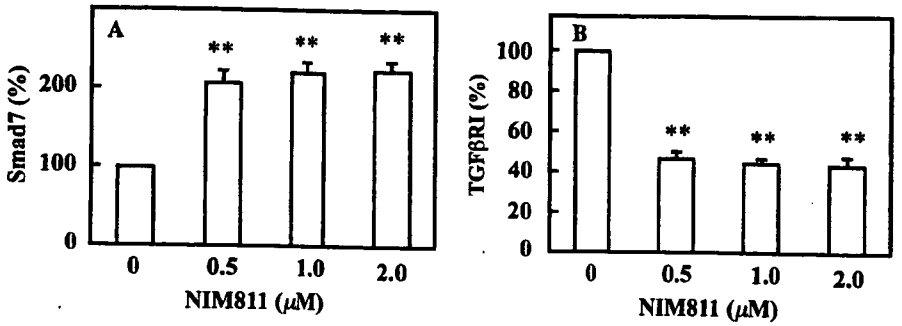

Fig. 7. Real-time reverse transciptase-polymerase chain reaction (RT-PCR) analysis of Smad7 and tumour growth factor-β (TGF-β)-RI mRNA expression. Total RNA was extracted from hepatic stellate cells (HSCs) treated with NIM811 (0.5, 1, or 2 mM) or mock treated. Samples were analysed by real-time RT-PCR, and all PCR reactions were normalized for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. NIM811 enhanced the expression of Smad7 (A), and it reduced the expression of TGF-β-RI (B). The ratio of expression in the absence of NIM811 was used as a control (100%). The data represent mean ± SEM from three independent experiments. **Statistically significant differences ($P < 0.01$) compared with HSCs in the absence of NIM811.

Previous work has demonstrated that, in addition to stimulating collagen production, activated HSCs inhibit the degradation of interstitial collagens by collagenases such as MMP-1, indicating that matrix degradation is inhibited during the progression of fibrosis (29–31). TIMP-1 has been reported to regulate cell growth and apoptosis independent of the inhibition of matrix degradation (32). We demonstrated that NIM811 suppressed the growth of HSCs in a concentration-dependent manner without apoptosis (Fig. 4). Taken together, these results indicate that NIM811 has therapeutic potential for liver fibrosis through suppression of collagen production and enhancement of collagenase activity.

To explore the mechanism by which NIM811 suppresses collagen production and cell proliferation, and enhances collagenase activity, we examined the effects of NIM811 on intra-cellular signalling cascades, such as MAPK cascades, which play important roles in collagen production and cell proliferation in HSCs (33). It is intriguing that NIM811 enhanced the activation of JNK and p38 but not ERK1/2 (Fig. 5). In contrast, cyclosporine suppressed the activation of JNK and p38, as we reported previously (9). It was shown that cyclosporine exerts its immunosuppressive effects through both the calcineurin-dependent nuclear factor of activated T cells (NFAT) pathway and the calcineurin-independent activation pathway for JNK and p38 (34). NIM811, an analogue of cyclosporine, does not activate the NFAT pathway because it cannot bind to cyclophilin A (13). The different effects of NIM811 and cyclosporine on JNK and p38 might be attributable to the absence of an effect of NIM811 on the NFAT pathway.

In addition to MAPKs, TGF-β signalling cascades strongly stimulate collagen production by HSCs (28). TGF-β binds to TGF-β-RII on the cell membrane, and then TGF-β-RII phosphorylates TGF-β-RI at the serine and threonine residues located in its glycine-/serine-rich domain (35). The phosphorylated TGF-β-RI phosphorylates Smad2 and Smad3 at a C-terminal SSXS motif and these form a complex with their common partner Smad4. These Smad proteins translocate to the nucleus and activate the transcription of target genes such as collagen (35). In this study, we demonstrated that Smad2 and Smad3 were constitutively phosphorylated in activated HSCs, as reported previously (36), and that NIM811 suppressed the phosphorylation of Smad2 and Smad3 (Fig. 6). These results suggest that NIM811 may inhibit the kinase activity of TGF-β-RII and/or TGF-β-RI. Several molecules such as Smad7 (28, 37), immunophilin FK506-binding protein (FKBP) 12 (38) and Smad anchor for receptor activation (SARA) (39) are associated with TGF-β-R and regulate TGF-β signalling. We found that NIM811 enhanced the expression of Smad7, and suppressed that of TGF-β-RI, indicating that NIM811 inhibits the TGF-β signalling pathways, at least partially through blockade at the receptor level. We also found that cyclosporine had similar effects on Smad2, Smad3, Smad7 and TGF-β-RI (unpublished data). As mentioned above, NIM811 had effects opposite to those of cyclosporine on JNK and p38, although both showed similar effects on collagen production and cell proliferation, suggesting that NIM811 and cyclosporine exhibit antifibrogenic effects mainly by blockade of TGF-β signalling pathways.

Because NIM811 lacks the ability to bind to cyclophilin A (13), NIM811 exerts its pharmacological effects by binding to other cyclophilins, such as cyclophilin B or D. Cyclophilins are a family of PPIases that catalyse the cis-trans interconversion of peptide-bound amino-terminal proline residues, facilitating

changes in protein conformation (40). There are more than 10 subtypes of cyclophilin and they are involved in numerous cellular processes, including transcriptional regulation, immune response, protein secretion and mitochondrial function (40, 41). Watashi *et al.* (42) recently reported that NIM811 suppressed the replication of an HCV replicon *in vitro*, whereas tacrolimus did not show this effect. Notably, NIM811 exerts its antiviral effects via binding cyclophilin B, which is a functional regulator of HCV RNA polymerase (43). NIM811 has also been reported to have cytoprotective properties depending on interference with the interaction with cyclophilin D, which regulates the mitochondrial permeability transition (13). Kon *et al.* (44) reported that NIM811 prevented acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. In order to explain the detailed working mechanism of NIM811, cyclophilins interacting with NIM811 are important factors; however, we have not determined which cyclophilin is utilized by NIM811 to exert its anti-fibrogenic and anti-proliferating activity. We are now in the process of identifying the target cyclophilin.

In conclusion, we demonstrated that NIM811, as well as cyclosporine, had anti-fibrogenic effects. NIM811 has no immunosuppressive activity and, in consideration of the toxicity data, seems more favourable for clinical use because of the absence of significant changes in kidney-specific parameters following 10 days of 50 mg/kg of oral NIM811, whereas the same dose regimen of cyclosporine produced signs of renal dysfunction (45). NIM811 would be a plausible candidate for prevention of the progression of HCV-related graft-cirrhosis after liver transplantation because of its anti-viral and anti-fibrogenic effects *in vitro*. Further studies *in vivo* will be required to determine whether NIM811 is effective for the treatment of hepatic fibrosis.

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BASIC STUDIES

Methylprednisolone injection via the portal vein suppresses inflammation in acute liver failure induced in rats by lipopolysaccharide and D-galactosamine

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Keywords
D-galactosamine – lipopolysaccharide – liver failure – methylprednisolone – portal vein – steroid

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Abstract

Background: We have reported that hepatic arterial steroid injection is an effective therapy to rescue patients from fulminant or severe acute hepatic failure. We speculate that a high concentration of steroid suppresses inflammatory processes in the liver directly by restraining activated inflammatory cells, including macrophages. To analyse the detailed mechanism, steroid injection via the portal vein was performed in an experimental model of liver damage. *Methods:* Rats subjected to lipopolysaccharide and D-galactosamine injection were treated with a methylprednisolone injection via the tail vein or the portal vein. The survival rate, serum levels of inflammatory cytokines and apoptotic cell counts in the liver were analysed. *Results:* The survival rate was significantly improved by steroid injection, especially via the portal vein. Serum values of alanine aminotransferase, tumor necrosis factor-α and interferon-γ were reduced in the treated groups, especially the group given portal venous injections. Apoptotic cell counts in the liver were significantly lower in the group injected with steroid via the portal vein. *Conclusion:* In the model rats, high concentrations of steroid in the liver acted on inflammatory cells and suppressed inflammatory cytokines and liver cell death. The mechanism is suggested to be the same for arterial steroid injection therapy in patients with acute hepatic failure.

Acute liver damage occasionally progresses to severe liver failure with extremely high mortality, even today (1–3). Plasma exchange and haemodiafiltration have been used as artificial liver support systems for affected patients but are only partially effective (4, 5). Although liver transplantation is valuable for the rescue of patients and improving prognosis (6, 7), it is accompanied by an extraordinary financial burden, and the possibility of life-threatening complications to living donors cannot be eliminated completely.

Recently, we reported that transcatheter arterial steroid injection therapy (TASIT), by which methylprednisolone is administered directly into the liver through a catheter located in the hepatic artery, dramatically suppressed liver injury and reduced the mortality of patients with severe acute hepatic failure (8). More than 75% of the patients treated with TASIT were cured without liver transplantation; however, although 24% of the patients without TASIT recovered, 35% underwent liver transplantation and 41% died. Because corticosteroid can suppress the effects of activated macrophages and other inflammatory cells (9, 10), it has been used for the treatment of severe acute hepatic failure by oral or intravenous administration; however, this therapy has not shown a satisfactory clinical effect and has not been definitively established (11, 12). We speculate that a sufficient concentration of corticosteroid cannot be achieved in the liver when corticosteroid is administered through a peripheral vein because of the injured circulation that accompanies severe hepatic damage. Therefore, it is possible that TASIT can supply a sufficient

concentration of steroid to the liver, even in such a poor condition.

To estimate the effect of exposure of the injured liver to high concentrations of steroid, we carried out experiments in a rat model of acute liver failure induced by lipopolysaccharide (LPS) and D-galactosamine (GalN) (13). It is well known that the liver damage in this model is caused by the activation of lymphocytes and macrophages and that cytokines released from these inflammatory cells accelerate the progression of liver cell destruction, including apoptosis of hepatocytes (14, 15). Although, in the case of TASIT, steroid is injected through a catheter located in the hepatic artery, via a puncture of the femoral artery, we decided to inject steroid via the portal vein in this study because of the technical difficulty of the arterial approach in rats. Because the blood supply to the liver tissue from the portal vein is twice in volume that from the hepatic artery, a sufficient or a greater drug supply to the liver tissue should be achieved using the portal vein approach (16). In this study, using the rat model of liver damage, the survival period and serum values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) were compared among three groups: rats without steroid treatment, with steroid injection via the tail vein and with injection via the portal vein. Liver damage was examined histologically, and apoptotic cell counts were estimated. Our results showed that steroid injection therapy via the portal vein was superior in suppressing hepatic inflammation and improving the survival rate in this animal model.

Materials and methods

Animal treatment

Eight- to 10-week-old male Wister rats weighing 175–225 g were maintained under controlled conditions with free access to standard chow and water. During all the experimental procedures, the animals were given human care in compliance with institutional guidelines according to the '*Guide for the Care and Use of Laboratory Animals*' published by the National Institutes of Health. One-hundred microgram per rat of LPS and 500 mg/kg of GalN (Sigma, St Louis, MO, USA), each of which was dissolved in 500 μL of phosphate buffered saline (PBS), were injected intraperitoneally into the rats. Two hours after the injection of LPS and GalN, animals were anaesthetized with pentobarbital sodium, and then 10 mg of methylprednisolone sodium succinate (mPSL) (Pfizer, New York, NY, USA) dissolved in 100 μL of PBS was injected into the portal vein or the tail vein. Control animals were injected with the same amount of PBS into the portal vein or the tail vein. Seven rats were examined in each group.

Histological examination

Liver tissue samples were isolated 10 h after the injection of mPSL or PBS, fixed in 10% formalin and embedded in paraffin. Serial sections (5 μm thick) were cut from each block. Liver injury was evaluated morphologically in hepatic sections stained with haematoxylin and eosin. Apoptotic cells were detected using an apoptosis detection kit (Wako, Osaka, Japan). The TdT-mediated dUTP nick end labelling (TUNEL) method was performed using deparaffinized liver sections (17). The number of positive cells in 10 high-performance fields (HPF) was counted on each section by three independent examiners who did not have information about the sections, and the average value of positive cell counts from 10 HPF from three examiners was used for statistical analysis.

Transaminase and cytokine assays

Blood samples were taken from the tail veins at 10 h after the injection of mPSL or PBS. The serum levels of AST and ALT were measured by a colorimetric assay (Fuji Film, Tokyo, Japan). TNF-α and IFN-γ were measured using ELISA kits (Endogen, Rockford, IL, USA).

Statistical analysis

The results are expressed as mean ± SD. Significant difference between two groups was assessed using unpaired two-tailed *t*-tests. A value of $P < 0.05$ was considered to be significant. Survival curves were compared using the log-rank test.

Results

Effect of methylprednisolone sodium succinate injection on the survival rate of animals with acute liver failure

The time course of our experimental procedure is summarized in Figure 1. We set the time of mPSL administration at 2 h after LPS and GalN challenges, when the inflammatory processes were thought to have started but not to have progressed fully and irreversibly to fatal liver failure. In our experimental model of acute liver damage induced by LPS and GalN, the spontaneous survival rate was 20% at 17 h of observation. The control animals without steroid treatment began to die 4–5 h after the chemical

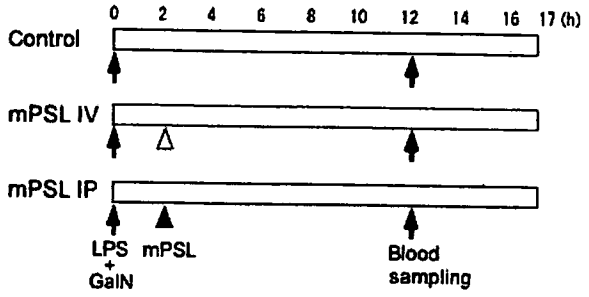

Fig. 1. Time course of experiments. Two hours after the intraperitoneal injection of 100 μg of lipopolysaccharide (LPS) and 500 mg/kg of D-galactosamine, 10 mg of methylprednisolone (mPSL) was administered into the tail vein (mPSL IV) or portal vein (mPSL IP). Blood for transaminase and cytokine measurements was taken at 10 h after mPSL injection and the survival rate of animals was estimated at 17 h from the beginning of experiments. Open triangles, mPSL injection via tail vein; closed triangles, mPSL injection via portal vein.

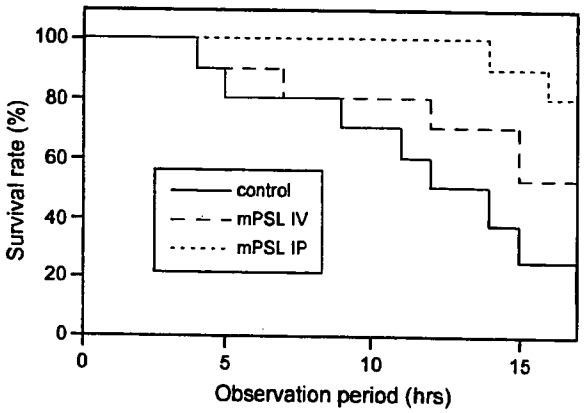

Fig. 2. Survival rate of animals with acute liver damage treated with or without methylprednisolone (mPSL). Two hours after the injection of D-galactosamine and lipopolysaccharide, mPSL was administered into the tail vein (mPSL IV) or portal vein (mPSL IP). Animals with mPSL injection via the tail vein showed lower mortality than the controls (50% at 17 h) and animals injected with mPSL via the portal vein showed the highest survival rate (80% at 17 h). The difference among the groups was significant (log-rank test: $P = 0.0083$). ($n = 10$ in each group).

injection and the number of dead animals increased with the passage of time (Fig. 2). The rats with mPSL injection via the tail vein showed a similar tendency, but the rate of death was lower than the control animals, and 50% of the animals survived throughout the observation period. However, the mortality of animals with an mPSL injection via the portal vein was significantly lower than that of the other groups. All the animals in this group survived for 12 h after the chemical injection. The final survival rate of this group was 80% after the complete 17-h observation period and surviving animals showed an almost normal appearance, in contrast to the surviving animals in the other groups, which looked ill.

Effect of methylprednisolone sodium succinate injection on serum levels of aspartate aminotransferase, alanine aminotransferase, tumor necrosis factor-α and interferon-γ

Liver damage was estimated by the serum levels of transaminases. AST and ALT levels in the control animals were extremely high (Fig. 3) but were reduced by mPSL treatment, both the mPSL-treated groups having significantly lower transaminase levels than the controls (decrease of about 50%). However, no difference in the levels was evident between the treated groups (injection via the tail vein and the portal vein). Next, cytokine levels were determined in the animals (Fig. 4). Serum TNF-α and IFN-γ levels in the groups injected with mPSL were much lower than those in the control animals, and the levels were lower in the group treated with mPSL via the portal vein than that via the tail vein. TNF-α and IFN-γ levels in the group injected with mPSL via the portal vein were about 30 and 25% of those in the control group respectively, and the difference between the groups was significant.

Effect of methylprednisolone sodium succinate injection on histological improvement

As mentioned above, the effect of mPSL on the levels of inflammatory cytokines depends on the injection route. It is possible that the considerable reduction of these cytokines results in the suppression of hepatic inflammation and improvement of survival rate. Therefore, liver damage in the animals was evaluated histologically. In control animals without mPSL treatment, zonal necroses were observed (Fig. 5A). These pathological changes were attenuated in the group injected with mPSL via the tail vein and extensive necroses were not found but confluent necroses were scattered (Fig. 5B). Further histological improvement was seen in the group injected with mPSL via the portal vein (Fig. 5C). A few spotty necroses with disarranged hepatic cords were observed.

In both the experimental liver damage model and human patients with fulminant hepatitis, it is known that a substantial increase in apoptotic cells may be observed in the injured liver and this may be evidence of progression of liver cell death. We analysed the number of apoptotic cells in each group of model animals. TUNEL-positive cells were counted in 10 HPF in each liver section from each group of animals.

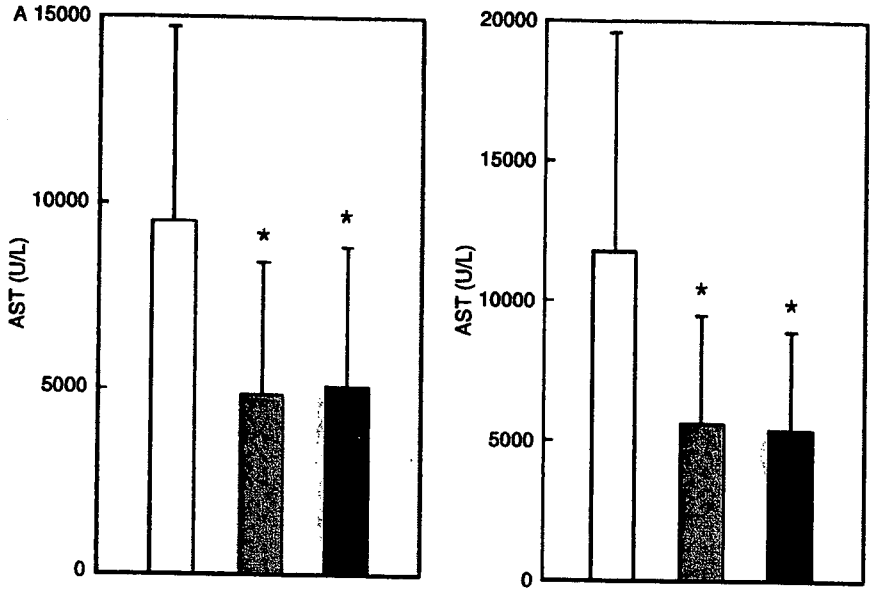

Fig. 3. Effect of methylprednisolone (mPSL) on serum transaminase levels. Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in mPSL treated groups were significantly lower than those of the control group but no difference was observed between the group injected with mPSL via the tail vain and via the portal vein. Open squares, control; shaded squares, mPSL injection via the tail vein; closed squares, mPSL injection via the portal vein. (A) AST. (B) ALT. Values are expressed as mean ± SD. ($n = 7$ in each group; *significantly different from the control; $P < 0.05$).

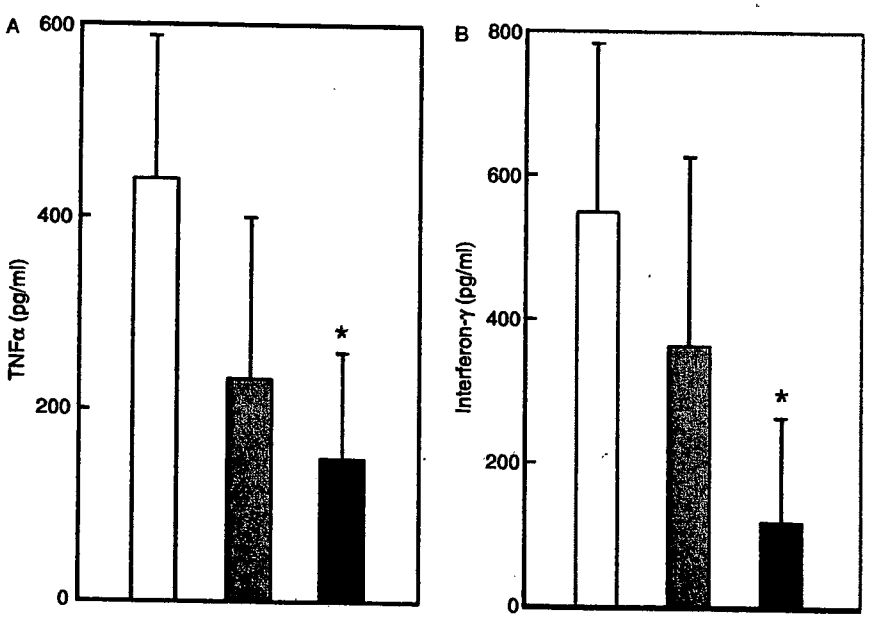

Fig. 4. Effect of methylprednisolone (mPSL) on serum tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) levels. mPSL treatments using two different routes reduced serum concentrations of TNF-α and IFN-γ and the effects of steroid were more pronounced in the group injected with mPSL via the portal vein than via the tail vein. Open squares, control; shaded squares, mPSL injection via the tail vein; closed squares, mPSL injection via the portal vein. (A) TNF-α. (B) IFN-γ. Values are expressed as mean ± SD ($n = 7$ in each group; *significantly different from the control; $P < 0.05$).

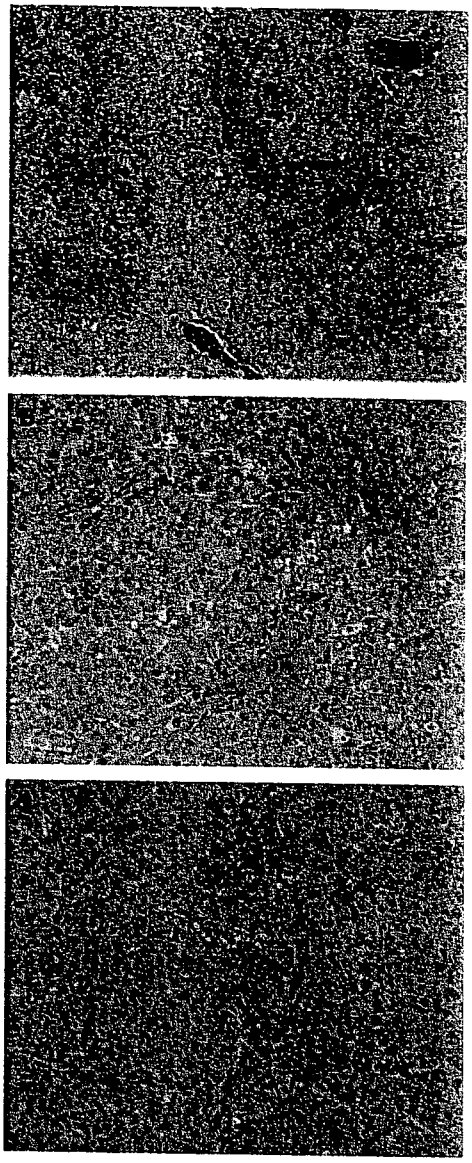

Fig. 5. Histological features of liver sections. A representative example from each group is shown (magnification: A, × 100; B, × 200; C, × 200). The control animals with injection of D-galactosamine and lipopolysaccharide without steroid treatment showed extensive eosinophilic areas, suggestive of zonal necroses (A). Extensive necroses were not found but confluent necroses were scattered in the animals injected with methylprednisolone (mPSL) via the tail vein (B). A few spotty necroses with disarranged hepatic cords were seen in the animals injected with mPSL via the portal vein (C).

As shown in Figure 6, numerous apoptotic cells were observed in the liver sections of animals treated without steroid. The number of apoptotic cells decreased substantially in both groups of mPSL-treated animals. In the group treated with mPSL via the tail vein, the

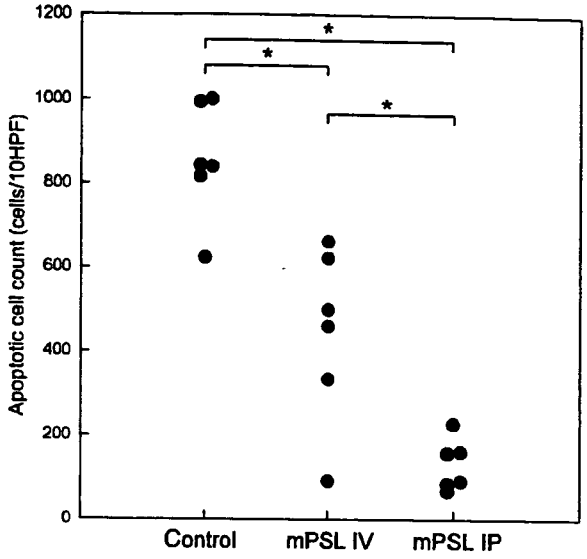

Fig. 6. Effect of methylprednisolone (mPSL) treatment on the number of apoptotic cells in liver. TdT-mediated dUTP nick end labelling (TUNEL) staining was performed to detect apoptotic cells in liver sections. TUNEL-positive cells were counted in 10 high-performance fields (HPF) for every group. Significantly fewer apoptotic cells were observed in the group treated with mPSL via the tail vein (mPSL IV) than the controls and the reduction was more pronounced in the group treated with mPSL via the portal vein (mPSL IP) ($n = 6$ in each group; *significantly different; $P < 0.05$).

number of apoptotic cells decreased significantly and was around half that of the control group. This reduction was more pronounced in the group treated with mPSL via the portal vein; the number of apoptotic cells was less than a quarter that of the control group and the difference was significant compared with the controls and the animals injected with mPSL via the tail vein.

Discussion

In this study, we showed that steroid injection via the portal vein led to an improvement of the survival rate, serum levels of transaminases and inflammatory cytokines and histological findings in model animals of acute liver failure induced by LPS and GalN. Steroid injection via the tail vein could also reduce serum transaminase levels; however, the effect of this treatment on other parameters was inferior to that of steroid injection via the portal vein.

The effects of steroid on suppression of transaminase elevation and improvement of survival have been reported in rodent models of acute liver damage (18–22); for instance, dexamethasone administered

1 h before LPS and GalN injection reduced the lethality from 60 to 10% in mice (21). However, all the steroidal effects reported were observed only when steroid was administered 5 min to 4 h before or at the same time as endotoxin challenge, and steroid treatment 2 h after the challenge did not lead to a sufficient improvement of the survival rate (22). The aim of this study was to confirm the effect of steroid in TASIT using an experimental animal model, with the steroid administered after the onset of liver inflammation, rather than before, to mimic the clinical condition of fulminant hepatitis. In this study, the effect of mPSL on the survival rate was weak when injected via the tail vein, and this insufficient effect of steroid is consistent with a previous report, when the drug was given after the onset of liver inflammation. From this point of view, the substantial improvement of the survival rate induced by mPSL injection via the portal vein is worthy of special mention, because mPSL was given after the onset of liver inflammation in this study.

The improvement of serum transaminase levels was almost equal for the groups given a steroid injection, via the tail vein and via the portal vein; however, in terms of the survival rate, the group injected via the portal vein showed a superior outcome. The difference between the two groups may be attributable not only to the suppression of destruction of hepatocytes but also to other essential biological systems. TNF-α and IFN-γ levels in the various groups suggest that at least one of these biological systems could be significantly disturbed by the activation of these cytokines, which play multiple roles in the progression of minor liver damage to fatal liver failure (23). It has been postulated that abnormalities of the microcirculation in the liver tissue make a substantial contribution to progression to liver failure (24). The fact that fibrin deposition in the sinusoids and destruction of sinusoidal endothelial cells are evident in patients with fulminant hepatitis and in animal models of liver failure suggests that impairment of the microcirculation plays an important role in progression to liver failure by exposing liver cells to hypoxia and leading to abnormalities in systemic coagulation, which results in multiple organ malfunction (25, 26). Cytokines, including TNF-α, IL-2 and IL-4, have been reported to be involved in the process of endothelial cell destruction (27–29); a high concentration of steroid in the liver induced by injection via the portal vein potentially improves the liver microcirculation by suppressing these cytokines in the injured organ.

The effects of steroid on suppression of inflammatory cells have been intensively investigated, including apoptosis and selection of lymphocytes, cytokine production by inflammatory cells, respiratory burst and migration of macrophages (10, 30–32). These effects of steroid are accomplished at relatively low concentrations, i.e., 10^{-6} M or so. This pharmacological characteristic is not applicable to the fact that 'pulse therapy' with a high dose of steroid is needed to produce a pronounced effect on the suppression of aggressively flared autoimmune disease (33). This phenomenon suggests that a high concentration of steroid has the potential to regulate inflammatory processes via unknown mechanisms, and such high concentrations were also achieved in our experiments by injection via the portal vein.

We demonstrated that, in the rat model of liver failure, steroid injection via the portal vein improved the survival rate and decreased apoptosis, probably by suppressing the production and activation of cytokines in the liver through the high concentration of steroid. Improvement of the liver microcirculation might be involved in this process. Further studies are necessary to elucidate the underlying mechanisms of these processes.

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Re-evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease

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Abstract. Nonalcoholic fatty liver disease (NAFLD) is one of the most frequent causes of abnormal liver dysfunction, and its prevalence has markedly increased. We previously evaluated the expression of fatty acid metabolism-related genes in NAFLD and reported changes in expression that could contribute to increased fatty acid synthesis. In the present study, we evaluated the expression of additional fatty acid metabolism-related genes in larger groups of NAFLD (n=26) and normal liver (n=10) samples. The target genes for real-time PCR analysis were as follows: acetyl-CoA carboxylase (ACC) 1, ACC2, fatty acid synthase (FAS), sterol regulatory element-binding protein 1c (SREBP-1c), and adipose differentiation-related protein (ADRP) for evaluation of *de novo* synthesis and uptake of fatty acids; carnitine palmitoyltransferase 1a (CPT1a), long-chain acyl-CoA dehydrogenase (LCAD), long-chain L-3-hydroxyacyl-coenzyme A dehydrogenase α (HADHα), uncoupling protein 2 (UCP2), straight-chain acyl-CoA oxidase (ACOX), branched-chain acyl-CoA oxidase (BOX), cytochrome P450 2E1 (CYP2E1), CYP4A11, and peroxisome proliferator-activated receptor (PPAR)α for oxidation in the mitochondria, peroxisomes and microsomes; superoxide dismutase (SOD), catalase, and glutathione synthetase (GSS) for antioxidant pathways; and diacylglycerol O-acyltransferase 1 (DGAT1), PPARγ, and hormone-sensitive lipase (HSL) for triglyceride synthesis and catalysis. In NAFLD, although fatty acids accumulated in hepatocytes, their *de novo* synthesis and uptake were up-regulated in association with increased expression of ACC1, FAS, SREBP-1c, and ADRP. Fatty acid oxidation-related genes, LCAD, HADHα, UCP2, ACOX, BOX, CYP2E1, and CYP4A11, were all overexpressed, indicating that oxidation was enhanced in NAFLD, whereas the expression of CTP1a and PPARα was decreased. Furthermore, SOD and catalase were also overexpressed, indicating that antioxidant pathways are activated to neutralize reactive oxygen species (ROS), which are overproduced during oxidative processes. The expression of DGAT1 was up-regulated without increased PPARγ expression, whereas the expression of HSL was decreased. Our data indicated the following regarding NAFLD: i) increased *de novo* synthesis and uptake of fatty acids lead to further fatty acid accumulation in hepatocytes; ii) mitochondrial fatty acid oxidation is decreased or fully activated; iii) in order to complement the function of mitochondria (ß-oxidation), peroxisomal (ß-oxidation) and microsomal (ω-oxidation) oxidation is up-regulated to decrease fatty acid accumulation; iv) antioxidant pathways including SOD and catalase are enhanced to neutralize ROS overproduced during mitochondrial, peroxisomal, and microsomal oxidation; and v) lipid droplet formation is enhanced due to increased DGAT expression and decreased HSL expression. Further studies will be needed to clarify how fatty acid synthesis is increased by SREBP-1c, which is under the control of insulin and AMP-activated protein kinase.

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Abbreviations: NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase, SREBP-1c, sterol regulatory element-binding protein 1c; ADRP, adipose differentiation-related protein; CPT1a, carnitine palmitoyltransferase 1a; LCAD, long-chain acyl-CoA dehydrogenase; HADH, long-chain L-3-hydroxyacyl-coenzyme A dehydrogenase; UCP2, uncoupling protein 2; ACOX, straight-chain acyl-CoA oxidase; BOX, branched-chain acyl-CoA oxidase; CYP, cytochrome P-450; PPAR, peroxisome proliferator-activated receptor; DGAT1, diacylglycerol O-acyltransferase 1; HSL, hormone-sensitive lipase; SOD, superoxide dismutase; GSS, glutathione synthetase; ROS, reactive oxygen species; AMPK, AMP-activated protein kinase

Key words: nonalcoholic fatty liver disease, fatty acid, oxidation, reactive oxygen species

Introduction

Nonalcoholic fatty liver disease (NAFLD), which is characterized by triglyceride accumulation in hepatocytes (hepatic steatosis), is one of the most frequent causes of

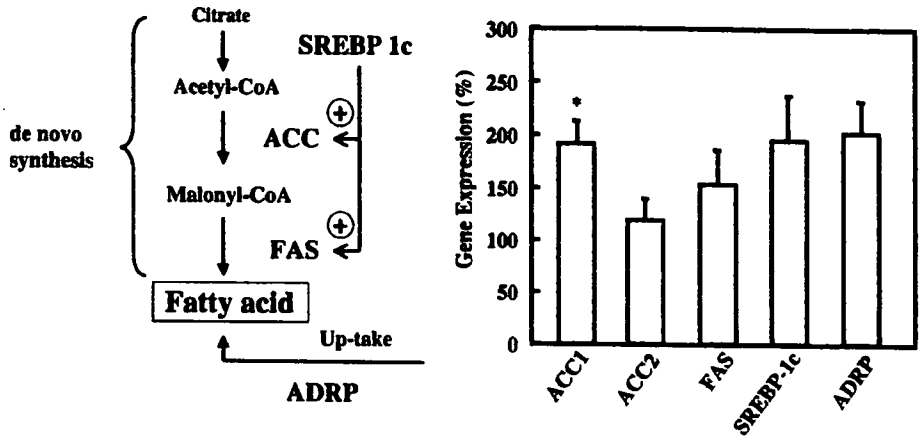

Figure 1. Real-time RT-PCR analysis for gene expression of *de novo* synthesis and uptake of fatty acids in NAFLD. *p<0.05, a statistically significant difference as compared with the normal liver. ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; SREBP-1c, sterol regulatory element-binding protein 1c; ADRP, Adipose differentiation-related protein (adipophilin).

abnormal liver function (1-3). The prevalence of NAFLD in the general population is estimated to be between 14 and 24% (4-6), and it has markedly increased in all segments of the population including children. Furthermore, nonalcoholic steatohepatitis (NASH), a severe form of NAFLD which is accompanied by hepatitis and fibrosis (7), can progress to cirrhosis and hepatic failure (8). It has been reported that >20% of patients with NASH develop cirrhosis, half of which subsequently die of liver failure (8). NAFLD is often associated with obesity and/or insulin resistance; however, the precise cause of NAFLD remains unclear. It is important, therefore, to characterize lipid metabolism, particularly fatty acid metabolism, in NAFLD.

Fatty acids in the liver are derived from *de novo* synthesis and plasma-free fatty acids. Acetyl-CoA is an essential substrate for *de novo* synthesis and is ultimately converted to stearic acid (C18:0), which also can be desaturated to oleic acid in hepatocytes. Plasma fatty acids are also actively taken up by a specific transporter. Up-regulation of synthesis and/or uptake can result in fatty acid accumulation. Fatty acids in hepatocytes are metabolized by either of 2 pathways; oxidation to generate ATP (e.g. ß-oxidation in the mitochondria) or esterification to produce triglycerides, which are either incorporated into lipoproteins for export or stored as lipid droplets within the hepatocytes. Defects in one or both of these pathways can lead to hepatic steatosis.

We previously evaluated the expression of genes related to fatty acid metabolism and reported that *de novo* synthesis of fatty acids was up-regulated in spite of their accumulation in hepatocytes of patients with NAFLD (9). In this study, using more samples from NAFLD and control livers, we further investigated fatty acid metabolism in NAFLD by re-evaluating the expression of genes involved in *de novo* synthesis, uptake, oxidation, antioxidant pathways, and triglyceride synthesis and catalysis.

Patients and methods

Tissue samples were obtained by liver biopsy from 26 patients with histologically diagnosed NAFLD, including 4 patients with NASH, who were admitted to the Kyushu University Hospital between 2004 and 2006. As a control, normal liver was also obtained by biopsy from 10 men whose liver function tests and histological findings were completely normal. Real-time RT-PCR was performed as previously reported (9). Total RNA was prepared with Trizol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized from 1.0 μg RNA with GeneAmp™ RNA PCR (Applied Biosystems, Branchburg, NJ, USA) using random hexamers. Real-time RT-PCR was performed using LightCycler-FastStart DNA Master SYBR-Green 1 (Roche, Tokyo, Japan), according to the manufacturer's instructions. The reaction mixture (20 μl) contained LightCycler-FastStart DNA Master SYBR-Green 1, 4 mM $MgCl_2$, 0.5 μM of the upstream and downstream PCR primers, and 2 ml of the first-strand cDNA as a template. The target genes and their primers are shown in Table I. To control for variations in the reactions, all PCRs were normalized against ß-actin expression. All results are shown as the mean ± SEM. Comparisons were made by the Mann-Whitney U test.

Results

Expression of genes related to de novo synthesis and uptake of fatty acids. In the process of fatty acid synthesis, acetyl-CoA carboxylase (ACC) converts acetyl-CoA, an essential substrate of fatty acids, to malonyl-CoA. Fatty acid synthase (FAS) then utilizes both acetyl-CoA and malonyl-CoA to form palmitic acid (C16:0). In NAFLD, the expression of ACC1 and FAS was ~2-fold and 1.5-fold higher, respectively, than that in the normal liver (Fig. 1). Both ACC and FAS are positively regulated by a transcriptional factor, sterol regulatory element-binding protein 1c (SREBP-1c) (1). In NAFLD, SREBP-1c expression was also higher than that in the normal liver (Fig. 1). In addition to *de novo* synthesis, fatty acids in hepatocytes are transferred from serum by adipose differentiation-related protein (ADRP, adipophilin) (10). ADRP expression in NAFLD was 2-fold higher than in the normal liver (Fig. 1).

Gene expression related to ß-oxidation in mitochondria. Carnitine palmitoyltransferase 1a (CPT1a) is a regulatory

Table I. Primers used for analysis for expression of fatty acid metabolism-related genes.

Gene	Forward primer 5' Reverse primer 3'	GenBank™ accession no.
ACC1	GAGGGCTAGGTCTTTCTGGAAG CCACAGTGAAATCTCGTTGAGA	NM-198834
ACC2	GCCAGAAGCCCCCAAGAAAC CGACATGCTCGGCCTCATAG	NM-001093
FAS	AGCTGCCAGAGTCGGAGAAC TGTAGCCCACGAGTGTCTCG	NM-004104
SREBP-1c	GCGGAGCCATGGATTGCAC CTCTTCCTTGATACCAGGCCC	NM-004176
ADRP	GGGATCCCTGTCTACCAAGC AGATGTCGCCTGCCATCACC	NM-001122
CPTIa	TGAGCGACTGGTGGGAGGAG GAGCCAGACCTTGAAGTAGCG	NM-001876
LCAD	GGTGTTCATCAGTAATGGGTCAT CACTGTCTGTAGGTGAGCAACTG	NM-001608
HADHα	GCTAGACCGAGGACAGCAAC CCTGCTTGAGACCAACTGCT	NM-000182
UCP2	CACCAAGGGCTCTGAGCATG TCTACAGGGGAGGCGATGAC	NM-003355
ACOX	TCCTGCCCACCTTGCTICAC TTGGGGCCGATGTCACCAAC	NM-004035
BOX	GGGCATTCCACATCCGGTTG TGGCTCCTGAGCAGATCAGC	NM-003500
CYP2E1	ATGTCTGCCCTCGGAGTGA GATGTCCTTCCAGGTAGGTCC	NM-000773
CYP4A11	AGGAGCTCCAACAGGACCAG CCTGATGGCTGAAGGCACAC	NM-000778
PPARα	CCAGTATTTAGGAAGCTGTCCTG CGTTGTGTGACATCCCGACAG	NM-005036
SOD	AGGCCGTGTGCGTGCTGAAG CACCTTTGCCCAAGTCATCTGC	NM-000454
Catalase	CCTTTCTGTTGAAGATGCGGCG GGCGGTGAGTGTCAGGATAG	NM-001752
GSS	AGAACGCTGCCTTCCTGGAG CAGTAGCACCAGAGCATTGGG	NM-000178
DGAT1	GGCATCCTGAACTGGTGTGTG GAGCTTGAGGAAGAGGATGGTG	NM-012079
PPARγ	GAACAGATCCAGTGGTTGCAG GGCATTATGAGACATCCCCAC	NM-138712
HSL	TACCGCAGCCTAGTGCACAC AGATGGTCTGCAGGAATGGC	NM-005357
ß-actin	GCAAGAGAGGCATCCTCACC CGTAGATGGGCACAGTGTGG	NM-001101

enzyme in mitochondria that transfers fatty acids from the cytosol to mitochondria prior to ß-oxidation. ß-oxidation is then catalyzed by enzymes such as long-chain acyl-CoA dehydrogenase (LCAD) and long-chain L-3-hydroxyacyl-coenzyme A dehydrogenase α (HADHα). In NAFLD, CPT1a expression was decreased by 50%, and expression of LCAD and HADHα was significantly increased 6-fold and 3-fold, respectively, compared with that in the normal liver (Fig. 2). Uncoupling protein 2 (UCP2), a mitochondrial inner-membrane protein is emerging as a potential regulator of mitochondrial reactive oxygen species (ROS) production (11). It mediates a proton leak across the inner membrane

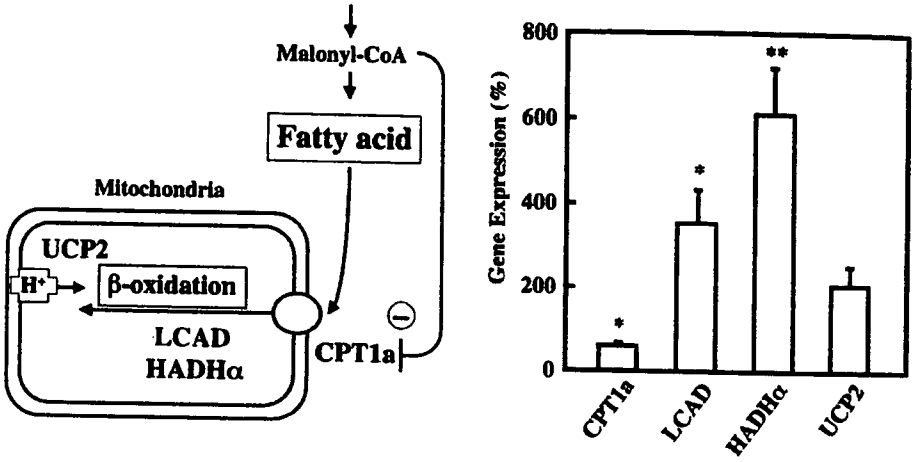

Figure 2. Real time RT-PCR analysis for gene expression of mitochondrial ß-oxidation in NAFLD. *p<0.05 and **p<0.01 indicate statistically significant differences as compared with the normal liver. CPT1a, carnitine palmitoyltransferase 1a; LCAD, long-chain acyl-CoA dehydrogenase; HADHα, long-chain L-3-hydroxyacyl-coenzyme A dehydrogenase α; UCP2, uncoupling protein 2.

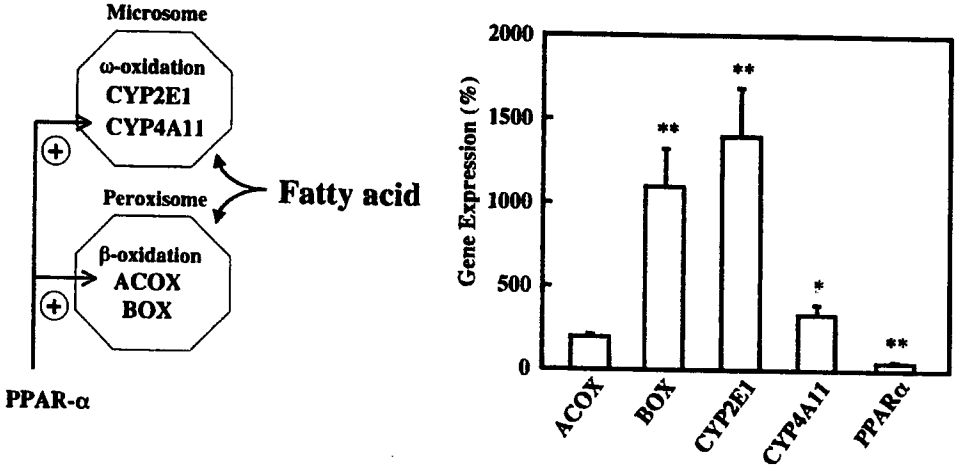

Figure 3. Real time RT-PCR analysis for gene expression of peroxisomal or microsomal oxidation in NAFLD. *p<0.05 and **p<0.01 indicate statistically significant differences as compared with the normal liver. ACOX, straight-chain acyl-CoA oxidase; BOX, branched-chain acyl-CoA oxidase; CYP, cytochrome P450; PPARα, peroxisome proliferator-activated receptor α.

and uncouples fuel oxidation from adenosine triphosphate (ATP) synthesis (12). UCP2 expression in NAFLD was 2-fold higher than in the normal liver.

Expression of other genes related to fatty acid oxidation. When cytosolic fatty acids accumulate due to impairment of oxidative capacity in mitochondria, alternative pathways in the peroxisomes (ß-oxidation) and in microsomes (ω-oxidation) are activated. In peroxisomal ß-oxidation, straight-chain acyl-CoA oxidase (ACOX) and branched-chain acyl-CoA oxidase (BOX) are responsible for the initial oxidation of very-long-chain fatty acyl-CoAs. In NAFLD, the expression of ACOX and BOX was increased 2-fold and 10-fold, respectively, compared with that in the normal liver (Fig. 3). In microsomal ω-oxidation, CYP2E1 and CYP4A11, which are inducible hepatic microsomal cytochrome P-450s, can initiate the autopropagative process of lipid oxidation. In NAFLD, the expression of CYP2E1 and CYP4A11 was significantly higher (14-fold and 4-fold, respectively) than in the normal liver (Fig. 3). Peroxisome proliferator-activated receptor (PPAR)α, a transcriptional factor, up-regulates the expression of a suite of genes that includes peroxisomal and mitochondrial ß-oxidation enzymes as well as CYP4A. In NAFLD, PPARα expression was significantly decreased by 50% compared with that in the normal liver (Fig. 3).

Expression of genes related to antioxidant pathways. ROS are formed during the process of fatty acid oxidation. They are eliminated by antioxidant enzymes such as superoxide dismutase (SOD) and catalase, and by compounds such as glutathione, which is produced by glutathione synthetase (GSS). In NAFLD, the expression of SOD and catalase was increased 5-fold and 10-fold, respectively, compared with that in normal liver, whereas GSS expression was unchanged (Fig. 4).

Expression of genes related to lipid droplet formation. Fatty acids are also metabolized by esterification to produce

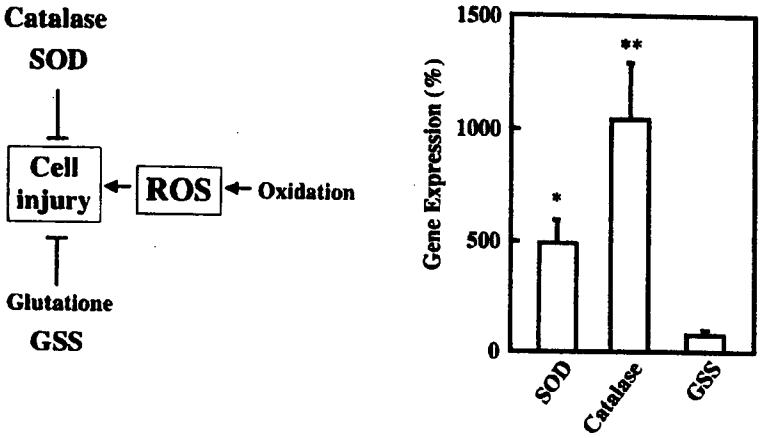

Figure 4. Real time RT-PCR analysis for expression of antioxidation-related genes in NAFLD. *p<0.05 and **p<0.01 indicate statistically significant differences as compared with the normal liver. SOD, superoxide dismutase; GSS, glutathione synthetase; ROS, reactive oxygen species.

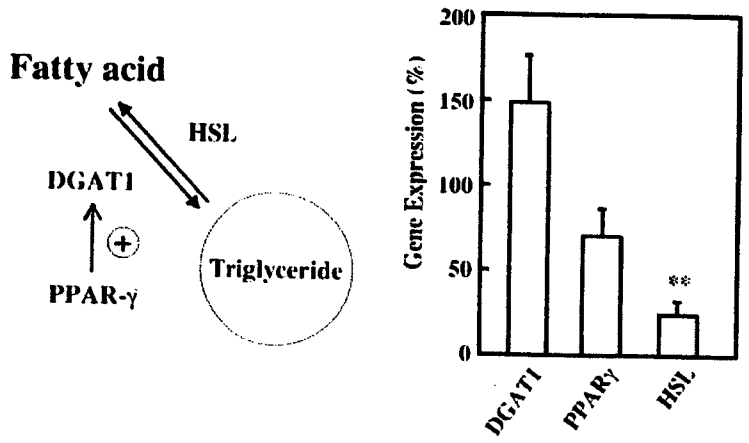

Figure 5. Real time RT-PCR analysis for expression of lipid droplet formation-related genes in NAFLD. **p<0.001 indicates statistically significant difference compared with the normal liver. DGAT1, diacylglycerol O-acyltransferase 1; PPARγ, peroxisome proliferator-activated receptor γ; HSL, hormone-sensitive lipase.

triglycerides, which are stored within hepatocytes as lipid droplets. Diacylglycerol O-acyltransferase 1 (DGAT1) is involved in triglyceride synthesis, and its expression in NAFLD was increased 2-fold compared to that in the normal liver (Fig. 5). Expression of PPARγ, which facilitates the storage of triglycerides in NAFLD, was 1.5 times greater than that of the controls. Hormone-sensitive lipase (HSL) is a key enzyme for catalyzing triglyceride accumulation in the form of lipid droplets. The expression of HSL was decreased by 80% in NAFLD as compared with the normal liver (Fig. 5).

Discussion

We previously reported a study on the expression of genes related to fatty acid metabolism in NAFLD (9). In the present study, we evaluated a wider range of genes and used a greater number of samples from both NAFLD and normal livers. Fatty acid metabolism in hepatocytes can occur by four mechanisms: a) *de novo* fatty acid synthesis and uptake of plasma-free fatty acids; b) fatty acid catalysis by oxidation in mitochondria, peroxisomes, and microsomes; c) neutralization of ROS derived from fatty acid oxidation; and d) conversion between fatty acids and triglycerides.

With respect to *de novo* fatty acid synthesis, the expression of ACC1 and FAS was increased in NAFLD, while expression of ACC2 was not. It has been reported that humans (20) and mice (21) with hepatic steatosis accumulate excess oleic acid (C18:1), the end-product of *de novo* fatty acid synthesis. This evidence, taken together with our results, suggests that fatty acid synthesis rates are increased in NAFLD despite the accumulation of fatty acids. In normal liver, fatty acid synthesis is positively regulated by transcriptional factor SREBP-1c, and in fatty acid overload, *de novo* fatty acid synthesis is suppressed through down-regulation of SREBP-1c (1). In NAFLD, the expression of SREBP-1c was increased 2-fold, indicating that negative feedback regulation via SREBP-1c failed to occur.

In addition to an increase in *de novo* fatty acid synthesis, fatty acid uptake from serum can contribute to accumulation of fatty acids in NAFLD. ADRP has been suggested to play a role in fatty acid transport, although its function is not fully understood. It has been reported that ADRP-knockout mice

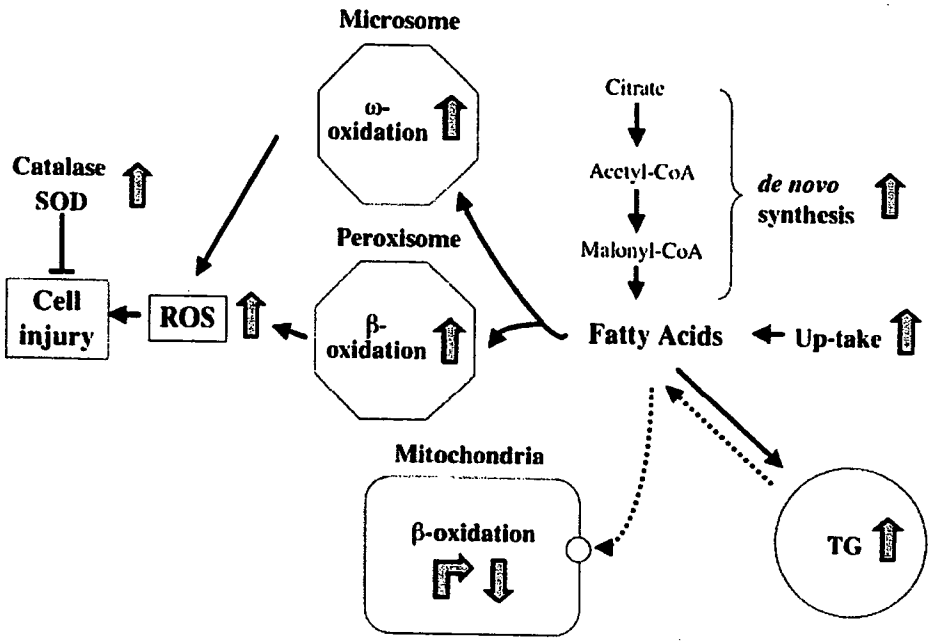

Figure 6. Summary of the present analysis of expression of fatty acid metabolism-related genes in NAFLD.

are resistant to the development of fatty liver (10). In NAFLD, the expression of ADRP was increased 2-fold and our findings are in agreement with the results of immunohistochemical studies in NAFLD by Motomura *et al* (13). It is very intriguing that not only *de novo* fatty acid synthesis was increased but also fatty acid uptake from serum, despite the excess hepatic accumulation of fatty acids in NAFLD.

Oxidation of fatty acids occurs mainly in the mitochondria. In this study, the expression of CPT1a was decreased, whereas that of LCAD, HADHα, and UCP2 was increased. CPT1a is a membrane transporter of fatty acids (acyl-CoA) from the cytoplasm into the mitochondrial matrix and is a primary regulatory enzyme involved in mitochondrial ß-oxidation. The down-regulation of CTP1a expression in NAFLD is attributable to an increase in malonyl-CoA (14), since the expression of ACC1 which converts acetyl-CoA to malonyl-CoA, was increased (Fig. 1). Therefore, it is possible that ß-oxidation in the mitochondria might be reduced in NAFLD. On the other hand, increased expression of LCAD and HADHα suggests enhancement of ß-oxidation. Furthermore, up-regulation of UCP2 expression suggests that excess ROS production occurs by increased ß-oxidation because UCP2 potentially reduces ROS production (15,16). When ß-oxidation reaches maximal levels, a lack of unesterified CoA could inhibit CPT and thereby prevent further entry of acyl groups to the mitochondria, thus serving as an intramitochondrial control on ß-oxidation (17). ß-oxidation in mitochondria was at maximal levels, thereby resulting in decreased expression of CTP1a.

When there is an excess of fatty acids in hepatocytes, alternative pathways of fatty acid oxidation are activated, such as ß-oxidation in peroxisomes and ω-oxidation in the endoplasmic reticulum (microsomes). The peroxisomal acyl-CoA oxidases ACOX and BOX are the first and rate-limiting enzymes of ß-oxidation pathways in peroxisomes (18), and their expression was increased in this study, indicating that peroxisomal ß-oxidation is compensatively enhanced in NAFLD. In the endoplasmic reticulum, ω-oxidation by CYP2E1 and CYP4As occurs (19). We demonstrated that the expression of both CYP2E1 and CYP4A11 was up-regulated in NAFLD. Increased activity of CYP2E1 in the liver is associated with factors commonly observed in NAFLD; obesity, diabetes, and hyperlipidemia. CYP4As are also assumed as key intermediaries in adaptive responses to the perturbations of hepatic lipid metabolism that accompany fasting, diabetes, and overnutrition. Recent evidence obtained from CYP2E1-null mice demonstrates that there is a compensatory increase in CYP4A activity (20). Therefore, in NAFLD, situations where CYP2E1 is fully activated may lead to increased CYP4A11 expression. On the whole, it appears that accumulation of fatty acids in NAFLD enhances oxidation not only in mitochondria but also in peroxisomes and microsomes.

PPARα is a major transcriptional activator of genes involved in mitochondrial ß-oxidation, such as peroxisomal ACOX and microsomal CYP4As (18,21). In our study, the expression of PPARα was unexpectedly reduced in NAFLD. When we compared the expression of ACOX to that of BOX, which is not regulated by PPARα, we found that the enhancement of expression of ACOX was less than that of BOX (2-fold vs. 10-fold, respectively) (Fig. 3). Similarly, CYP4A11 showed less enhancement of expression than did CYP2E1 (4-fold vs. 14-fold, respectively). The difference in the expression of oxidation enzymes in peroxisomes and microsomes might be attributable to the decreased expression of PPARα. We initially found that HOMA-IR, an index of insulin resistance, was negatively correlated with PPARα expression, suggesting that the decreased expression of PPARα may be attributable to insulin resistance, which often accompanies NAFLD. Recent studies have demonstrated that

PPARα agonists reduce hepatic steatosis in animal models (22,23). Further study will be needed to clarify the mechanism of down-regulation of PPARα and the effects of PPARα activation as a treatment for NAFLD.

We investigated the expression of genes related to anti-oxidant pathways including SOD, catalase, and GSS, because it was expected that ROS overproduction would occur as a result of enhanced mitochondrial and peroxisomal ß-oxidation and microsomal ω-oxidation, as described above. As we expected, the expression of SOD and catalase was dramatically enhanced. In contrast, the expression of GSS, which produces glutathione, was unchanged. Although we do not know precisely why GSS levels were unchanged, the antioxidant effects of glutathione can also be regulated by glutathione peroxidase, which, together with glutathione, neutralizes ROS.

Excess lipid droplet formation in NAFLD is indicative of increased triglyceride synthesis in hepatocytes, and in the present study, we observed increased expression of DGAT1. The expression of PPARγ, which is a transcriptional factor that facilitates adipogenesis by various mechanisms including induction of DGAT1 expression, was unexpectedly unchanged in NAFLD. It has been reported that the expression of PPARγ is markedly increased in fatty liver (24). Conversely, adipogenesis resulting in triglyceride storage occurs under conditions where there is a decrease in PPARα activity and fatty acid oxidation (21), implying that cross-talk occurs between PPARγ and PPARα. In this study, the expression of both PPARγ and PPARα was decreased, suggesting that the cross-talk between these receptors might be impaired in NAFLD. Expression of HSL was also greatly down-regulated, indicating that lipolysis, in contrast to lipogenesis, is inhibited in NAFLD. Further study is needed to clarify the mechanisms that regulate expression of HSL and protein kinase A, which is a major regulator of HSL expression (25).

In summary, our results in patients with NAFLD indicate that: i) *de novo* synthesis of fatty acids is increased, although fatty acids have already been accumulated in hepatocytes, and is accompanied by increased fatty acid uptake from serum; ii) mitochondrial fatty acid oxidation is decreased or fully activated to improve fatty acid accumulation; iii) in order to complement the function of mitochondria (ß-oxidation), peroxisomal (ß-oxidation) and microsomal (ω-oxidation) oxidation is up-regulated; iv) antioxidant pathways including SOD and catalase are enhanced to neutralize overproduced ROS by enhanced oxidation; and v) lipid droplet formation is enhanced (Fig. 6). Four cases of histologically proven NASH were included in the present study, and the gene expression profiles did not differ between patients with NASH and those with NAFLD (data not shown). Since ROS are believed to be a major cytotoxic factor in NASH (19,26,27), it is assumed that uncompensated ROS overproduction due to enhanced oxidation might lead to the transition from simple obesity to NASH, a condition in which excessive ROS production can cause mitochondrial failure leading to apoptosis and oncosis (necrapoptosis) (28-30).

Eleven of the NAFLD patients in this study, who were candidates as donors for liver transplantation received a strict low-calorie diet, exercise, and 400 mg/day bezafibrate (a ligand of PPARα) for 4-8 weeks prior to the operations (31). We found that this treatment normalized dysfunctional expression of genes related to fatty acid metabolism, i.e. ACC1 and FAS expression were decreased and CTP1a and PPARα expression were increased (data not shown). Therefore, treatments that target the expression of fatty acid metabolism-related genes may be beneficial in NAFLD.

Finally, as discussed above, several disorders of fatty acid metabolism were recognized in NAFLD, and we assume that unregulated enhancement of *de novo* fatty acid synthesis is a primary disorder in NAFLD. Fatty acid synthesis by ACC1 and FAS is tightly regulated by SREBP-1c and its expression is also regulated negatively by AMP-activated protein kinase (AMPK) and positively by insulin (1,32). Obesity, which is often accompanied by NAFLD, causes decreasing serum levels of adiponectin and increasing levels of TNFα. It has been reported that decreased adiponectin and/or increased TNGα activity results in decreased SREBP-1c expression (33,34). Insulin resistance, a condition in which insulin-signaling pathways are suppressed, was also commonly observed in NAFLD which presumably caused a decrease in SREBP-1c expression. We are now investigating AMPK expression and insulin-receptor substrates which are key molecules in the insulin signaling cascade affecting lipid metabolism (35).

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Effects of lamivudine on serum albumin levels correlate with pretreatment HBV-DNA levels in cirrhotic patients

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Abstract

Background: Lamivudine treatment has been recently demonstrated to increase the serum albumin levels in cirrhotic patients with hepatitis B virus (HBV) infection, but the precise mechanism remains unclear. We hypothesized that the improvement of hypoalbuminemia by lamivudine may be attributable to the reduction of HBV replication itself, rather than to cessation of hepatitis. In order to confirm this hypothesis, in this study we evaluated factors which correlated with the increase in serum albumin levels. Fifty-four patients (Child-Pugh A/B/C, 35/9/10) with HBV-related liver cirrhosis who had been treated with lamivudine for more than 12 months were evaluated. We analyzed the correlation between the increase in serum albumin levels at month 12 after starting treatment (Δ-albumin) and various pretreatment variables. We also analyzed the correlation between Δ-albumin and the reduction in serum levels of HBV-DNA (Δ-HBV-DNA) or alanine aminotransferase (Δ-ALT) at month 12.

Results: The average Δ-albumin was 0.38 g/dL and only serum HBV-DNA levels before treatment correlated significantly with Δ-albumin. We also analyzed the correlation in patients whose alanine aminotransferase levels were normalized after 12 months so that the possible influence of breakthrough hepatitis could be excluded. Even among this subgroup of patients, there was no significant correlation between Δ-albumin and either pretreatment alanine aminotransferase

levels or Δ-ALT. In contrast, in patients whose serum HBV-DNA was undetectable at month 12, we found a significant correlation between Δ-albumin and both pretreatment serum HBV-DNA levels and Δ-HBV-DNA.

Conclusion: Our results demonstrated that albumin levels are associated with pretreatment HBV-DNA but not with alanine aminotransferase levels.

Background

Chronic hepatitis B is an important cause of morbidity and mortality resulting from cirrhosis-related liver failure and hepatocelluar carcinoma (HCC) [1-3]. Lamivudine, a nucleoside analogue with potent antiviral effects against hepatitis B virus (HBV), has been shown to be effective both in patients with chronic hepatitis and also those with liver cirrhosis [4-6]. In cirrhotic patients, decreased HBV-DNA loads following lamivudine treatment result in decreased serum levels of alanine aminotransferase (ALT), increased serum albumin levels, and improvement of the Child-Pugh score [7-13]. The underlying mechanism for the increase in albumin levels after lamivudine treatment has not been determined. It has been suggested that the improvement of hypoalbuminemia may be attributable to the cessation of hepatic inflammation. However, earlier treatments such as glycyrrhizin, ursodeoxycholic acid [14,15], predonisolone [16], and Stronger Neo-Minophagen C therapy [17], all of which reduce ALT levels in viral cirrhotic patients, do not result in improvement of hypoalbuminemia. Furthermore, it has been shown that there is no significant correlation between serum ALT levels and HBV-DNA loads in patients with HBV [18-20]. We hypothesized that the improvement of hypoalbuminemia by lamivudine may be attributable to the reduction of HBV replication itself, rather than to cessation of hepatitis. In order to confirm this hypothesis, we evaluated several laboratory parameters in cirrhotic patients treated with lamivudine that could influence serum albumin levels.

Results

Fifty-four cirrhotic patients with HBV infection were analyzed (Table 1, see Materials and methods). Before the treatment, there was no significant correlation between either serum ALT or albumin levels and HBV-DNA loads in our patients (data not shown). Following lamivudine treatment, the levels of HBV-DNA and ALT rapidly decreased while albumin levels simultaneously increased (Figure 1). HBV-DNA levels decreased significantly from 6.59 ± 0.18 log copies/mL to 2.98 ± 0.12 log copies/mL at 3 months after treatment ($p < 0.01$), and decreased further to 2.87 ± 0.14 log copies/mL and 2.94 ± 0.18 log copies/mL at 6 and 9 months, respectively. Similarly, ALT levels also decreased significantly from 102.1 ± 10.4 U/L to 42.0 ± 2.7 U/L at 3 months after treatment ($p < 0.01$), and to 38.8 ± 4.1 U/L and 33.1 ± 2.4 U/L at 6 and 9 months, respectively. However, at 12 months there was a slight increase in both HBV-DNA and ALT levels (3.17 ± 0.21 log copies/mL and 44.3 ± 8.6 U/L, respectively), although the differences between values at 9 and 12 months were not statistically significant. The serum levels of albumin increased from 3.56 ± 0.09 g/dL to 3.76 ± 0.08 g/dL at 3 months after treatment, and increased further to 3.89 ± 0.08 g/dL ($p < 0.05$) and 3.95 ± 0.08 ($p < 0.01$) g/dL at 6 and 9 months, respectively. At 12 months, albumin levels remained steady at 3.94 ± 0.08 g/dL.

To identify the factors associated with increased serum albumin levels, correlations between the increase in serum albumin levels at 12 months after the start of treatment (Δ-albumin) and basic variables before treatment were examined using the data for all patients. In this anal-

Table 1: Characteristics of the patients

	Child A	Child B	Child C	Total
n	35	9	10	54
Male/female	26/9	7/2	5/5	38/16
Age	53.0 ± 9.1	54.9 ± 4.6	49.5 ± 9.1	52.6 ± 8.8
Albumin (g/dL)	3.85 ± 0.43	3.12 ± 0.38	2.94 ± 0.57	3.56 ± 0.6
Bilirubin (mg/dL)	0.90 ± 0.43	1.25 ± 0.35	3.09 ± 1.28	1.37± 1.07
ALT (U/L)	118.2 ± 125.5	62.7 ± 43.2	80.6 ± 96.8	102.0 ± 113.0
Platelet (× 10^4/μL)	11.8 ± 5.3	7.3 ± 3.2	6.3 ± 2.5	10.0 ± 5.2
HBeAg (+/-)	17/18	6/3	6/4	29/25
HBV-DNA (log copies/mL)				
< 5.0	1	1	2	4
5.0 ≤ x < 7.0	21	4	3	28
≥ 7.0	13	4	5	22