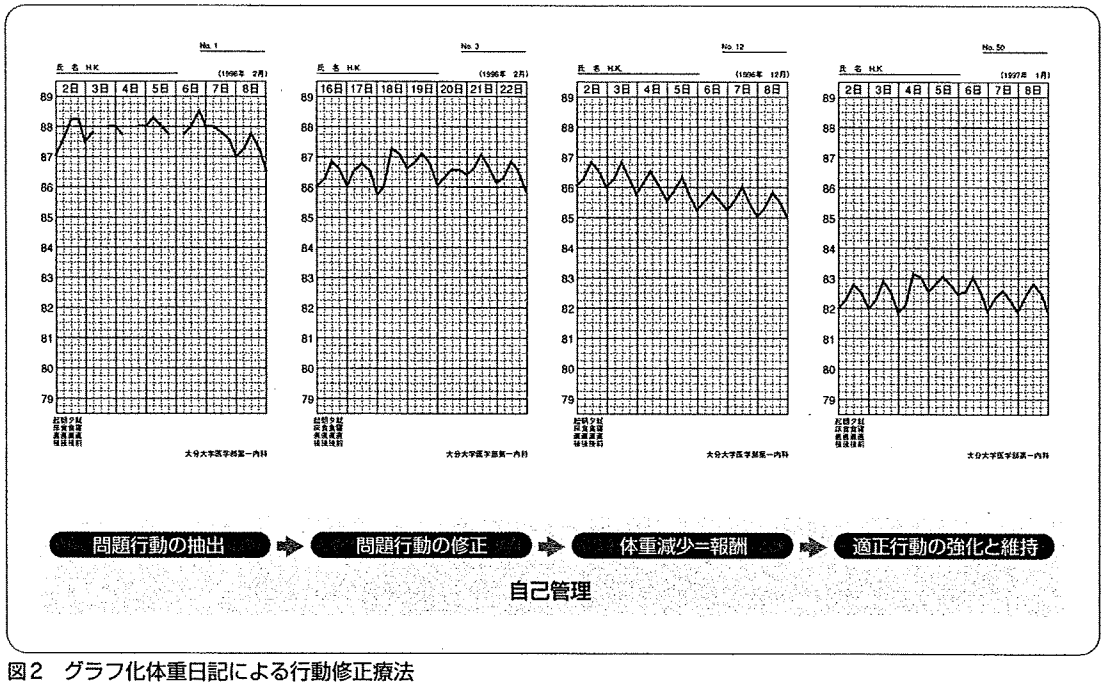

図2　グラフ化体重日記による行動修正療法

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ヵ月

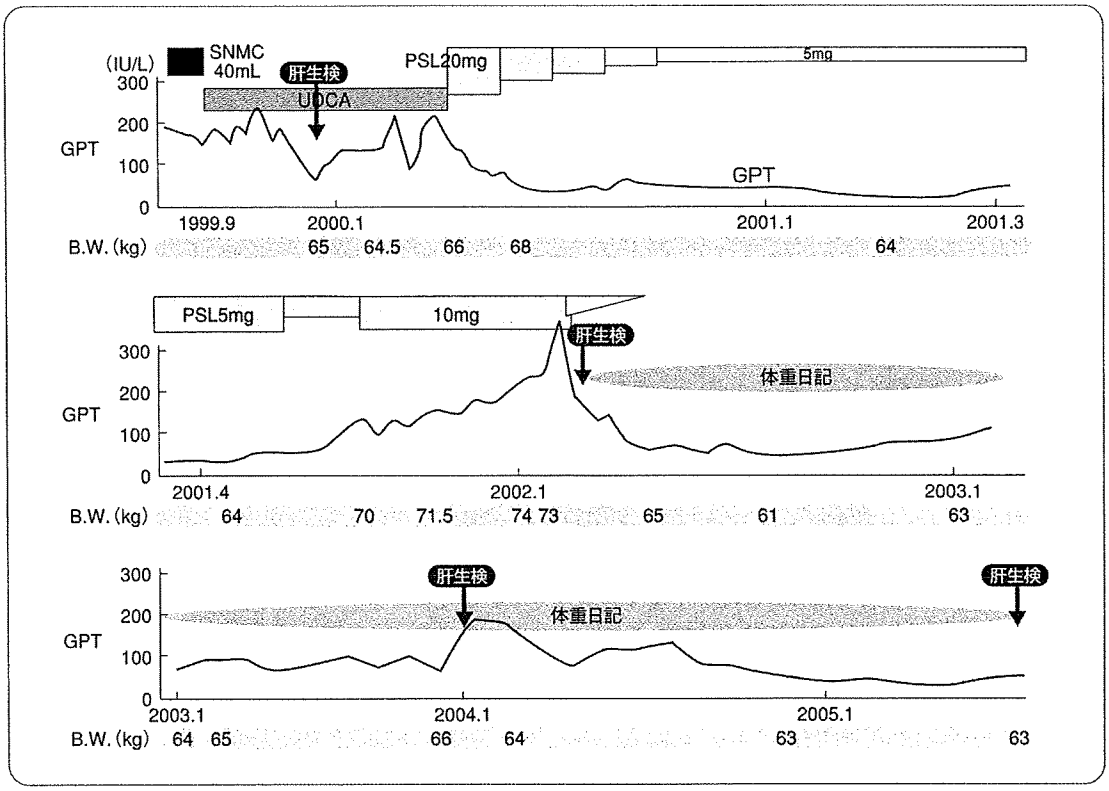

図3　臨床経過
50y.M. 肝庇護剤が効果が不十分で，体重制御や種々の治療により改善した．

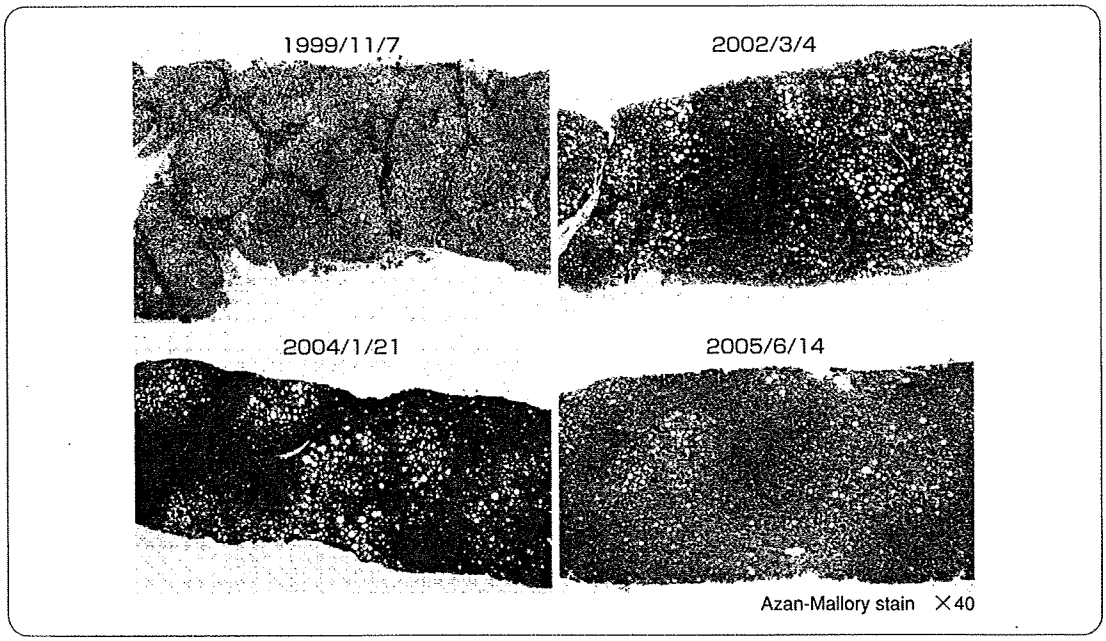

図4　肝病理組織像の推移
グラフ化体重日記による体重の制御を中心とした治療で肝の線維化は改善している．

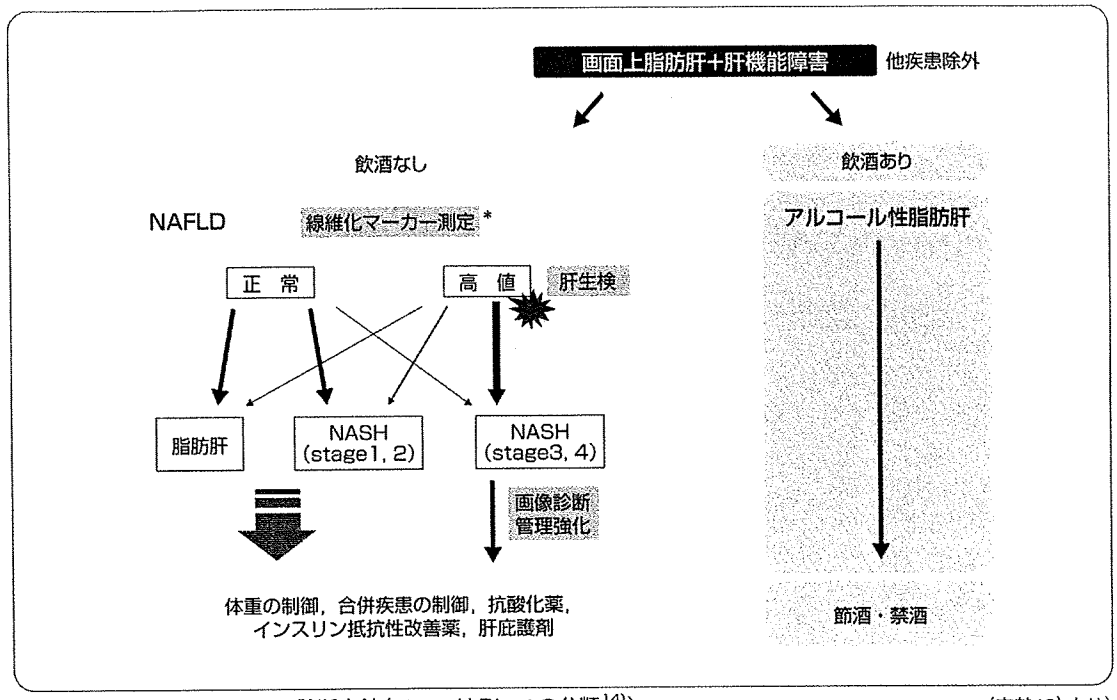

図5 当科におけるNAFLDの診断方針(stageはBluntの分類[14])
(文献13)より)
*：40歳未満ではヒアルロン酸は線維化が進んでいても正常のことが多いのでⅣ型コラーゲンを指標にする．

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．

参考文献

1) Day CP, James OF : Steatohepatitis : a tale of two "hits" ?. Gastroenterology, 114 : 842-845, 1998.

2) Dixon JB, Bhathal PS, Hughes NR, et al : Nonalcoholic fatty liver disease : Improvement in liver histological analysis with weight loss. Hepatology, 39 : 1647-1654, 2004

3) Huang MA, Greenson JK, Chao C, et al : One-year intense nutritional counseling results in histological improvement in patients with non-alcoholic steatohepatitis : a pilot study. Am J Gastroenterol, 100 (5) : 1072-1081, 2005.

4) Fujimoto K, Sakata T, Etou H, et al : Charting of daily weght pattern reinforces maintaince of weght reduction in moderately obese paients. Am J Med Sci, 303 : 145-150, 1992.

5) 吉松博信：肥満症の行動療法. 糖尿病・代謝症候群. 医学のあゆみ (別冊), 門脇　孝, 小川佳宏, 下村伊一朗 (編), 医歯薬出版, 827-834, 2004.

6) Tanaka M, Itoh K, Abe M, et al : Irregular pattern in the daily weight chart at night predict body weight regain. Exp Biol Med, 229 : 940-945, 2004

7) Laurin J, Lindor KD, Crippin JS, et al : Ursodeoxycholic acid or clofibrate in the treatment of non-alcholic-induced steatohepatitis : A pilot study. Hepatology, 23 : 1464-1467, 1996.

8) Lindor KD, Kowdley KV, Heathcote EJ , et al : Ursodeoxycholic acid for treatment of nonalcoholic steatohepatitis : Results of a randomized trial. Hepatology, 39 : 770-778, 2004.

9) Dufour JF, Onera CM, Gonvers JJ, et al : Radomized placebo-controlled trial of ursodeoxycholic acid with vitamin E in nonalcoholic steatoheptitis. Clin Gastroenterol Hepatol, 12 : 1537-1543, 2006.

10) Fan JG, Zhong L, Tia LY, et al : Effect of usodeoxycholic acid and/or low-calorie diet on steatohepatitis in rat with obesity and hyperlipidemia. World J Gastroenterol, 11 : 2346-2350, 2005.

11) Okan A, Astarcioglua H, Tankurt E, et al : Effect of ursodeoxycholiccid on hepatic steatosis in rats. Digestive Disease and Science, 11 : 2389-2397, 2002.

12) 中島俊彰, 八木みすみ, 木本順子, 他：強力ネオミノファーゲンシーと体重減少が有効であった非アルコール性脂肪性肝炎 (NASH) の1例. MINOPHAGEN MEDICAL REVIEW, 49 : 77-80, 2004.

13) 織部淳哉, 清家正隆, 本田浩一, 他：NASHにおける病理組織像と臨床背景の関連.消化器科, 37 : 175-181, 2003.

14) Blunt EM : Nonalcoholic steatohepatitis : definition and pathology. Semin Liver Dis, 21 : 3-16, 2001.

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Original Article

Amino acid substitutions in the S region of hepatitis B virus in sera from patients with acute hepatitis

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Background: An increase in the number of acute hepatitis patients with hepatitis B virus (HBV) of non-indigenous genotypes may reduce the efficacy of vaccination against HBV.

Methods: We have determined the amino acid (aa) sequences in the major hydrophilic region (MHR) in the S region of HBV in patients with acute hepatitis B and compared those with the ones from HBV strains used for the production of HBV vaccines commercially available in Japan.

Results: Of 48 patients studied, 11 were infected with genotype A, 11 with genotype B and 26 with genotype C HBV. The aa sequences of the nine genotype A isolates were the same as the aa sequence of J02205 which is used for the production of one of the commercially available recombinant vaccines. The aa sequences of the 11 genotype B isolates differed from the aa sequence of J02205 in two or three amino acids. Of the 26 genotype C isolates, 22 had the same aa sequence as X01587 which is used for the production of another recombinant vaccine. The remaining genotype C isolates had aa substitutions at aa131, which have a potential to alter the hydropathy and the three-dimensional structure of the MHR. The differences among the three current HBV vaccines in aa sequences in the MHR theoretically alter the hydropathy and three-dimensional structure.

Conclusion: Our results suggest that the transmission of HBV isolates with different genotypes or with aa substitutions in the MHR might reduce the efficacy of currently available HBV vaccines in the protection of HBV infections.

Key words: genotype, hepatitis B virus, major hydrophilic region, vaccine

INTRODUCTION

ABOUT 300 MILLION people in the world are chronically infected with hepatitis B virus (HBV). Chronic infection may eventually lead to liver cirrhosis or hepatocellular carcinoma.[1–4] To prevent the transmission of this virus, vaccination has been introduced in many countries. Indeed, universal vaccination has not only reduced the number of infected individuals, but also the number of deaths related to HBV.[5,6]

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In Japan, in 1985, a national project was started to vaccinate children born to HBV-infected mothers. The chances of vertical transmission from HBV-carrying mothers have decreased. Recently, the prevalence of HBV in Japan has decreased to approximately 0.6%.[7]

Because the number of individuals infected with HBV has decreased, the number of patients with acute hepatitis B, mainly caused by horizontal transmission from HBV carriers, should also have decreased. However, in Japan, the number of patients with acute hepatitis B has recently increased (Yatsuhashi H. *et al.*, 2004, unpubl. data).

The increase in the number of patients with acute hepatitis B may, in part, be the result of patients carrying novel HBV genotypes imported from abroad. For

example, in recent years, genotype A HBV has often been detected in patients with acute hepatitis B.[8,9]

Genotype A HBV is transmitted from individuals who live in or have immigrated from other countries to Japan. Its infection is characterized by a high viral load and a long hepatitis B surface antigen (HBsAg) positivity period. The transition of acute hepatitis B with genotype A HBV infection to the chronic state has been reported recently.[8,10] Decreasing the transmission rate of genotype A HBV is therefore important for the control of the disease. Introducing universal vaccination for adolescents or adults is a measure to be considered.

The effectiveness of universal vaccination depends on the reactivity of vaccines against HBV. HBsAg binds antibody to hepatitis B surface antigen (anti-HBs) produced against HBV vaccines mainly via the 'a' determinant region (aa124–aa149). This region contains common antigenic epitopes of all subtypes (adw, adr, ayw, ayr) of HBsAg and lies in the major hydrophilic region (MHR) between aa99 and aa169. Amino acid (aa) substitutions in the MHR, particularly in the 'a' determinant region, can alter B cell epitopes of HBsAg, leading to immunological escape from the host immunity induced by either vaccination or previous infection.[11] Therefore, if HBV prevalent in Japan has aa substitutions in the MHR, the effect of universal vaccination may be reduced.

In Japan, three types of HBV vaccine (Bimmugen, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan; Heptavax, Merck & Co., Whitehouse Station, NJ, USA; and Meinyu, Meiji Dairies, Tokyo, Japan) are now available. Efficacy and immunogenicity of vaccines are not always comparable or identical.[12,13] Whether giving a single vaccine effectively prevents the transmission of all genotypes of HBV is an important but still unsolved problem. Elucidating the aa substitutions in the MHR may give a clue to this problem.

The purpose of the present study is to determine the difference of the aa sequences in the MHR of HBV among isolates from patients with acute hepatitis and also the difference of the aa sequences among viral strains used for the production of anti-HBV vaccines, and to find ways to use currently available vaccines as effective prophylaxes.

METHODS

Patients

FROM 1992 TO 2001, serum samples were collected from 48 patients diagnosed with acute hepatitis B in our institutions. Only patients whose serum samples were stored at the onset of hepatitis were included in this study. All the 48 patients ran a self-limited clinical course. No patients subsequently developed fulminant hepatic failure or chronic sequelae.

The criteria for the diagnosis of acute hepatitis B were the following: (i) an acute onset of liver injury without a history of liver dysfunction and positivity for HBsAg in serum; and (ii) immunoglobulin M (IgM) antibody to HBV core antigen (anti-HBc) at a titer of more than 2.5 of cut-off index. Coinfection with a hepatitis A virus or a hepatitis C virus was excluded by serological tests. None of the patients had previously received any vaccination against HBV.

Serum samples from the 48 patients with acute hepatitis B were examined virologically, and the results were examined for correlations with clinical characteristics. Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of our institutions.

Determination of HBV-DNA

Hepatitis B virus DNA level was determined using transcription-mediated amplification (TMA) and a hybridization protection assay (Chugai Diagnostics Science, Tokyo, Japan) using the protocol of Kamisango *et al.*[14] The range of detection using TMA was from 3.7 log genome equivalents (LGE)/mL (i.e. $10^{3.7}$ copies/mL corresponding to 5000 copies/mL) to 8.7 LGE/mL ($10^{8.7}$ copies/mL). In seven of 34 studied serum samples, the level of HBV-DNA was lower than 3.7 LGE/mL and these were categorized as 3.7 LGE/mL.

Genotyping HBV

Hepatitis B virus genotypes were determined using commercial enzyme immunoassay kits (Smitest HBV Genotyping kit; Genome Science, Fukushima, Japan). In brief, DNA extracted from serum was amplified by polymerase chain reaction (PCR) using three sense primers (i.e. s1: 5'-ACCAACCCTCTGGGATTCTTTCC-3', s2: 5'-ACCAATCCTCTGGGATTCTTCCC-3', and s3: 5'-AGCAATCCTCTAGGATTCCTTCC-3' [nt 2902–2924]) and an antisense primer (i.e. as1: 5'-GAGCCTGA GGGCTCCACCC-3' [nt 3091–3073]) biotinylated at the 5' end; their sequences were deduced from conserved sequences in the pre-S1 region of HBV. The biotin-labeled and amplified HBV-DNA was denatured in an alkaline solution, and tested for hybridization to probes specific for one of the seven HBV genotypes (A-G) immobilized on wells of a 96-well