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 (β-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

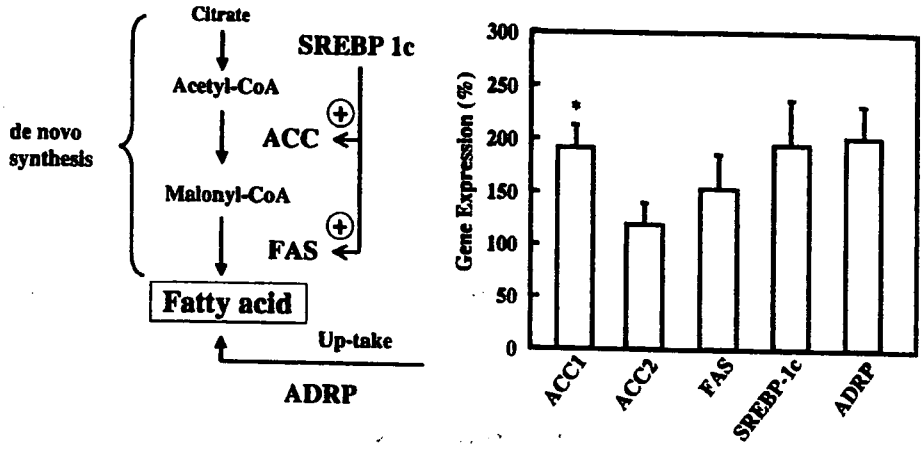

Figure 1. Real-time RT-PCR analysis for gene expression of *de novo* synthesis and uptake of fatty acids in NAFLD. *p<0.05, a statistically significant difference as compared with the normal liver. ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; SREBP-1c, sterol regulatory element-binding protein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

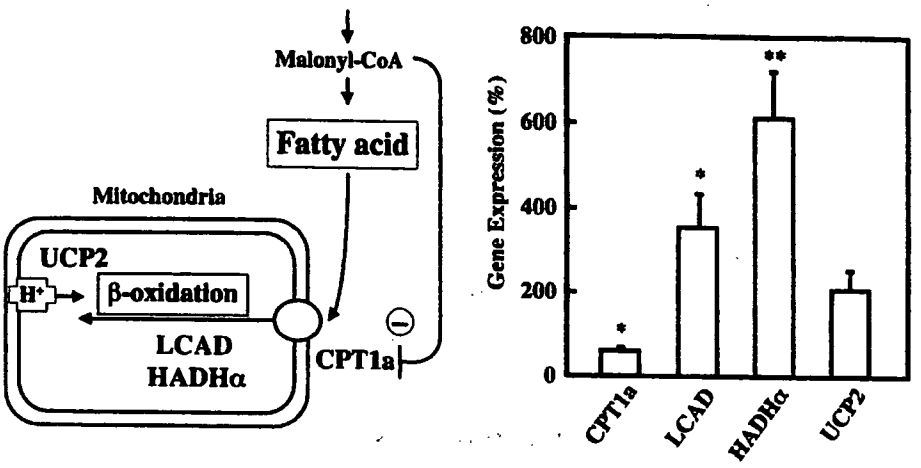

Figure 2. Real time RT-PCR analysis for gene expression of mitochondrial ß-oxidation in NAFLD. *p<0.05 and **p<0.01 indicate statistically significant differences as compared with the normal liver. CPT1a, carnitine palmitoyltransferase 1a; LCAD, long-chain acyl-CoA dehydrogenase; HADHα, long-chain L-3-hydroxyacyl-coenzyme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

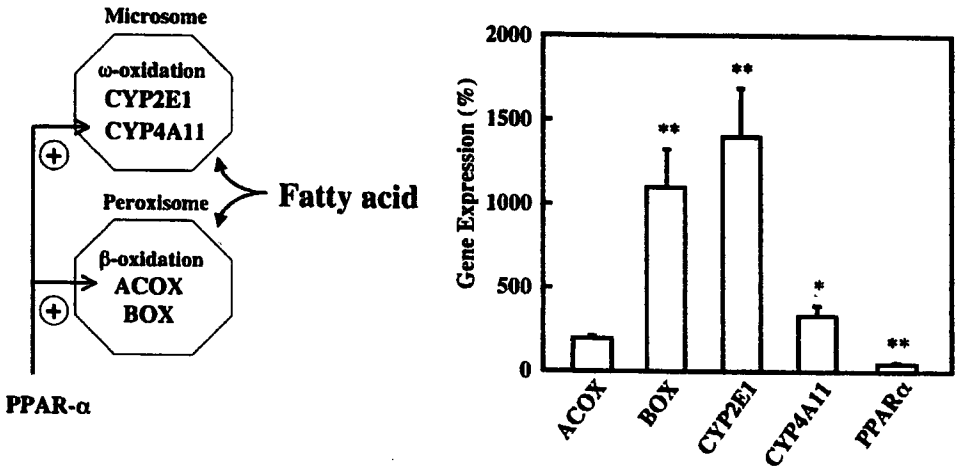

Figure 3. Real time RT-PCR analysis for gene expression of peroxisomal or microsomal oxidation in NAFLD. *p<0.05 and **p<0.01 indicate statistically significant differences as compared with the normal liver. ACOX, straight-chain acyl-CoA oxidase; BOX, branched-chain acyl-CoA oxidase; CYP, cytochrome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

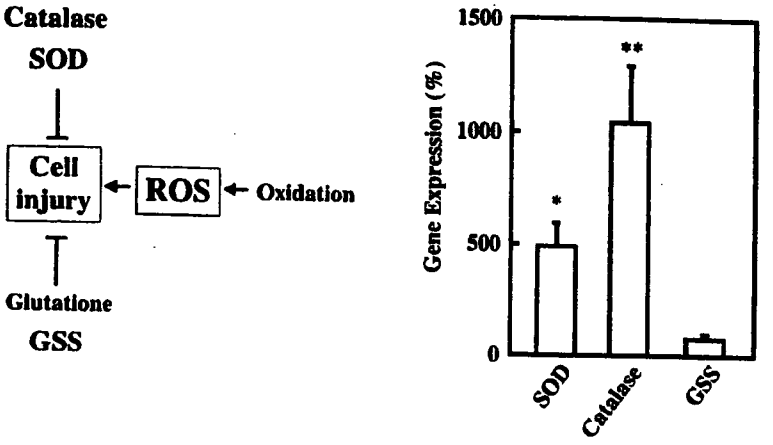

Figure 4. Real time RT-PCR analysis for expression of antioxidation-related genes in NAFLD. *p<0.05 and **p<0.01 indicate statistically significant differences as compared with the normal liver. SOD, superoxide dismutase; GSS, glutathione synthetase; ROS, reactive oxygen species.

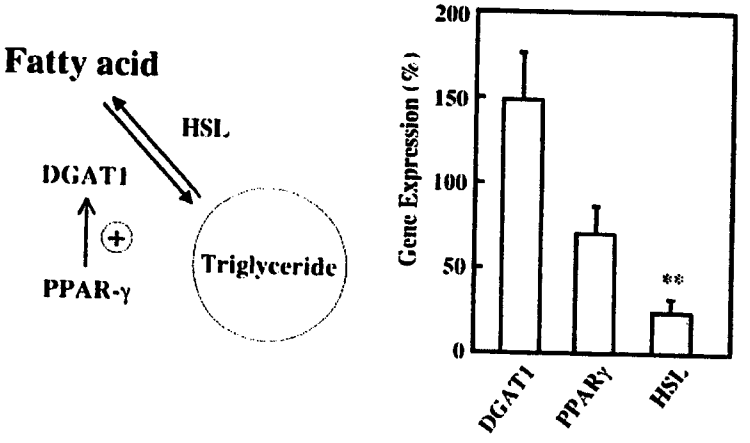

Figure 5. Real time RT-PCR analysis for expression of lipid droplet formation-related genes in NAFLD. **p<0.001 indicates statistically significant difference compared with the normal liver. DGAT1, diacylglycerol O-acyltransferase 1; PPARγ, peroxisome proliferator-activated receptor γ; HSL, hormone-sensitive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

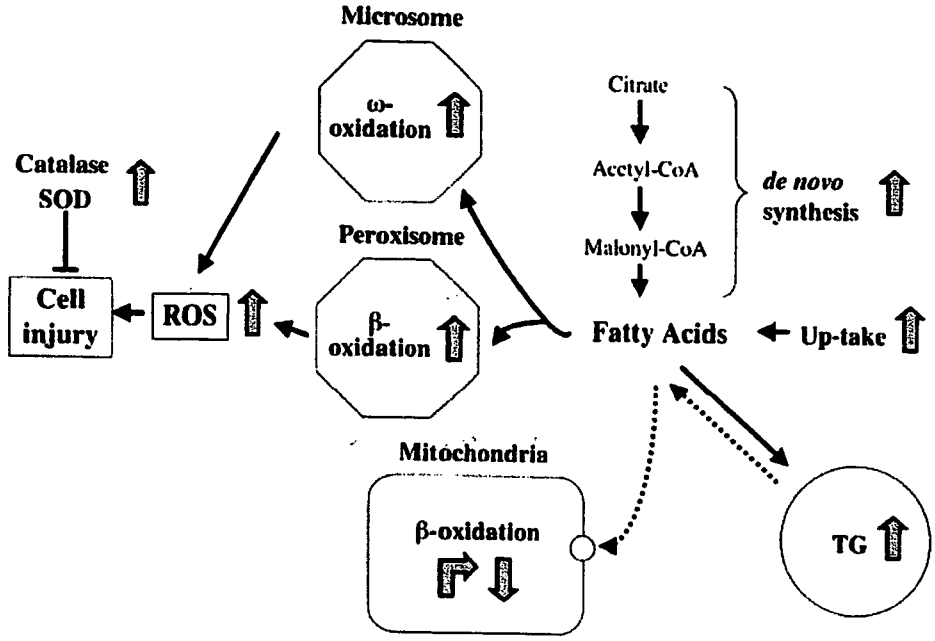

Figure 6. Summary of the present analysis of expression of fatty acid metabolism-related genes in NAFLD.

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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Comparative Hepatology

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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

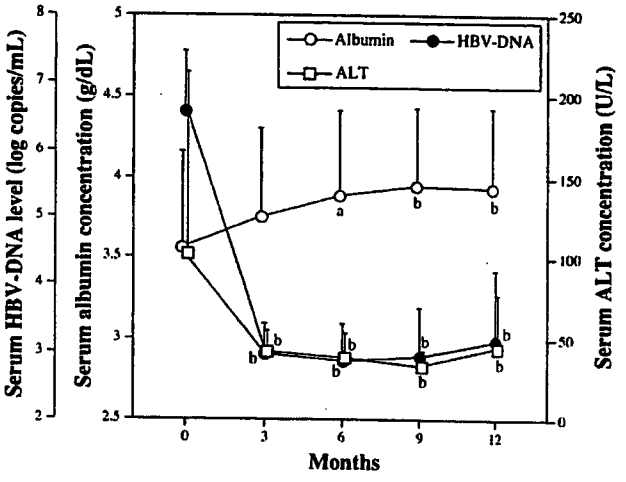

Figure 1
Time course of albumin, HBV-DNA, and ALT levels in lamivudine treatment. The average serum levels of albumin (closed circles), HBV-DNA (open squares), and ALT (open circles) at 3-month intervals from the start of lamivudine therapy are plotted. Soon after the start of treatment, serum albumin levels increased rapidly and simultaneously with a decrease in HBV-DNA and serum ALT levels. The data represent mean + SD (a, b; $p < 0.05$ and $p < 0.01$ vs. 0 month, respectively).

ysis, only HBV-DNA load correlated significantly with Δ-albumin ($t = 2.66$, $r^2 = 0.120089$, $p = 0.0103$), whereas age, sex, HBeAg, ALT, bilirubin, platelet count, and Child-Pugh classification did not (Table 1).

Although we found no correlation between Δ-albumin and pretreatment serum ALT levels for the entire patient population, the possibility remained that breakthrough hepatitis or continuous elevation of ALT might interfere with Δ-albumin. Indeed, two patients showed breakthrough hepatitis, where ALT levels increased to over 100 U/L, and 20 patients still showed abnormally high ALT (> 35 U/L) at 12 months after treatment. We next evaluated the correlation between Δ-albumin and pretreatment serum ALT levels among the 32 patients in whom serum ALT levels were normalized (< 35 U/L) at 12 months after the start of therapy. As shown in Figure 2A, there was no significant correlation between Δ-albumin and pretreatment serum ALT levels in this subgroup of patients ($r = 0.083$, $p = 0.64$). We also evaluated the correlation between Δ-albumin and reduction in ALT levels at month 12 after starting treatment (Δ-ALT) in this group, but there was still no significant correlation between Δ-albumin and Δ-ALT ($r = 0.0685$, $p = 0.67$) (Figure 2B).

Furthermore, we evaluated the correlation between Δ-albumin and serum HBV-DNA levels before treatment

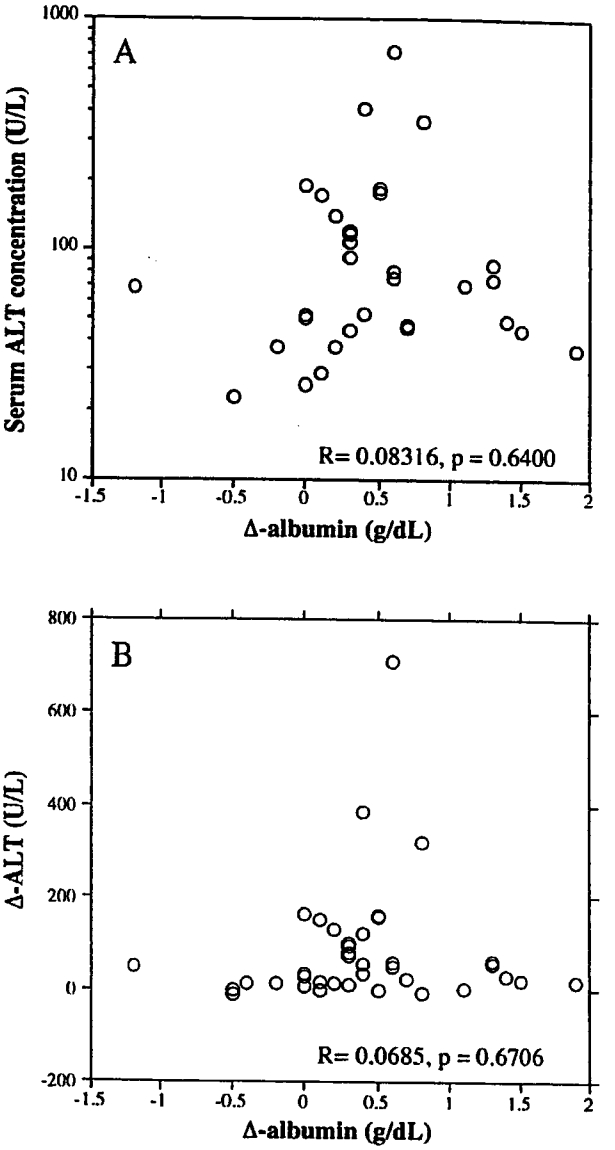

Figure 2
Correlation between ALT levels before treatment and Δ-albumin (A), and Δ-ALT and Δ-albumin (B). In patients whose serum ALT levels were normalized at 12 months after treatment, there was no significant correlation Δ-albumin and pretreatment serum ALT levels (A). There was also no significant correlation Δ-albumin and Δ-ALT (B).

among the 41 patients in whom serum HBV-DNA levels were undetectable at 12 months post-treatment. In this analysis, we found a significant correlation between Δ-albumin and the serum levels of HBV-DNA before the start of therapy ($r = 0.42459$, $p < 0.0001$) (Figure 3A). We also evaluated the correlation between Δ-albumin and reduction in HBV-DNA levels at month 12 after starting

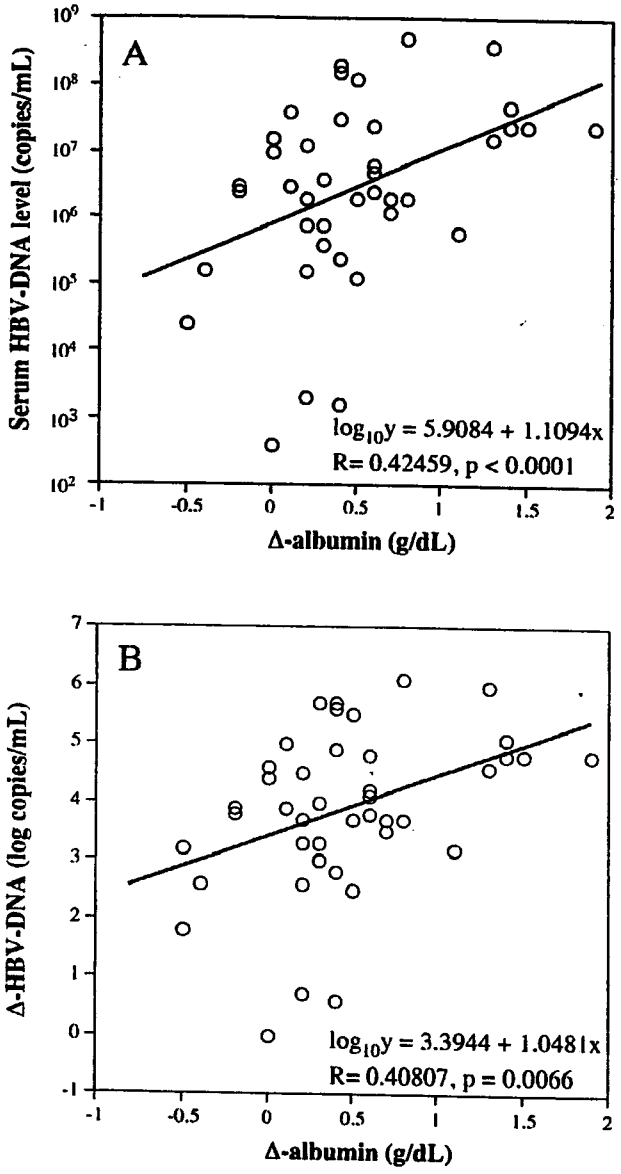

Figure 3
Correlation between HBV-DNA levels before treatment and Δ-albumin (A), and Δ-HBV-DNA and Δ-albumin (B). Patients whose serum HBV-DNA was undetectable at 12 months after treatment, there was a significant correlation between Δ-albumin and both pretreatment serum HBV-DNA levels (A) and Δ-HBV-DNA (B).

treatment (Δ-HBV-DNA) in this group, and we again found that Δ-albumin significantly correlated with Δ-HBV-DNA ($r = 0.40807$, $p = 0.0066$) (Figure 3B).

Discussion

This study demonstrated the followings: 1) HBV-DNA, but not ALT levels, before lamivudine treatment was associated with increased serum albumin levels at 12 months after treatment (Δ-albumin); 2) Even among those patients who showed cessation of hepatitis following treatment, there was no correlation between either pretreatment ALT levels or Δ-ALT and Δ-albumin; 3) In contrast, in the analysis of subjects with undetectable HBV-DNA levels after treatment, there was significant correlation between both pretreatment HBV-DNA levels and Δ-HBV-DNA and Δ-albumin. Taken together, these results suggest that the improvement of hypoalbuminemia following lamivudine treatment is attributable to a reduction of HBV replication, but not to cessation of hepatitis.

We do not deny the idea that cessation of hepatitis, which is represented by lowering of serum ALT levels, contributed to and increase of serum albumin levels. In true, we think that replicative HBV and inflammation are closely related; however, in our study, HBV reduction statistically showed more effect improving serum albumin levels than decreasing the inflammation marker ALT. This may happen perhaps because, in cirrhotic patients, fibrosis is the main pathological change (compared with inflammation), and the correlation between, on the one hand, serum albumin or HBV-DNA levels and, on the other hand, ALT levels was in some degree weakened as the cirrhotic change proceed. Therefore, in cirrhotic patients, Δ-ALT is within a narrower range and ALT levels cannot influence albumin levels significantly.

How does lowering of HBV load induces the increase of albumin levels in an inflammation-independent manner? Hui et al. [7] recently showed that emergence of phenotypic resistance of HBV-DNA was associated with a rapid decline in serum albumin levels following prolonged lamivudine treatment, although they did not report whether a correlation existed between serum ALT levels and serum HBV loads. In a series of studies in woodchucks and Hep G2 cells, Kosovsky et al. demonstrated that HBV replication inversely correlated with cell proliferation and DNA synthesis by hepatocytes [21-23]. Yang et al. has analyzed gene expression profiles of HepG2 cells with or without HBV [24]. However, whether HBV replication directly influences the ability of infected hepatocytes to synthesize protein is still unclear and further studies are needed.

Our results indicate that increased serum albumin levels should be expected in cirrhotic patients following lamivudine treatment, and that this occurs independently of serum ALT levels and Child-Pugh's score before treatment, as shown by the lack of a correlation between those variables and Δ-albumin. Previous studies of lamivudine treatment for liver cirrhosis showed that fatalities occur because of acute liver failure after discontinuation of lamivudine [25,26] or emergence of lamivudine-resistance mutants [27,28]. Recent reports, however, indicate that

Table 2: Correlations between Δ-albumin and basic variables before treatment

	t	R^2	P-value
Age	-0.14	0.000398	0.8873
ALT	0.67	0.008536	0.5064
Bilirubin	-0.04	0.000036	0.9659
Platelet	-0.87	0.014279	0.3894
HBV-DNA	2.66	0.120089	0.0103
HBeAg (+/-)	-	-	0.6201
Sex (male/female)	-	-	0.4251
Child-Pugh's classification	-	-	0.0968

prolonged use of lamivudine for cirrhotic patients is safe and effective [5,29,30]. Furthermore, since adefovir is effective for treating resistant mutants [31-33], lamivudine therapy should be encouraged. Hypoalbuminemia, which causes ascites, edema, and hydrothorax, lowers the quality of life of cirrhotic patients [34,35]. High viral load of HBV is associated with higher mortality and morbidity in cirrhotic patients in consequence of high occurrence or recurrence rate of HCC [36,37]. Lamivudine is effective for preventing or delaying occurrence of liver failure and HCC through lowering HBV, and therefore can be a first choice drug for patients with high HBV levels regardless of serum ALT levels.

Methods

Patients

A total of 54 cirrhotic patients with HBV infection were evaluated, including 38 males and 16 females, ranging in age from 28 to 71 years (mean 52.6 years) (Table 1). Informed consent was obtained from each patient prior to their entering the study. Liver cirrhosis was diagnosed based on liver biopsy (n = 11), laboratory data, ultrasonography, and/or computed tomography. Patients were classified as Child-Pugh class A, B and C (35, 9, and 10 patients, respectively). For all patients, the existence of serum HBV-DNA was confirmed by TMA assay ($10^{3.7}$–$10^{8.7}$ genome equivalents/mL; 3.7–8.7 log genome equivalents [LGE]/mL) (Chugai Diagnostic Science, Tokyo, Japan) or by a Roche Monitor kit ($10^{2.6}$–$10^{7.6}$ copies/ml; 2.6–7.6 log copies/mL) (Roche Diagnostics, Tokyo, Japan) before treatment. HBe-Ag was positive in 29 patients and negative in 25 patients. Patients with fatty liver, viral hepatitis C, a history of alcohol abuse, or autoimmune disorders such as autoimmune hepatitis and primary biliary cirrhosis were excluded. None of the patients had a prior history of treatment for hepatocellular carcinoma.

Patients had been treated with lamivudine (100 mg, once a day) without interruption for more than twelve months at Kyushu University Hospital and its affiliated hospitals. Basic laboratory data, such as platelet counts, serum ALT levels, bilirubin, albumin, serum HBV-DNA load (Roche Monitor kit: Roche Diagnostics) and HBe-Ag were determined at least every 3 months.

Statistical analysis

Data are expressed as mean ± SD, and statistical comparisons were performed using chi-squared test for categorical data and one-way ANOVA for numeric data. In cases where the serum HBV-DNA load was less than 2.6 log copies/mL, it was entered as 2.6 log copies/mL. For the analysis of correlations between two continuous variables, a simple regression model was used. For the analysis of discontinuous variables, such as sex and HBe-Ag, statistical differences were confirmed using Mann-Whitney U test or Kruskal-Wallis test.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

MN and ME participated in the experimental design and writing of the manuscript. JH participated in the experimental design. KK performed most of the analysis. YT, EK, JS, AM, TM, NF, HN, HS, KT, KA, and SS collected and supplied the clinical data of patients.

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Original Paper

Fertile Females with Nonalcoholic Fatty Liver Disease (NAFLD) have Higher Levels of ALT than Postmenopausal Females: Implications for the Influence of Fertility on NAFLD

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KEY WORDS:
Nonalcoholic fatty liver disease (NAFLD); Nonalcoholic steatohepatitis (NASH); Insulin resistance; Sex steroids; Estrogen; Fertility; Postmenopause

ABBREVIATIONS:
Nonalcoholic Steatohepatitis (NASH); Nonalcoholic Fatty Liver Disease (NAFLD); Cytochrome P450 2E1 (CYP2E1); Body Mass Index (BMI); C-peptide (CPR); Fasting Blood Sugar (FBS)

ABSTRACT

Background/Aims: Insulin resistance recently has been reported to play a major role in nonalcoholic fatty liver disease (NAFLD). We evaluated the influence of fertility on fatty liver injury in fertile and postmenopausal women with insulin resistance.

Methodology: We investigated 152 patients with noninsulin-dependent diabetes mellitus without insulin treatment; 46 males, 52 fertile women and 54 postmenopausal women. All had liver damage and/or steatosis recognized by ultrasonography. We measured the fasting serum levels of C-peptide and insulin, as markers of insulin resistance, and the serum levels of ALT. The severity of liver steatosis was judged by ultrasonography.

Results: Fertile females had significantly higher levels of ALT and demonstrated a more significant correlation between serum levels of ALT and C-peptide or insulin than did the postmenopausal females or males. Fertile females with moderate to severe steatosis had significantly higher levels of ALT than those with mild or no steatosis, although such a significant difference was not found in postmenopausal females or males.

Conclusions: We demonstrate that fertility is an important factor in fatty liver damage of NAFLD with insulin resistance, suggesting that estrogen may exacerbate nonalcoholic steatohepatitis.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is a worldwide disorder with the potential for progression to cirrhosis, and is generally associated with obesity, diabetes, hypertension and hyperlipidemia (1,2). The progression from simple fatty liver to more severe forms of NAFLD is known as nonalcoholic steatohepatitis (NASH), the histological findings of which are very similar to those of alcoholic liver injury (3). It has been determined that the patients with NAFLD or NASH have insulin resistance and hyperinsulinemia (3,4). Increased serum insulin induces the lipolysis of adipose tissue and elevates the serum levels of free fatty acid (FFA) (3). Therefore, many investigators accepted the hypothesis that the liver injury is caused by the oxidative stress, which usually develops through the mitochondrial oxidation (β-oxidation) of excessive FFA. Unexpectedly, it was revealed recently that β-oxidation and the mitochondrial respiratory chain are impaired (5,6), although whether mitochondrial impairment is a result of enhanced β-oxidation (excess radical production) is not clear. In contrast, several reports have shown that hepatic cytochrome P450 2E1 (CYP2E1) activity was significantly enhanced in patients with NASH, as well as with alcoholic liver disease, and this enhancement also may cause production of cytotoxic radicals (3,7,8). However, it is also the case that many people with both fatty liver and hyperinsulinemia maintain normal liver function (9). This discrepancy suggests that other additional factors can influence the progression of NAFLD.

In the process of searching for potential factors, we noted the uneven gender distribution among patients with NASH (10,11). Most earlier reports showed a high prevalence of female in NASH, indicating that female sex steroids may promote NASH. If estrogen is a key factor for NAFLD or NASH progression, liver injury in fertile women should be greater than in postmenopausal women, although estrogen is well known as an anti-oxidant (12). In this study, to clarify the influence of estrogen on NAFLD, we compared the de-

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gree of the liver injury in fertile and postmenopausal patients. In this study, we investigated the patients with diabetes mellitus complicated with NAFLD and aimed to clarify the influence of gender or menopausal status on the degree of liver injury.

METHODOLOGY

Patients and Methods

Between 1998 and 2003, 282 patients with diabetes mellitus (160 males and 132 females) were hospitalized in our department, Kyushu University Hospital. Patients with insulin dependent diabetes mellitus (4 males and 6 females), habitual alcohol drinkers (105 males and 8 females), patients with chronic viral hepatitis and patients with complications of cancer were excluded. Three pregnant women and five fertile patients with abnormalities of ovulation also were excluded. The remaining 46 males and 106 females (52 fertile and 54 postmenopausal women) were investigated in this study. The body mass index (BMI) was calculated using the height and the weight on the day of admission. The serum levels insulin or C-peptide (CPR), or both, were measured after overnight fasting within five days of admission. On the same day, the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), alkaline phosphatase (ALP), fasting blood sugar (FBS), total cholesterol, triglyceride, albumin and HbA1c also were measured.

Although abdominal ultrasonography was performed for all patients, the results of five patients were excluded from the analysis because they underwent the examination over seven days after admission. The severity of fatty liver on ultrasonography was judged by two expert examiners as follows: When the echo level of the liver was elevated compared with that of the kidney cortex, the patient was diagnosed as having fatty liver. If the wall of the portal vein at the umbilical portion provided a clear image, the disease was classified as mild. When the wall of portal vein was partially or wholly undetectable at the same position, according to the echo level elevation of liver parenchyma, the disease was classified as moderate or severe fatty liver, respectively.

Statistical Analysis

Data of the basal characteristics of the patients are presented as mean ± standard deviation (SD). Student's unpaired *t* test was used to compare the pairs of subgroups. Sheffe's F test following the analysis of variance (ANOVA) was used to analyze the influence of the severity of steatosis on liver injury. Stepwise multiple linear regression analysis was used to investigate independent variables that might influence the serum levels of ALT. Independent variables tested included BMI, CPR, insulin, FBS, cholesterol, triglyceride and HbA1c. Stepwise multiple linear regression analysis was performed for each subgroup, males, females, fertile women and postmenopausal women.

RESULTS

When we compared the laboratory data concerning liver function and glucose tolerance between male and female patients, there was no significant difference except for the serum levels of albumin (**Table 1**). The comparisons of serum levels of cholesterol and triglyceride, and BMI, also showed no significant differences between the genders. Upon dividing the female patients into fertile and postmenopausal groups, the serum levels of ALT in fertile females were significantly higher than those in postmenopausal females, while the FBS was significantly lower (**Table 2**). Although the difference was not significant, the results

TABLE 1 Characteristics of the Patients

	Male	Female	All
Number	46	106	152
Age	54.5±14.7	49.3±14.4	50.9±14.6
BMI (kg/m²)	25.1±6.6	26.5±6.5	26.1±6.5
AST (U/L)	20.9±9.7	25.0±16.2	23.8±14.7
ALT (U/L)	26.4±23.8	31.4±27.3	29.9±26.3
γ-GTP (U/L)	37.2±19.6	37.5±31.9	37.4±28.7
ALP (U/L)	242.3±66.5	240.0±91.8	240.4±84.8
Cholesterol (mg/dL)	205.4±46.0	213.7±40.7	211.3±42.3
Triglyceride (mg/dL)	183.4±125.0	162.1±100.8	168.6±108.7
FBS (mg/dL)	184.4±75.6	167.7±66.6	172.7±69.6
Albumin (g/dL)	3.9±0.6	4.1±0.4*	4.1±0.5
HbA1c (%)	9.2±2.2	8.6±2.2	8.8±2.2
CPR (ng/mL)	1.9±1.3	2.2±1.3	2.1±1.3
Insulin (μU/mL)	8.3±8.8	11.3±8.5	10.4±8.6
Steatosis on US			
Normal	19	18	37
Mild	13	43	57
Moderate	9	31	40
Severe	3	10	13

*$p<0.05$ *vs.* male.

TABLE 2 Characteristics of the Patients, Comparing Fertile and Postmenopausal Women

	Fertile women	Postmenopausal women
Number	52	54
Age	38.3±10.8	60.0±7.9**
BMI (kg/m²)	26.5±6.9	26.6±6.1
AST (U/L)	28.1±21.3	22.1±8.0
ALT (U/L)	37.4±34.6	25.7±15.9*
γ-GTP (U/L)	40.9±37.3	34.3±25.5
ALP (U/L)	221.1±82.0	257.7±97.9*
Cholesterol (mg/dL)	214.7±47.8	212.7±33.1
Triglyceride (mg/dL)	149.0±75.1	174.3±119.2
FBS (mg/dL)	152.7±67.2	182.1±63.3*
Albumin (g/dL)	4.1±0.4	4.2±0.4
HbA1c (%)	8.3±2.7	8.9±1.7
CPR (ng/mL)	2.1±1.3	2.4±1.3
Insulin (μU/mL)	10.5±8.5	12.1±8.4
Steatosis on US		
Normal	10	8
Mild	21	22
Moderate	13	18
Severe	5	5

*$p<0.05$ *vs.* fertile women, **$p<0.001$ *vs.* fertile women.

TABLE 3 Stepwise Multiple Linear Regression Model for Independent Predictors for Serum Levels of ALT

Category	Variables	SE	β	*p* value
Male	BMI	0.678	0.452	0.0002
	Insulin	0.438	0.309	
Female	CPR	2.084	0.550	<0.0001
Fertile women	CPR	3.710	0.682	<0.0001
Postmenopausal women	CPR	1.476	0.556	0.0001

β: standardized coefficient.

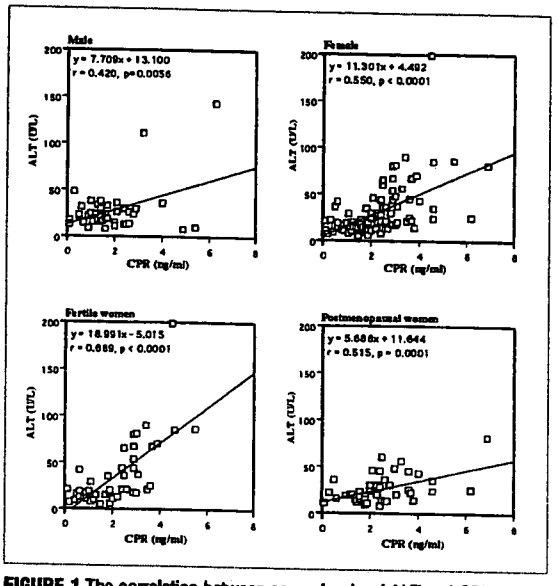

FIGURE 1 The correlation between serum levels of ALT and CPR was examined separately in males and females (upper two panels). When female patients were divided into fertile and postmenopausal groups, the former showed both a stronger degree of correlation and much higher levels of ALT than the latter (lower two panels). The fertile females had about threefold higher levels of ALT compared to the menopausal females with the same level of insulin.

of HbA1c, CPR and insulin also showed that the postmenopausal group had poorer glucose tolerance, which indicated that the postmenopausal females had more severe insulin resistance. On the other hand, BMI and the severity of fatty liver on ultrasonography were similar between the two groups. In order to confirm that this difference was not merely related to age, the male patients were divided into two groups according to age and were compared for the same factors, which revealed that there was no difference between the younger and the older males (data not shown).

Many investigators have reported that insulin resistance is an important factor in the progression of NASH, and it is accepted that the serum levels of CPR and insulin of fasting patients correlate well with the degree of insulin resistance. In order to confirm that the influence of insulin resistance is similar regardless of gender or menopause, stepwise multiple regression analysis was performed for males, females, and fertile and postmenopausal women (**Table 3**).

For all females, and for fertile and postmenopausal women, CPR was evaluated as a variable that was significantly correlated with the serum levels of ALT, whereas BMI and insulin were evaluated in males. By further analysis of the correlation between the serum levels of ALT and those of CPR or insulin, it was revealed that the degree of the correlation varied among the subgroups. As shown in **Figure 1**, both male and female patients showed a weak but significant correlation between the serum levels of CPR and those of ALT, and the correlation curves were similar. In dividing females into fertile and postmenopausal groups, however, their correlation curves were apparently different. Furthermore, the correlation, particularly in the fertile group, was stronger than that of all females or males. These findings indicated that a fertile patient would have about a threefold higher ALT level compared to a postmenopausal patient with the same CPR level. In addition, the correlation curve of postmenopausal women was almost equal to that of all males. We obtained similar results from analysis of the correlation between the serum levels of insulin and those of ALT (**Figure 2**).

We also analyzed the correlation between the serum levels of ALT and the severity of steatosis, another factor known to be related to NASH progression. In males and postmenopausal women, although the average level of ALT increased according to the progression of fatty liver, the difference was not significant (**Figure 3**). In the fertile group, however, the patients with moderate to severe fatty liver showed significantly higher levels of ALT than those without steatosis or with mild fatty liver.

DISCUSSION

Recent studies revealed that other factors, in addition to steatosis, are involved in the progression of NAFLD or NASH; such factors are so-called "second hit" (3) because many people with fatty liver and hyperinsulinemia maintain normal liver function (9). As shown in our study, the severity of fatty liver did not correlate significantly with the serum levels of ALT in males and postmenopausal women. Previous reports have suggested several candidates as "second hit" factors, such as insulin resistance, cytokines, oxidative stress, and so on (3,13). Among these, it has been proven that most patients with NAFLD or NASH have insulin resistance (1,3,14), however, the combination of steatosis and insulin resistance is not sufficient to explain the mechanism for all patients. Therefore, it has been suggested that oxidative stress and subsequent lipid peroxidation may be the ultimate factors which cause inflammation and fibrosis in the liver. Nevertheless, this final step for progression of NHLD or NASH has not yet been proven directly.

Before embarking on this study we formed the hypothesis that the liver damage might be moderate in fertile patients because of the well known anti-oxidative properties of estrogen. There is a large body of ev-

idence that oxidative stress is implicated in chronic liver disease and serves as a link between hepatic injury and fibrosis. It has been reported that estradiol inhibits liver inflammation and fibrosis caused by CCl_4 or dimethylnitrosamine in animal models (15,16). Shimizu *et al.* also suggested that the rapid progression to cirrhosis in men and postmenopausal women with hepatitis C might be due to the low level of estradiol (17). In addition to its anti-oxidant effect, it has been reported that estrogen reduces the expression of cell adhesion molecules *in vivo* and *in vitro*, which indicates that estrogen might have an anti-inflammatory role (18,19).

In our study, both fertile and postmenopausal women showed good correlations between the serum levels of ALT and CPR or insulin, which indicates that hyperinsulinemia or insulin resistance is a key factor in the progression of NAFLD. We also found that HOMA index (FBS x insulin /405) also had weaker correlation to the serum level of ALT than CPR or insulin (data not shown), although HOMA index is not a good marker of insulin resistance when the level of FBS is over 170mg/dL. Although our findings are in agreement with previous reports (3), our results are contrary to our hypothesis that the degree of liver injury would be moderate in fertile females with higher estrogen levels. Fertile females clearly showed a stronger correlation between ALT levels and CPR levels than did postmenopausal females, indicating that estrogen is possibly an aggravating factor.

If estrogen is one of the 'second hit' factors of NAFLD or NASH, how might it affect the progression of the disease? Several lines of evidence have been reported that estrogen suppresses lipid oxidation in the liver and increases the activity of CYP2E1. Lipid oxidation is reduced in pregnant women compared with healthy non-pregnant females, who in turn have lower lipid oxidation than postmenopausal females (20). In addition, serum estradiol correlated negatively with lipid oxidation (20). Furthermore, oral estrogen replacement in postmenopausal females suppresses hepatic lipid oxidation more significantly than transdermal replacement, because of a first pass effect (21). With regards to CYP2E1, its activity in female mice is higher than in male mice, and the activity in male mice was significantly increased by treatment with estradiol (22). The situation of both suppression of lipid oxidation and enhancement of CYP2E1 by estrogen is very similar to that observed in NASH, as well as alcoholic liver disease. Toremifene, an estrogen receptor antagonist, recently has been reported to alleviate ethanol induction of CYP2E1, resulting in protection of female rats from alcoholic liver injury (23). These evidences suggest that estrogen possibly exacerbates liver damage in NAFLD or NASH despite its anti-oxidative properties, and we now are evaluating the effect of withdrawing estrogen by ovariectomy in NASH model mice.

Because the menopause clearly means a dramatic withdrawal of estrogen, indicating that androgenic

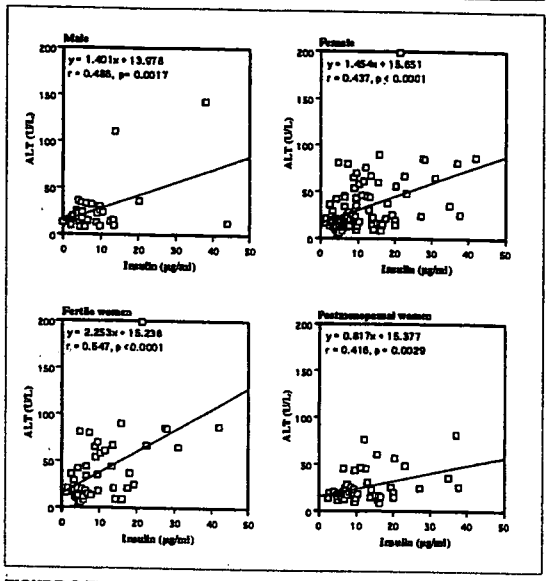

FIGURE 2 The correlation between the serum levels of ALT and insulin was examined separately in males and females (upper two panels). The correlations were significant but weaker in females when compared with the correlation between the serum levels ALT and CPR. Comparing fertile and postmenopausal women, similar results were obtained to those in Figure 1 (lower two panels).

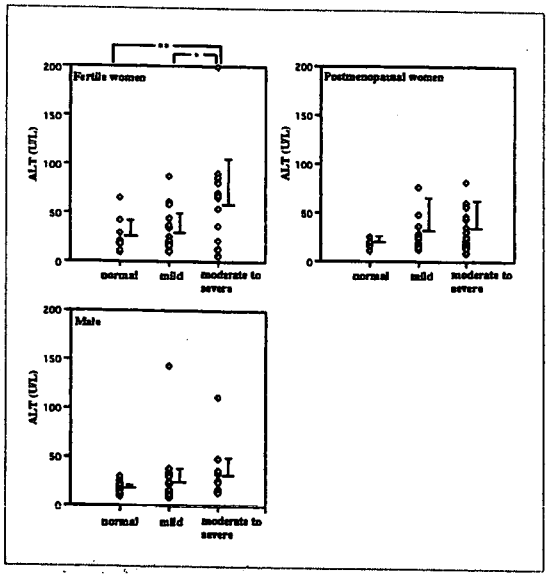

FIGURE 3 The influence of the severity of fatty liver on the serum levels of ALT was examined. In males and postmenopausal women, the average ALT levels tended to be high according to the progression of steatosis in all subgroups, but the difference was not significant. In fertile women, however, the patients with moderate to severe fatty liver showed significantly higher levels of ALT than those without steatosis or with mild fatty liver.

effects become relatively increased, we have to pay attention to androgenic roles in NFALD or NASH. It has been reported that male rats show higher levels of hepatic peroxisome proliferator-activated receptor (PPAR) alpha protein, the activation of which simulates oxidation, and that this difference is abol-