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Life Sciences 85 (2009) 617–624

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Synthetic triglyceride containing an arachidonic acid branch (8A8) prevents lipopolysaccharide-induced liver injury

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ARTICLE INFO

Article history:
Received 28 May 2009
Accepted 21 July 2009

Keywords:
Endotoxin
Lipopolysaccharide (LPS)
Macrophages
Nitric oxide (NO)
Tumor necrosis factor (TNF)-α
Nuclear factor (NF)-κB
Cannabinoid receptors
Alcoholic liver disease
Non-alcoholic steatohepatitis (NASH)

ABSTRACT

Aims: In this study, we investigated the effect of synthetic triglyceride containing an arachidonic acid branch (8A8) on lipopolysaccharide (LPS)-induced production of tumor necrosis factor (TNF)-α and nitric oxide (NO) in macrophages, and LPS-induced liver injury in the rat.

Main methods: RAW264.7 macrophages were co-incubated with 8A8 and LPS (100 ng/ml), and TNF-α mRNA/protein levels, nuclear factor (NF)-κB DNA binding activity, expression of inducible-type NO synthase (NOS2), and NO_2 production were measured. Male Wistar rats were given a single intraperitoneal injection of 8A8 prior to an intravenous injection of LPS (5 mg/kg), and liver histology, apoptotic cell death, serum TNF-α levels, and hepatic TNF-α mRNA were then evaluated.

Key findings: LPS-induced increases in TNF-α production in RAW264.7 macrophages were blunted by 8A8 in a dose-dependent manner, with 40% inhibition at 100 ppm. Further, 8A8 dose-dependently prevented LPS-induced increases in TNF-α mRNA levels, as well as NF-κB DNA binding activities, in RAW264.7 macrophages. LPS-induction of NOS2 and NO_2 release from these cells was also decreased by 8A8 in a dose-dependent manner. *In vivo*, LPS-induced liver injury, including hepatocyte apoptosis, was largely prevented when 8A8 (100 μl/kg) was given 30 min prior to LPS. Indeed, 8A8 blunted increases in both serum TNF-α and hepatic TNF-α mRNA levels significantly.

Significance: LPS-induced liver injury was prevented by 8A8 most likely through the inhibition of TNF-α and NO production from hepatic macrophages, suggesting a potential usefulness of 8A8 as an immunomodulating nutrient for prevention/treatment of endotoxin-related organ injuries including alcoholic liver disease and non-alcoholic steatohepatitis (NASH).

Introduction

Endotoxin (lipopolysaccharide; LPS) is a component of the outer wall of gram-negative bacillus, which plays a pivotal role in a broad spectrum of pathophysiological conditions comprising from local inflammatory responses to septic shock. Since the majority of bacterial flora in the gut consists from gram-negative rods, impairment of intestinal mucosal barrier results in the increase in endotoxin levels in the portal blood, triggering inflammatory responses in the liver. Lines of evidence clearly indicate that gut-derived LPS activates hepatic macrophages (Kupffer cells), which produce toxic mediators such as tumor necrosis factor (TNF)-α, eicosanoids, and free radicals, thereby causing hepatocellular injury in alcoholic liver diseases (Enomoto et al. 2000; Thurman 1998). More recently, the similar hypothesis is implicated in non-alcoholic steatohepatitis (NASH) that quite resembles pathological features of alcoholic liver disease despite the lack of heavy drinking (Solga and Diehl 2003). A neutralizing antibody to TNF-α ameliorates both alcoholic and non-alcoholic models of steatohepatitis in rodents (Iimuro et al. 1997; Li et al. 2003). However, clinical application of an anti-TNF antibody and a TNF receptor chimera are limited for severe alcoholic hepatitis with indefinite efficacy, and the long-term outcome has been reported to be even worse when used in combination with corticosteroid (Naveau et al. 2004). Anti-TNF strategies with safer and more efficient ways are theoretically promising for the prevention and treatment of steatohepatitis both in alcoholics and non-alcoholics.

Triglyceride, one of the major nutrients, consists of glycerol and three fatty acid chains with various lengths and combinations. Triglyceride and related compounds are not only the source of free fatty acids and their metabolites, but exerts various physiological functions by themselves. Recently, 2-arachidonoyl glycerol (2-AG) has been recognized as an endogenous cannabinoid receptor agonist (Mechoulam et al. 1995; Sugiura et al. 1995). Since cannabinoid receptors (CB1 and CB2) are

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doi:10.1016/j.lfs.2009.07.013

widely expressed in peripheral tissues including liver, 2-AG is postulated to act as an intrinsic regulator of hepatic inflammation and fibrogenesis through the cannabinoid receptor-mediated pathway (Julien et al. 2005; Mallat and Lotersztajn 2008). Here in this study, we investigated the effect of synthetic triglycerides containing one arachidonic acid and two caprylic acid branches (8A8 and 88A; Fig. 1A), which are potent cannabinoid receptor agonists, on LPS-induced production of TNF-α and nitric oxide (NO) in RAW264.7 macrophages. Further, we evaluated the preventive effect of 8A8 on LPS-induced liver injury in the rat.

Materials and methods

Materials

Synthetic triglycerides containing one arachidonic acid and two caprylic acid branches (SUN8A8-E and 88A; molecular weight 631 Da, Fig. 1A) were prepared enzymatically, and kindly provided by Suntory Co. Ltd. (Osaka, Japan). Lipopolysaccharide (LPS, from E. coli 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals were of analytical grade unless otherwise specified.

Cell culture and treatment in vitro

RAW264.7 macrophages, obtained from the American Type Culture Collection (ATCC, Rockville, MD), were cultured in Dulbecco's modified essential medium (D'MEM, GIBCO®, Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, GIBCO®, Invitrogen Corp.) and antibiotics (100 IU/ml penicillin G and 100 μg/ml streptomycin sulfate) in a humidified air containing 5% CO_2 at 37 °C. Cells were cultured on regular 60 mm polystyrene dishes unless otherwise specified. Every experiment was performed in duplicate, and then repeated for 6 times to obtain the average data. Cells were incubated with LPS (100 ng/ml) in the presence/absence of 8A8 and 88A [dissolved in 2 volumes of dimetylsulfoxide (DMSO)] at various concentrations ranging from 10 to 1000 ppm (vol/vol). The final concentration of DMSO in the media did not exceed 0.2%.

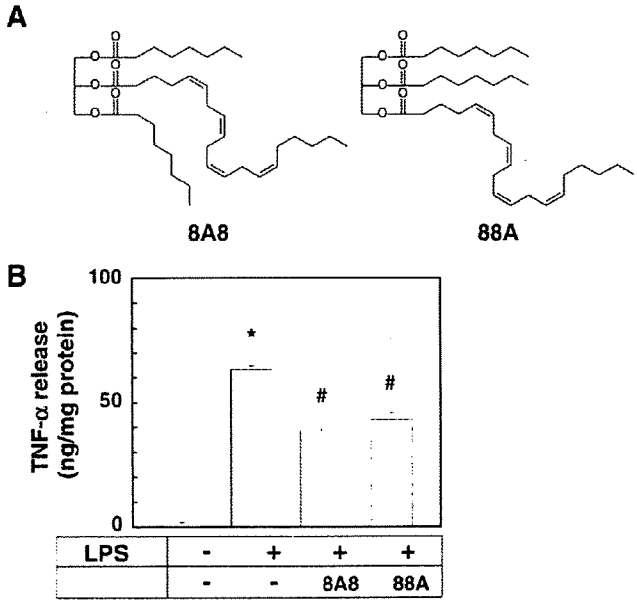

Fig. 1. The effect of 8A8 and 88A on LPS-induced production of TNF-α in cultured macrophages. Chemical structures of 8A8 and 88A, the synthetic triglycerides containing one arachidonic acid and two caprylic acid branches, are shown (A). RAW264.7 macrophages were incubated with 100 ng/ml LPS in the presence of 8A8 and 88A (100 ppm) for 6 h, and concentrations of TNF-α in the media were measured by ELISA (B). Data express mean ± SEM from 6 individual samples (*; $p<0.05$ vs. controls, #; $p<0.05$ vs. LPS alone by ANOVA on ranks and Student–Newman–Keuls post hoc-test).

Animal experiments

Specific pathogen-free female Wistar rats were purchased from Charles River Japan Inc. (Saitama, Japan). All animals received humane care and the experimental protocol was approved by the Committee of Laboratory Animals according to institutional guidelines. All animals were allowed free access of water and laboratory chow diet and housed for several days prior to experiments. Rats weighing 200–250 g were given a single intraperitoneal injection of 8A8 [10–100 μl/kg body weight (BW), dissolved in olive oil] 30 min prior to a single intravenous injection of LPS (5 mg/kg BW). Animals were sacrificed under light ether anesthesia 90 min and 24 h after injection of LPS by exsanguinations from inferior vena cava to obtain liver and serum samples. Serum and liver tissue samples were kept frozen at −80 °C until assayed. For histological analysis, liver specimens were fixed with 10% buffered formalin, embedded with paraffin, and hematoxylin–eosin (H-E) staining and esterase staining were performed. Specimens were photographed using a microscope (BH-2, Olympus Corp., Tokyo, Japan) equipped with a digital imaging system (VB-6010, Keyence Corp., Osaka, Japan). Pathological evaluation was performed by one of the co-authors by a blinded fashion.

Measurement of aminotransferases, TNF-α and NO_2 levels

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured spectrophotometrically by a standard enzymatic method (KAINOS Laboratories Inc., Tokyo, Japan). Tumor necrosis factor (TNF)-α levels in the culture media and serum were determined using enzyme-linked immunosorbent assay (ELISA) kits (BioSource International Inc., Camarillio, CA). NO_2 in the culture media was quantified colorimetrically by Griess reaction using a commercial kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan).

Cell viability assay and detection of apoptotic cell death

Cells were plated in 96-well microplates (Sumitomo Bakelite Co., Tokyo, Japan) at a density of 5×10^4 cells/well in the complete culture medium. Number of viable cells was measured spectrophotometrically using a commercial kit (Cell counting Kit-8; Dojindo Molecular Technologies, Inc.) according to the manufacturer's instructions. Experiments were performed in duplicated wells for each condition, and repeated for 6 times.

Apoptotic cell death was visualized by Hoechst 33342 fluorescent-dye staining. Briefly, the whole cells in the culture were harvested and collected by centrifugation at 200×g for 10 min. Cells were fixed with 1% glutaraldehyde solution overnight, re-suspended in phosphate buffer solution, and then stained with 167 μM Hoechst 33342 (Dojindo Molecular Technologies, Inc.). Specimens were observed and photographed under a fluorescent microscope (Axiovision 3.1/Axioplan 2 imaging, Carl Zeiss Microimaging GmbH, Germany).

RNA preparation and real-time reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from cultured cells and frozen tissue samples were prepared using Trizol reagent (Invitrogen Corp.) and Quick Prep™ total RNA extraction kit (Amersham Pharmacia Biotech, Piscataway, NJ), respectively. The concentration and purity of isolated RNA were determined by measuring optical density at 260 and 280 nm. Further, the integrity of RNA was verified by electrophoresis on formaldehyde-denaturing agarose gels.

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA (1 μg) were reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Super-ScriptII, Invitrogen Corp.) and an oligo dT [12–18] primer (Invitrogen Corp.) at 42 °C for 1 h. Obtained cDNA was amplified using TaqMan gene expression assays for mouse TNF-α (Mm00443258_ml) and TaqMan Universal PCR master mix (PE Applied Biosystems, Foster City, CA). The quantitative PCR analysis was carried out using ABI PRISM 7500 Sequence Detection System (PE Applied Biosystems), and the threshold cycle (C_T) values were obtained. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used for normalizing the expression data.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from RAW264.7 cells 45 min after incubation with LPS and/or 8A8, according to the method of Dignam (Dignam et al. 1983) with slight modifications. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). DNA binding activities for nuclear factor-κB (NF-κB) in the nuclear extracts were detected by electrophoretic mobility shift assay (EMSA) as previously described elsewhere (Shibuya et al. 2002). Briefly, double-strand oligonucleotide containing consensus sequence of NF-κB element (5′-AGTTGAGGGGACTTTCCCAGGC-3′, Santa Cruz Biotechnology, Santa Cruz, CA) was labeled with ^{32}P using T4 polynucleotide kinase and $\gamma^{32}P$-ATP. Binding reactions were carried out for 20 min on ice in a buffer containing 10 μg nuclear extracts, 5 μg poly[d(I-C)], 10 mM Hepes, pH 7.6, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 5 mM $MgCl_2$, and 20,000 cpm of ^{32}P-labeled probe. DNA–protein complexes were separated by non-denaturing polyacrylamide gel (5% in 0.4× Tris-borate–EDTA buffer) electrophoresis. Gels were dried and exposed to X-OMAT films (Kodak, Rochester, NY).

Western blotting

Whole cell protein extracts were prepared by homogenizing cells in a buffer containing 20 mM Hepes (pH7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 10% glycerol, 1% Triton X-100, and protease/phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μl/ml leupeptin, 4 μg/ml aprotinin, 17.5 mM β-glycerophosphate, 1 μM NaF, 100 μM Na_3VO_4; Sigma Chemical Co.), followed by a centrifugation at 15,000 rpm for 10 min. Protein concentration was determined by Bradford assay using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Thirty micrograms of protein was separated in 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and electrophoretically transferred onto polyvinylamide fluoride membranes. After blocking with 5% non-fat dry milk in Tris-buffered saline-0.05% Tween 20, membranes were incubated overnight at 4 °C with rabbit polyclonal anti-nitric oxide synthase (NOS)-2, (Santa Cruz Biotechnology Inc., Santa Cruz, CA), followed by a secondary horseradish peroxidase-conjugated polyclonal goat anti-rabbit IgG antibody (Dako-Cytomation Norden A/S). Subsequently, specific bands were visualized using the ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were then stripped, and re-probed for GAPDH using a rabbit polyclonal anti-GAPDH antibody (Santa Cruz Biotechnology Inc.) as an internal control.

TUNEL assay and immunohistochemistry

To detect apoptotic cell death in tissue, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was performed using a commercial kit (DeadEnd™ Fluorometric TUNEL System, Promega Corp., Madison, WI) according to the manufacturer's instructions. Subsequently, localization of macrophages was visualized by immunohistochemistry for F4/80. After blocking with normal horse serum for 60 min, tissue sections were incubated with a rabbit polyclonal anti-F4/80 antibody (Santa Cruz Biotechnology Inc.). After rinsing the primary antibody, the sections were incubated with a secondary rhodamine-conjugated anti-rabbit IgG antibody. Specimens were observed and images were captured under a fluorescent microscope (Axiovision 3.1/Axioplan 2 imaging, Carl Zeiss Microimaging GmbH, Germany). TUNEL-positive staining was detected from green nuclear fluorescence, and localization of total nuclei was determined by blue nuclear fluorescence of 4′,6-diamidino-2-phenylindole (DAPI), whereas F4/80-positive macrophages were detected by red, rhodamine fluorescence. Accordingly, nuclei of TUNEL-positive macrophages were visualized as yellowish fluorescence when images were merged. TUNEL-positive hepatocytes and macrophages were then counted in five 200× fields on each specimen to determine the average number, and repeated for 6 animals in each condition.

Statistical analyses

Data were expressed as means ± SEM. Statistical differences between means were determined using analysis of variance (ANOVA) or ANOVA on ranks followed by Student–Newman–Keuls multiple comparison procedure as appropriate. A value of $P<0.05$ was selected prior to experiments to reflect statistical significance.

Results

Effect of 8A8 and 88A on TNF-α production in RAW264.7 macrophages

To test whether 8A8 and 88A inhibit TNF-α production in macrophages, RAW264.7 cells were incubated with 100 ppm of 8A8 and 88A in the presence of LPS (100 ng/ml) for 6 h, and TNF-α concentrations in the culture media were determined by ELISA (Fig. 1B). As expected, 100 ng/ml LPS elicited production/release of TNF-α from RAW264.7 cells within 6 h tremendously, the values reaching almost 40-fold over controls. In contrast, the TNF-α levels in the media reached to the values only 60% of LPS alone when cells were incubated together with 100 ppm 8A8. Similarly, 88A also blunted production of TNF-α from LPS-stimulated RAW264.7 macrophages to the same extent.

Next, the dose-dependent effects of 8A8 on LPS-induced TNF-α production/release, as well as mRNA levels in RAW264.7 cells, were evaluated more precisely (Fig. 2A). LPS-induced increases in TNF-α production were blunted by 8A8 dose-dependently, with significant decreases from 30 ppm (Fig. 2A, upper panel). Further, LPS-induction of TNF-α mRNA was also blunted by 8A8 at a concentration of 100 ppm significantly (Fig. 2A, lower panel). Moreover, LPS-induced increases in NF-κB DNA binding activities were blunted by 8A8 dose-dependently (Fig. 2B), confirming that 8A8 inhibits production of TNF-α by decreasing transcription levels.

Effect of 8A8 on cell survival in RAW264.7 macrophages

To check whether 8A8 affects cell survival in RAW264.7 macrophages, cells were incubated with 8A8 for 6 h in the presence or absence of LPS (100 ng/ml), and viable cells were counted spectrophotometrically using WST assay. After incubation with 8A8, viability of cells was not decreased within the range up to 100 ppm both in the presence and absence of LPS (100 ng/ml), indicated that 8A8 up to 100 ppm inhibited TNF-α production in RAW264.7 macrophages without cell killing. In contrast, viable cells tended to decrease at concentrations above 300 ppm both in the presence and absence of LPS, the percentages of viable cells declining to almost 70% with 1000 ppm 8A8. Therefore, we evaluated the morphological changes of dying cells using Hoechst 33342 dye staining. Typical condensation and disruption of nuclei were observed in cells treated with 1000 ppm 8A8 for 6 h, indicating that high concentrations of 8A8 induce apoptotic cell death in RAW264.7 macrophages.

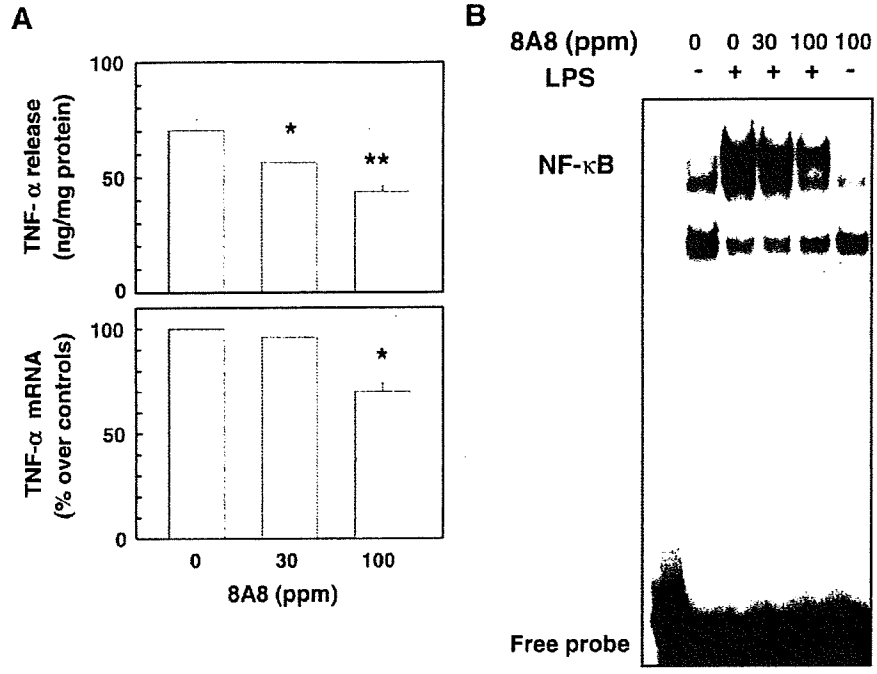

Fig. 2. Dose-dependent effect of 8A8 on LPS-induced TNF-α production and NF-κB activation in cultured macrophages. Experimental design as in Fig. 1 except that various doses of 8A8 (30–1000 ppm) were applied in the presence of LPS (100 ng/ml), and concentrations of TNF-α in the media were determined by ELISA (A, upper panel). Similarly, cells were incubated with LPS in the presence of various doses of 8A8 for 3 h, and TNF-α mRNA levels were quantified using real-time RT-PCR as described in details in Materials and methods (A, lower panel). Data represent mean ± SEM from 6 individual samples (*; $p<0.05$, **; $p<0.001$ vs. LPS loaded controls, by ANOVA and Student–Newman–Keuls post hoc-test). Nuclear extracts were prepared from cells incubated with LPS/8A8 for 45 min, and NF-κB DNA binding activities were detected by EMSA as described in details in Materials and methods (B).

Effect of 8A8 on LPS-induction of NOS and NO production in RAW264.7 macrophages

Next, we evaluated whether 8A8 inhibits production of NO in RAW264.7 macrophages. It is well known that inducible-type NOS (NOS2) plays a major role in LPS-induced production of NO in macrophages; therefore we first analyzed the effect of 8A8 on expression of NOS2 in these cells by Western blotting (Fig. 3A). Obvious induction of NOS2 was observed in RAW264.7 cells 6 h after incubation with LPS as expected, whereas 8A8 blunted LPS-induction of NOS2 levels almost 42%. Further, NO_2 levels in the culture media were measured colorimetrically by Griess reaction (Fig. 3B, lower panel). LPS-

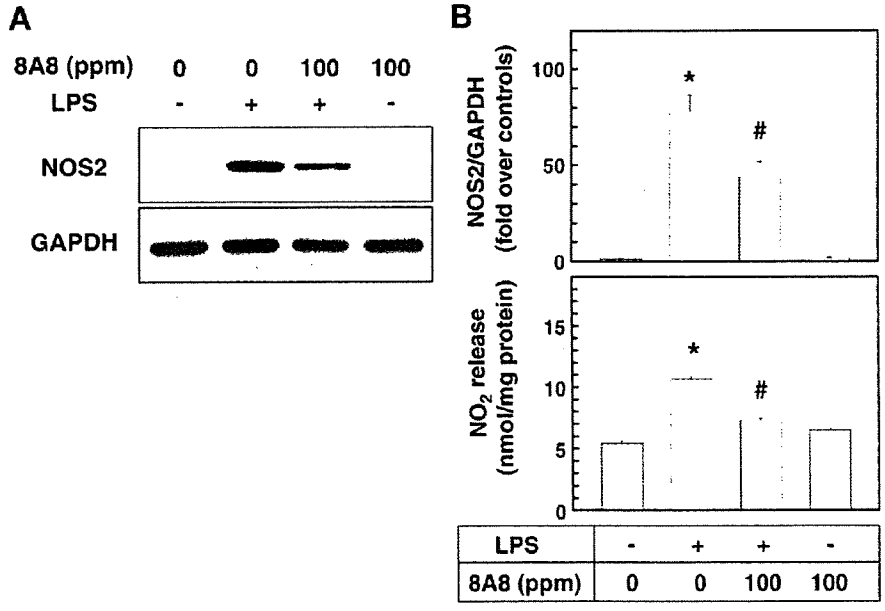

Fig. 3. The effect of 8A8 on induction of NOS in cultured macrophages. Experimental design as in Fig. 1 except that 8A8 (100 ppm) was applied in the absence or presence of LPS (100 ng/ml) for 6 h, and expression of inducible-type NOS (NOS2) in RAW264.7 macrophages were determined by Western blotting. Representative photographs of specific bands for NOS2 (130 kDa) and GAPDH (37 kDa) are shown (A). Densitometrical values of NOS2/GAPDH protein levels are plotted (B, upper panel). NO_2 levels in the cultured media 6 h after incubation with LPS/8A8 were measured colorimetrically by Griess reaction (B, lower panel). Data represent mean ± SEM from 6 individual samples (*; $p<0.05$ vs. controls, #; $p<0.05$ vs. LPS alone, by ANOVA on ranks and Student–Newman–Keuls post hoc-test).

induced increases in NO_2 levels in the media were also prevented by 8A8 significantly, the values being nearly 2/3 of LPS alone. Taken together, these findings indicate that 8A8 also inhibits LPS-induced production of NO from RAW264.7 macrophages.

Protective effect of 8A8 on LPS-induced liver injury

Since 8A8 inhibited LPS-induced production of TNF-α in macrophages in vitro, next we evaluated if 8A8 prevents LPS-induced liver injury in vivo. Rats were given an intraperitoneal injection of 8A8 (100 μl/kg) 30 min prior to LPS (5 mg/kg) injection via the tail vein. LPS-induced necro-inflammatory changes in the liver in 24 h as expected (Fig. 4A, upper panel); however, pretreatment with 8A8 prevented this LPS-induced liver damage remarkably (Fig. 4A, lower panel). Indeed, increases in serum aminotransferase levels 24 h after LPS injection were blunted by pretreatment with 8A8 significantly (Fig. 4B). It is noted that 8A8 per se had no effect on liver histology and serum aminotransferases (data not shown).

Next, we evaluated inflammatory infiltration in the liver using esterase staining. Massive accumulation of granulocytes in the hepatic lobules, especially into the necrotic area, was observed 24 h after LPS injection as expected (Fig. 5A, upper panel), whereas this phenomenon was minimized by simultaneous treatment with 8A8 (Fig. 5A, lower panel). Indeed, 8A8 blunted LPS-induced increases in granulocytes per field significantly to the levels almost 1/5 of LPS alone (Fig. 5B). Since high concentrations of 8A8 induced cell death in RAW267.8 macrophages in vitro, we also evaluated whether the doses of 8A8 used in vivo kills hepatic macrophages or not. While hepatic macrophages were slightly increased 24 h after injection of LPS, simultaneous injection of 0.1 ml/kg 8A8 did not decrease these cells. Further, macrophages in the hepatic lobules were not decreased in number by treatment of 8A8 alone (Fig. 5B), indicating that 8A8 used in vivo did not reach the cytotoxic concentrations observed in vitro.

Effect of 8A8 on LPS-induction of TNF-α and apoptotic cell death in the liver

To confirm that 8A8 inhibited LPS-induced production of TNF-α in vivo, serum and liver samples were collected 90 min after LPS injection. Serum TNF-α levels were elevated tremendously 90 min after LPS injection as expected; however, pretreatment with 8A8 blunted this increase in a dose-dependent manner, with significant inhibition at 30 μl/kg and higher (Fig. 6A). Moreover, LPS-induced increases in TNF-α mRNA levels in the liver were blunted over 40% by pretreatment with 100 μl/kg 8A8 (Fig. 6B). Collectively, these findings clearly demonstrated that 8A8 prevents LPS-induced liver injury in vivo, most likely through suppression of TNF-α production in hepatic macrophages.

To further evaluate the protective effect of 8A8 on LPS-induced liver injury, we perform dual staining of TUNEL and F4/80 immunostaining of liver specimens (Fig. 7). TUNEL-positive hepatocytes were increased dramatically 24 h after injection of LPS as expected (Fig. 7A, upper panel). Further, TUNEL- and F4/80-dual positive, apoptotic macrophages were also observed especially in inflammatory foci after LPS challenge, indicating that LPS-induced activation of hepatic macrophages causes suicidal cell killing. In sharp contrast, 8A8 inhibited increases in apoptotic hepatocytes significantly (Fig. 7A, lower panel). Interestingly, 8A8 prevented increases in the number of apoptotic macrophages as well as hepatocytes significantly (Fig. 7B), suggesting that the doses of 8A8 used in vivo study is rather protective against oxidative burst-induced self-killing of hepatic macrophages.

Discussion

In the present study, we demonstrated that 8A8, a synthetic triglyceride containing arachidonic acid branch, inhibits TNF-α production from macrophages both in vitro and in vivo, thereby preventing LPS-induced liver injury in the rat. In concentrations of

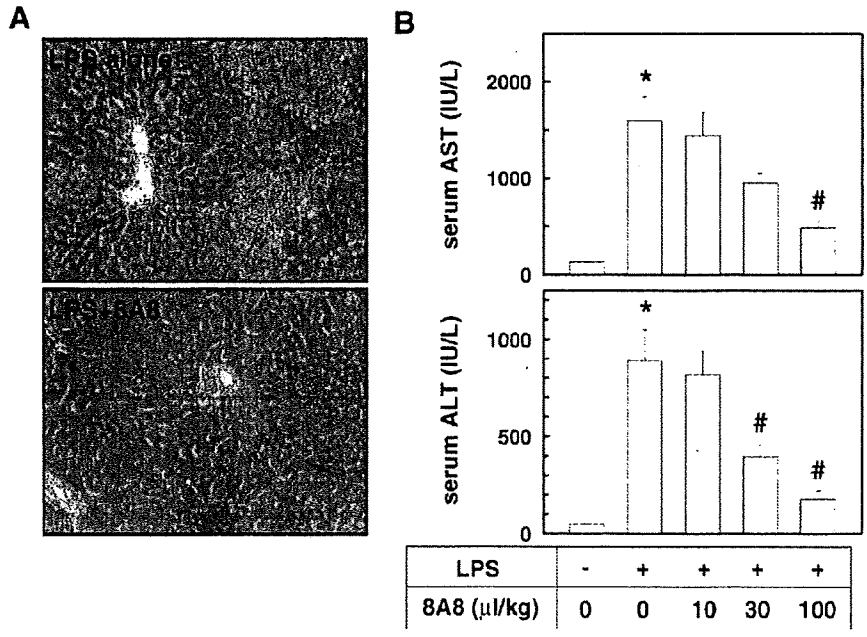

Fig. 4. Effect of 8A8 on LPS-induced liver injury in the rat. Rats were given an intraperitoneal injection of 8A8 (10–100 μl/kg BW) 30 min prior to a single intravenous injection of LPS (5 mg/kg) via the tail vein. Liver and serum samples were obtained 24 h after LPS injection. Representative photomicrographs of the liver histology from LPS alone (A, upper panel) and LPS in combination with 100 μl/kg 8A8 (A, lower panel) are shown (H-E staining, original magnification: ×100). Serum aminotransferase levels 24 h after injection of LPS in combination with various doses of 8A8 pretreatment are plotted ($n = 6$, mean ± SEM, *; $p < 0.05$ vs. controls, #; $p < 0.05$ vs. LPS alone by ANOVA on ranks and Student–Newman–Keuls post hoc-test).

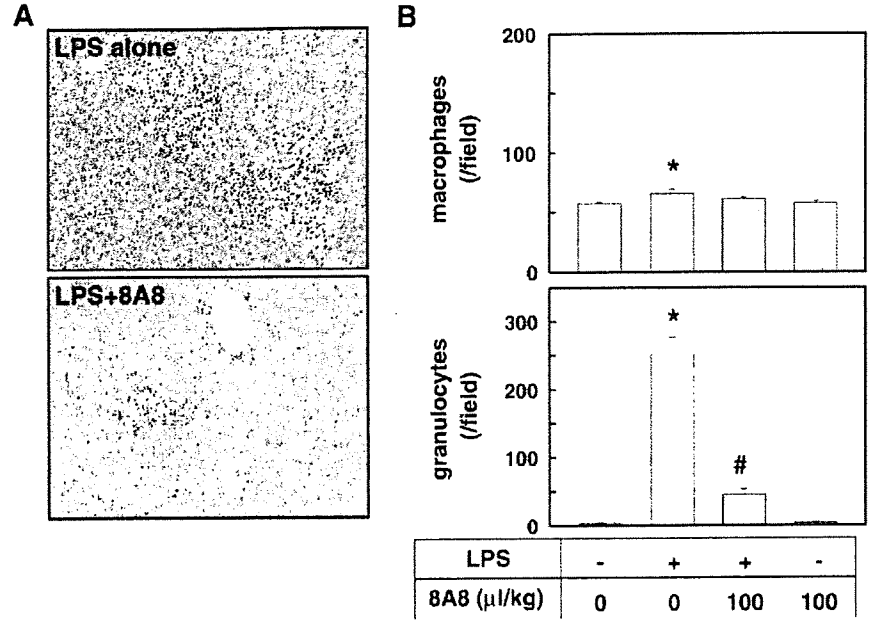

Fig. 5. Effect of 8A8 on LPS-induced hepatic inflammation in the rat. Experimental design as in Fig. 4 except that liver samples obtained 24 h after LPS injection were evaluated by esterase staining. Representative photomicrographs of the liver histology from LPS alone (A, upper panel) and LPS in combination with 100 μl/kg 8A8 (A, lower panel) are shown (esterase staining, original magnification: ×200). Note that granulocytes and macrophages were stained as purple and blue, respectively. Average number of macrophages and granulocytes per field 24 h after injection of LPS in combination with 8A8 pretreatment are plotted ($n = 6$, mean ± SEM, *; $p < 0.05$ vs. controls, #; $p < 0.05$ vs. LPS alone by ANOVA on ranks and Student–Newman–Keuls post hoc-test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

8A8 up to 100 ppm prevented LPS-induced activation of NF-κB (Fig. 2B), thereby blunting LPS-induced transcriptional up-regulation of TNF-α in RAW264.7 macrophages (Fig. 2A). Although higher concentrations of 8A8 above 300 ppm decreased viability of RAW264.7 cells by inducing apoptosis in vitro, the dose of 8A8 which significantly inhibited TNF-α induction in vivo did not decrease numbers in hepatic macrophages, indicating that 8A8 is capable to prevent LPS-induced TNF-α production from Kupffer cells without cell elimination. In addition, 8A8 also prevented production of NO by inhibiting induction of NOS2 in RAW264.7 macrophages (Fig. 3), suggesting that 8A8 most likely inhibits radical formation as well as inflammatory cytokine production from macrophages.

The precise mechanism of this inhibitory effect of 8A8 on TNF-α production in macrophages still remain unclear, however, one possibility is that 8A8 acts through cannabinoid receptors. It has been reported that macrophages predominantly express CB2 receptors (Munro et al. 1993). Here in this study, two synthetic triglycerides 8A8 and 88A, both of which have structural similarities to an intrinsic cannabinoid receptor agonist 2-AG, blunted LPS-induced production of TNF-α in RAW264.7 macrophages to the same extent (Fig. 1). The similar molar levels of 2-AG were also able to inhibit LPS-induced production of TNF-α in RAW264.7 macrophages significantly (data not shown). Taken together, it is hypothesized that 8A8 inhibits transcriptional up-regulation of TNF-α in macrophages most likely through the cannabinoid receptor-mediated signaling.

Another potential mechanism through which 8A8 and 88A prevents LPS-induced activation of macrophages is that these triglycerides may interact with toll-like receptors (TLR). TLRs are a family of pattern recognition receptors which play pivotal roles in innate immunity (Armant and Fenton 2002; Takeda and Akira 2005). Among these, TLR-4 is the major trans-membrane LPS recognition molecule in macrophages, and molecular interaction between LPS and

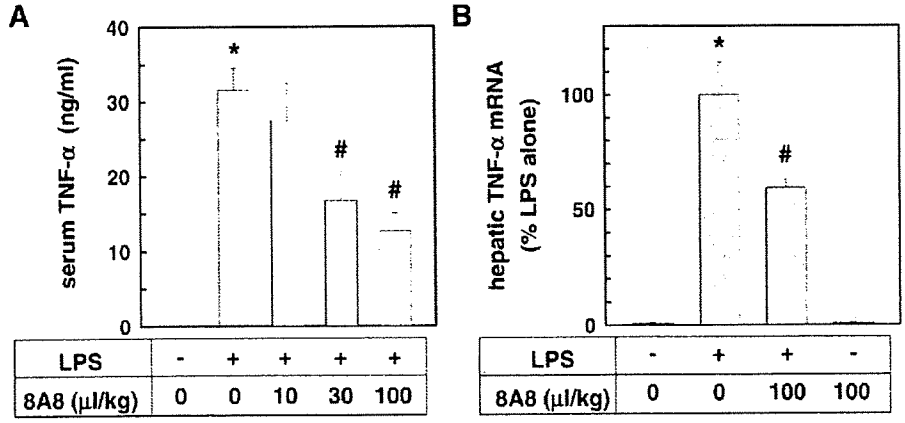

Fig. 6. Effect of 8A8 on LPS-induced increases in TNF-α in vivo. Experimental design as in Fig. 4 except that serum and liver samples were obtained 90 min after LPS injection. Serum TNF-α levels were measured by ELISA (A), and mRNA levels in hepatic tissue were determined by real-time RT-PCR (B). Data represent mean ± SEM from 6 animals for each treatment (*; $p < 0.05$ vs. controls, #; $p < 0.05$ vs. LPS alone by ANOVA on ranks and Student–Newman–Keuls post hoc-test).

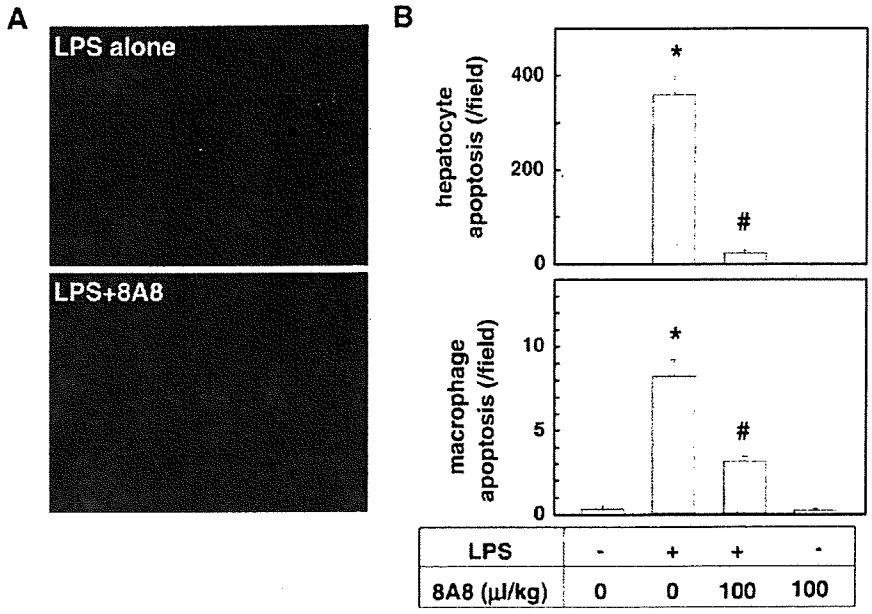

Fig. 7. Effect of 8A8 on LPS-induced increases in apoptotic hepatocytes/macrophages in vivo. Experimental design as in Fig. 4. Dual staining of TUNEL and F4/80 immunohistochemistry was performed using liver specimens 24 h after injection of LPS and/or 8A8. Representative photomicrographs from LPS alone (A, upper panel), and LPS + 8A8 (A, lower panel) are shown (original magnification; ×200). Green, blue, and red fluorescence indicate TUNEL-positive nuclei, total nuclei, and F4/80-positive macrophages, respectively. Note that yellowish nuclei indicate TUNEL-positive macrophages, in comparison with green nuclei of TUNEL-positive hepatocytes. Average number of TUNEL-positive hepatocytes and macrophages in the field (×200) are plotted (B). Data represent mean ± SEM from 6 animals for each treatment (*; $p<0.05$ vs. controls, #; $p<0.05$ vs. LPS alone by ANOVA on ranks and Student–Newman–Keuls post hoc-test). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

TLR-4 is critical for the activation of the downstream intracellular signaling cascade involving myeloid differentiation primary response protein (MyD)-88, interleukin-1 receptor-associated kinase (IRAK), tumor necrosis factor receptor-associated factor (TRAF)-6, and NF-κB (Kawai and Akira 2006). Since TLR-4 recognizes lipid A, the lipid moiety of LPS, it is hypothesized that TLR-4 also recognizes a sort of lipid molecules and/or fatty acids. Indeed, emerging lines of evidence indicated that free fatty acids are likely to be the regulators of TLR-mediated signaling (Lee et al. 2003; Schaeffler et al. 2009; Weatherill et al. 2005). In the present study, in turn, 8A8 prevented LPS-induced activation of NF-κB and subsequent transcriptional up-regulation of TNF-α in RAW264.7 macrophages (Fig. 2). Collectively, these findings suggest a possibility that 8A8 interferes LPS recognition by TLR-4 in a direct manner, thereby inhibiting macrophage activation and subsequent organ injury in the liver.

In terms of clinical significance, 8A8 is a potent immuno-nutrient, which most likely prevents a variety of endotoxin-related organ injuries. Originally, 8A8 and 88A were synthesized in order to develop innovational functional food ingredients, and the safety profiles of 8A8 indicated that chronic oral administration of 8A8 up to 2000 mg/kg for 13 weeks caused no toxic effect in the rat. Since gut-derived endotoxin is postulated to play a pivotal role in the development of NASH as well as alcoholic hepatitis, it is also promising to establish the clinical utility of 8A8 as a therapeutic nutrient for NASH and alcoholic liver disease.

Conclusion

In conclusion, 8A8 prevents LPS-induced liver injury through direct inhibition of TNF-α and NO production in macrophages. Synthetic triglycerides containing arachidonic acid branch, such as 8A8 and 88A, are promising dietary ingredients bearing immuno-modulating function for the prevention/treatment of endotoxin-related organ injuries, including alcoholic and non-alcoholic steatohepatitis.

Acknowledgements

This work was supported in part by Grant-in-Aid from the Ministry of Education, Science, Technology, and Culture of Japan (No. 19590791 to KI), and a grant from Research Conference on Alcohol and Health (sponsored by Suntory Co. Ltd.).

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Hepatology Research 2009; 39: 939–943 doi: 10.1111/j.1872-034X.2009.00530.x

Short Communication

Clinical significance of serum ornithine carbamoyltransferase in patients with non-alcoholic steatohepatitis

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Aim: Ornithine carbamoyltransferase (OCT) is reported to be a liver-specific marker for the evaluation of hapatocellular damage. In this study, we investigated its clinical significance in non-alcoholic steatohepatitis (NASH).

Methods: Serum OCT levels were measured by the ELISA (enzyme-linked immunosorbent assay) method. One hundred and twenty patients with NASH (18 liver cirrhosis induced by NASH and 9 NASH combined with hepatocellular carcinoma) were measured.

Results: The serum levels of OCT and the ratios of OCT : alanine amino transferase (ALT) and OCT : aspartate amino transferase (AST) were increased in parallel with the progression of NASH. Especially, OCT and both ratios were markedly increased in hepatocellular carcinoma. As for the relationship between fibrosis grade and OCT, the serum OCT levels and the ratio of OCT : ALT levels were increased in parallel with liver fibrosis. In NASH patients with ALT within normal range, about 30% showed elevation of OCT.

Conclusion: Serum OCT levels and the ratios of OCT : ALT and OCT : AST increase in parallel with the progression of NASH. It was suggested that OCT is a useful marker in the progression of NASH.

Key words: fibrosis, non-alcoholic steatohepatitis, ornithine carbamoyltransferase

INTRODUCTION

NON-ALCOHOLIC FATTY LIVER disease (NAFLD) has recently been recognized as a leading cause of abnormal liver function tests. Its spectrum ranges from simple fatty liver, which is usually a benign and non-progressive condition, to non-alcoholic steatohepatitis (NASH), which may progress to cirrhosis.[1,2] In addition, NASH is increasingly being recognized as a major cause of cryptogenic cirrhosis and as an indication for liver transplantation.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are used widely as serum markers in the diagnosis of liver diseases. However, it has been reported that ALT is not a predictor of activity and fibrosis severity in NASH.[3–6] In addition, NASH with normal ALT range has been reported.[3–5] We previously reported that the ratio of AST : ALT was increased and platelet count was decreased in NASH patients with advanced fibrosis, but there is no significant association between the progression of fibrosis and transaminase levels.[6] Liver biopsy is the only method for the diagnosis of NASH and the evaluation of progression.[1,2]

Ornithine carbamoyltransferase (OCT) is reportedly a liver-specific marker for the evaluation of hepatocellular damage. AST and ALT are not liver-specific, existing in a variety of organs such as heart, muscle, and kidney, while OCT is highly liver-specific and is a relatively abundant protein. Serum OCT levels were increased in alcoholic liver diseases.[7] In addition, the ratio of OCT : ALT was reported as a marker of hepatocellular carcinoma.[8,9] Murayama *et al.* showed the usefulness of OCT for evaluation in acute and chronic liver injuries.[9,10]

The etiology of NASH is still unknown, but several studies have reported that mitochondria dysfunction is

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Received 11 February 2009; revision 25 March 2009; accepted 26 March 2009.

an important element.[11,12] And, the localization of OCT is restricted to mitochondria.[13] Taken together, in this study we measured the serum OCT level in NASH patients and evaluated its clinical significance in NASH.

PATIENTS AND METHODS

ONE HUNDRED AND twenty Japanese patients histologically diagnosed as having NASH at Tokyo Women's Medical University between 1995 and 2008 were evaluated along with 80 healthy subjects serving as controls. All liver biopsy specimens were examined using hematoxylin-eosin, Mallory, and silver reticulin as stains. Fibrosis was scored using a 5-grade scale: F0, normal connective tissue; F1, foci of perivenular or pericellular fibrosis in zone 3; F2, perivenular or pericellular fibrosis confined to zones 3 and 2, with or without portal/periportal fibrosis; F3, bridging or septal fibrosis; F4, cirrhosis.[2,14,15] The diagnosis of NASH was established based on the following criteria: (i) histologically macrovesicular steatosis affecting at least 10% of hepatocytes and steatohepatitis including ballooning cells or perisinusoidal/pericellular fibrosis in zone 3;[2,14,15] (ii) intake of less than 20 g of ethanol per day, as confirmed by physicians and family members of the patients; and (iii) appropriate exclusion of other liver diseases such as alcoholic liver disease, viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, primary sclerosing cholangitis, and metabolic liver diseases. NASH patients were divided into three groups: (i) NASH patients without liver cirrhoisis (LC) and hepatocellular carcinoma (HCC), (ii) cirrhotic NASH (NASH-LC) without HCC, and (iii) NASH with HCC (NASH-HCC). Eighteen NASH-LC patients were proven as LC by liver biopsy. Nine NASH-HCC patients were diagnosed histologically or by detection of consistent findings on at least two radiologic modalities.

All patients underwent liver tests for measurement of the following laboratory parameters: AST, ALT, platelet count, hepatitis B serology (hepatitis B surface antigen, antibody to hepatitis B surface antigen, and antibody to hepatitis B core antigen), hepatitis C virus (HCV) serology (antibody to HCV and HCV-RNA polymerase chain reaction), and autoantibodies (antinuclear antibody (ANA), anti-smooth muscle antibody, and anti-mitochondrial antibody). The upper normal limit of the ALT level was set at 30 U/L in our hospital. In all NASH patients, diet and exercise were directed.

All control subjects were Japanese and were confirmed to have normal liver function and no viral hepatitis infection by blood test. In addition, fatty liver was ruled out by ultrasound. This group was formed by enrolling volunteers. Informed consent was obtained from all patients and healthy controls before their entry into the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by our institution's research committee.

Serum OCT levels were measured by ELISA as reported previously.[8,9] Briefly, 50 μL of the HRP-conjugated F(ab') fraction of anti-OCT monoclonal IgG (secondary antibody, Mo5B11), and 50 μL of standard solution or sample diluted 10-fold with assay buffer (250 mmol/L glycine-buffer pH 9.4, containing 0.1% bovine serum albumin, 50 mmol/L NaCl and 0.1% ProClin950) were added to an antibody-coated dish (first antibody, Mo3B11). After mixing, the dish was incubated for 2 h and then washed with washing solution (10 mmol/L phosphate-buffer pH 7.4, containing 0.1% BSA, 150 mmol/L NaCl and 0.1% ProClin950). Then, a substrate solution (200 μg/mL 3, 3', 5, 5'-teramethylbenzidine containing 0.001% H_2O_2) was added. After the coloring reaction (20 min) was terminated by adding a stop solution (0.5 mol/L H_2SO_4), absorbance at 450 nm was measured with a microplate reader.

Statistic analysis

Data were expressed as mean ± standard deviation (SD). Statistical comparison among the four groups (control, NASH, NASH-LC, NASH-HCC) was conducted using Dunn's test, with $P < 0.05$ considered statistically significant. The comparison between F0-2 patients and F3-4 patients was performed by Mann–Whitney *U*-test. The correlations between serum OCT levels and serum ALT levels or serum AST levels or platelet count were confirmed by Spearman's correlation test.

RESULTS

DETAILS CONCERNING THE patients and control subjects are shown in Table 1. Serum levels of OCT, AST and ALT were measured. Table 2 shows the mean serum AST, ALT, OCT levels, platelte counts, and the ratios of AST : ALT, OCT : AST and OCT : ALT in 93 NASH patients without HCC and LC, 18 NASH-LC patients and 9 NASH-HCC patients. There were significant associations between serum OCT levels and serum AST levels or ALT levels in all patients and controls (ALT, $r = 0.774$, $P < 0.01$; AST, $r = 0.843$, $P < 0.01$). The AST and ALT levels of NASH-HCC were slightly increased compared to those of NASH-LC and NASH. The ALT level of NASH-LC was slightly decreased compared to those of

Table 1 Patient profiles

	N	Age (years)	Sex (M : F)	BMI (kg/m^2)
Control	80	21–59	43:37	ND
NASH	93	18–81	46:47	27.8 ± 5.3
NASH-LC	18	43–87	6:12	27.4 ± 5.7
NASH-HCC	9	61–77	7:2	27.5 ± 3.0

BMI, body mass index (mean ± SD); ND, not done; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NASH-HCC, non-alcoholic steatohepatitis with hepatocellular carcinoma; NASH-LC, liver cirrhosis induced by NASH.

NASH and NASH-HCC. However, regarding AST and ALT, the differences among the three groups (NASH, NASH-LC, NASH-HCC) were not significant. In contrast, the serum OCT levels in NASH were higher than those of controls, and gradually increased with development of liver disease from NASH to LC and HCC. In addition, the ratios of OCT : ALT and OCT : AST were significantly increased in parallel with the progression of NASH, LC and HCC. Especially, serum OCT levels and the ratios of OCT : ALT and OCT : AST were markedly increased in HCC. Regarding the comparison between NASH-LC and NASH-HCC, the serum OCT level and the ratio of OCT : AST of NASH-HCC were significantly higher than those of NASH-LC (both, $P < 0.05$). The ratio of OCT : ALT of NASH-HCC tended to be higher than that of NASH-LC ($P < 0.1$), but the ratios of AST/ALT in NASH-LC and NASH-HCC were almost equal. Regarding the relationships between OCT or ratios and platelet counts, there was a significant association between the ratio of OCT : ALT and platelet count ($r = -0.285$, $P < 0.01$). However, the relationships between platelet counts and OCT/AST ratio or OCT were not significant. Further, there were no significant relationships between body mass index and both ratios or OCT. Among all NASH patients, 42 patients had a normal range of ALT, and among these 42 patients, 13 (31%) had OCT over 43 ng/mL (mean ± 1.96 SD in control = 43.2 ng/mL). Of these 13 patients, eight had F3 or F4 fibrosis.

Figure 1 shows the association between liver fibrosis and serum OCT levels, the ratios of OCT : AST and OCT : ALT. The serum OCT levels and the OCT : ALT ratios were increased in parallel with liver fibrosis. In the comparison between F0-2 patients and F3-4 patients, OCT and both ratios in F3-4 patients were significantly higher than those of F0-2 patients (F0-2 patients (mean) OCT, 56.1; OCT : ALT, 1.20; OCT : AST, 1.69; F3-4 patients OCT, 144.8; OCT : ALT, 2.08; OCT : AST, 2.16).

Table 2 Serum levels of liver-specific markers and their ratios in NASH

Disease	OCT (ng/mL)	AST (U/L)	ALT (U/L)	AST : ALT	OCT : AST	OCT : ALT	Plt (×10^4/μL)
Control ($n = 80$)	20.6 ± 12.6	18.4 ± 4.3	16.3 ± 7.0	1.22 ± 0.33	1.09 ± 0.62	1.30 ± 0.87	N.D.
NASH ($n = 93$)	73.1 ± 68.8[a]	41.7 ± 39.6[a]	68.4 ± 99.4[a]	0.77 ± 0.30[a]	1.75 ± 0.86[a]	1.37 ± 1.00	22.4 ± 6.8
NASH-LC ($n = 18$)	82.8 ± 74.3[a]	42.9 ± 20.9[a]	41.2 ± 21.3[a,b]	1.13 ± 0.41[b]	1.84 ± 0.93[a]	2.02 ± 1.15[b]	13.5 ± 6.4[b]
NASH-HCC ($n = 9$)	398.3 ± 583.8[a,b,c]	93.3 ± 90.7[a]	105.8 ± 114.8[a]	1.05 ± 0.38[b]	3.41 ± 1.41[a,b,c]	3.44 ± 1.62[a,b]	12.8 ± 4.3[b]

Data are expressed as mean ± standard deviation (SD). [a]$P < 0.05$ versus control. [b]$P < 0.05$ versus NASH. [c]$P < 0.05$ versus NASH-LC.
ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; NASH, non-alcoholic steatohepatitis; NASH-HCC, non-alcoholic steatohepatitis with hepatocellular carcinoma; NASH-LC, liver cirrhosis induced by NASH; ND, not done; OCT, ornithine carbamoyltransferase; Plt, platelate.

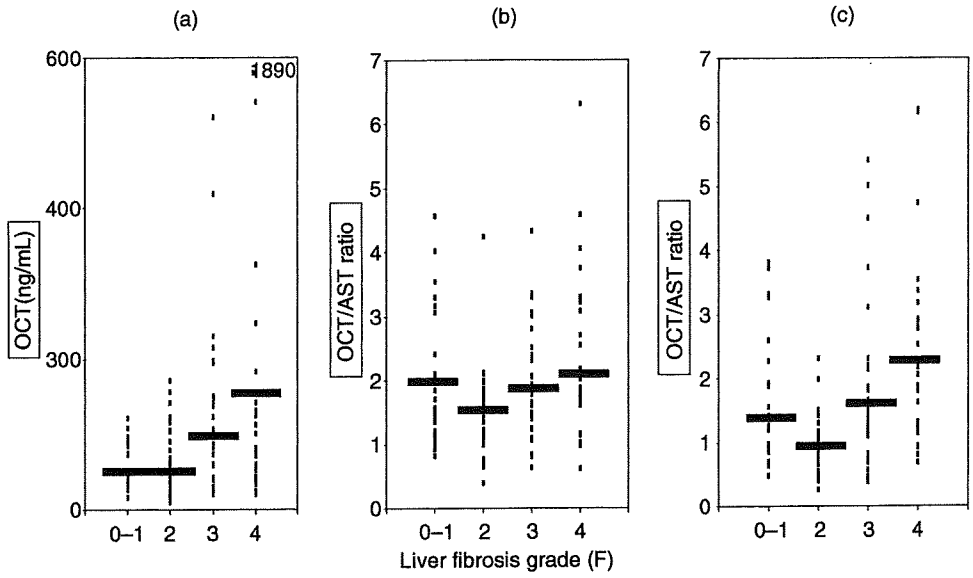

Figure 1 The relationship between liver fibrosis grade (F) and serum ornithine carbamoyltransferase (OCT) levels (a), OCT : alanine aminotransferase (ALT) ratio (b), OCT : aspartate aminotransferase ratio (c). The serum OCT levels and the ratio of OCT : ALT levels were increased in parallel with liver fibrosis. Bars express mean.

DISCUSSION

THIS IS THE first report of the measurement of OCT in NASH patients. An ideal biomarker should be simple, accurate, specific, inexpensive and readily available. OCT is highly liver-specific and a relatively abundant protein. Murayama and Watanabe[8,9] reported that OCT levels tended to be higher in HCC than in LC or CH patients induced by other etiologies, mainly viral hepatitis. It was confirmed that in NASH patients, OCT and the ratios of OCT : ALT and OCT : AST were increased in parallel with disease progression. Without liver biopsy, we can only speculate about the activity and progression of NASH by routine laboratory examinations. As the next step, we need to perform longitudinal analyses of serum OCT levels in NASH. Regarding HCC, the ratios of OCT : ALT and OCT : AST were markedly increased in NASH-HCC, suggesting that the increase of these ratios in HCC was a common phenomenon in various liver diseases. The reason why both ratios were increased in HCC is still unclear. One possibility is that cancer cells, expressing Fas-Ligand, might have induced apoptosis of hepatocytes.[16] Then, in apoptotic cell death, mitochondoria-related proteins were released. Another possibility is that the expression of enzymes might change in HCC. In any event, the ratios of OCT : ALT and OCT : AST were markedly increased in NASH-HCC, and there was significant association between fibrosis grade and OCT : ALT. Therefore, we believe that OCT and the ratios of OCT : ALT and OCT : AST are useful for monitoring the progression of NASH. We need to compare the sensitivity and specificity between these ratios and other tumor markers such as AFP or PIVKA-II.

Aminotransferase levels do not necessarily reflect the activity and progression of NASH. Fracanzani *et al.*[4] reported that more than half of NAFLD patients with persistently normal ALT have a potentially progressive liver disease. In our study, about 30% of NASH patients with a normal range of ALT show the elevation of OCT. In addition, the majority of these patients had severe fibrosis. Hashimoto *et al.*[17] reported that serum ALT levels in severe fibrosis of NASH were decreased. Taken together, in NASH patients with a normal rage of ALT and severe fibrosis, OCT might be a useful marker. It is unclear why OCT is frequently elevated in NASH patients with a normal rage of ALT and severe fibrosis. One possibility is that even if necrosis is minimum in NASH patients with severe fibrosis, mitochondria damage might continue.

The localization of OCT is restricted to mitochondria, which are mainly expressed in cytosol.[13] Some studies have reported that in alcoholic liver disease and NASH, oxidative stress induced endoplasmic reticulum stress and mitochondrial damage.[10,11,18,19] In

our preliminary data, the OCT : AST ratio of NASH was higher than that of viral hepatitis, suggesting that OCT might be a more useful marker in NASH, compared to in viral hepatitis.

As for the relationship between OCT and fibrosis grade, OCT levels and OCT : ALT ratios were increased in parallel with liver fibrosis. The relationship between the ratio of OCT : ALT and liver fibrosis was confirmed by that between the ratio of OCT : ALT and platelet count, which is associated with liver fibrosis grade. Murayama *et al.* reported in a chronic liver damage model that OCT was elevated compared to AST and ALT. The mechanism for this is still unknown. However, the expression of enzymes of parenchymal cells might change during the course of disease progression, as Murayama *et al.* reported that serum OCT levels were influenced by the state of Kupffer cell activation.[20]

AST and ALT do not necessarily reflect the activity and progression of NASH. In contrast, OCT testing is simple, inexpensive, and highly liver-specific, and serum OCT levels and the ratios of OCT : ALT and OCT : AST were elevated in concert with the progression of NASH. Thus, it was concluded that OCT might be a useful marker for revealing the progression of NASH.

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J Gastroenterol (2009) 44:976–98
DOI 10.1007/s00535-009-0085-z

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Influence of adiponectin gene polymorphisms in Japanese patients with non-alcoholic fatty liver disease

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Received: 6 December 2008 / Accepted: 8 May 2009 / Published online: 30 May 2009

Abstract
Purpose Single nucleotide polymorphisms (SNPs) of the adiponectin gene have been reported to be associated with insulin resistance and the prevalence of type-2 diabetes. We investigated the SNPs of adiponectin in non-alcoholic fatty liver disease (NAFLD) patients.
Methods One hundred nineteen patients histologically diagnosed as having NAFLD and 115 control subjects were examined. Adiponectin SNP sites were investigated at +45 of exon 2 and at +276 of intron 2; these sites have been thought to be associated with diabetes or insulin resistance.
Results Regarding the +276 SNP, the frequency of G/G tended to be higher in NAFLD patients than in controls, but not significantly. Among females only, however, the G/G frequency was significantly higher in NAFLD patients. As for the +45 SNP, in the severe fibrosis group, the frequency of G/G homozygotes was significantly higher than that in the mild fibrosis group, and G/G homozygotes of the +45 SNP proved by multivariate analysis to be an independent factor in severe fibrosis. In NAFLD patients with adiponectin +45 G/G, homeostasis model assessment-insulin resistance was significantly higher than in NAFLD patients without adiponectin +45 G/G.
Conclusion Adiponectin SNPs were found to be associated with the progression of liver fibrosis and insulin resistance, suggesting that adiponectin SNPs might play roles in the occurrence and progression of NAFLD.

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Keywords NAFLD · Adiponectin · SNP · Insulin resistance

Introduction

Non-alcoholic fatty liver disease (NAFLD) has recently been recognized as a leading cause of abnormal liver function tests. Its spectrum ranges from simple steatosis, which is usually a benign and non-progressive condition, to non-alcoholic steatohepatitis (NASH), which may progress to cirrhosis [1, 2]. Patients with NAFLD usually have insulin resistance syndrome, but Stefan et al. reported that hyperinsulinemia alone is not a major driving force for fatty liver [3, 4]. NASH's etiology remains unclear. Most investigators agree that its development requires underlying steatosis followed by a "second hit" that induces inflammation, fibrosis, or necrosis [2]. The interaction of cytokines with oxidative stress and lipid peroxidation has been postulated to play a key role in NASH [2, 5, 6].

Adiponectin is one of the adipo-cytokines associated with insulin resistance and type-2 diabetes [7, 8]. Several papers have reported a significant decrease in the serum levels of adiponectin in NASH patients [9, 10]. And the expression of adiponectin was lower in NASH liver than in those with simple steatosis [11]. Adiponectin is associated with liver fibrosis and inflammation [12, 13], suggesting that it might be associated with the pathogenesis of NASH.

Cytokine production rates vary among individuals [14]. Some of these differences may be related to polymorphisms in the cytokine genes themselves, or to polymorphisms in genes that regulate cytokine gene transcription. We have recently reported the relationships between tumor necrosis factor (TNF) gene polymorphisms and the progression to NASH [15]. Single nucleotide polymorphisms

(SNPs) of the adiponectin gene at +45 of exon 2 and +276 of intron 2 have been reported to be associated with insulin resistance and the prevalence of type-2 diabetes [16]. Other SNP sites of the adiponectin gene were reported [17, 18]. But many papers reported that adiponectin SNPs at +45 of exon 2 and at +276 of intron 2 were associated with diabetes, insulin resistance, and atherosclerosis, especially in East Asia [16, 19–21]. We therefore investigated these SNPs of the adiponectin gene, comparing them with the respective clinical and pathological findings.

Patients and methods

We evaluated 119 Japanese patients who were histologically diagnosed as having NAFLD at Tokyo Women's Medical University between 1995 and 2006, along with 115 healthy subjects who served as controls. The diagnosis of NAFLD was established based on the following criteria: (1) Histologically macrovesicular steatosis affecting at least 10% of hepatocytes. NAFLD activity score (NAS) was used to classify NAFLD into simple steatosis (NAS 0–2) or borderline (NAS 3–4) and steatohepatitis (NASH) (NAS $\geq$ 5) [22]. (2) Intake of less than 20 g of ethanol per day, as confirmed by physicians and family members with the patients. (3) Appropriate exclusion of other liver diseases such as alcoholic liver disease, viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, primary sclerosing cholangitis, and metabolic liver diseases.

Body mass index (BMI) was calculated by the standard formula: weight(kg)/[height(m)^2]. The diagnosis of type II diabetes mellitus (DM) was based on WHO criteria. Homeostasis model assessment-insulin resistance (HOMA-IR; fasting serum insulin μU/ml × fasting glucose mg/dl/405) was measured in NAFLD patients without DM [23]. None of the patients received drug treatment for NAFLD before the liver biopsy. A complete history was obtained, and physical examinations were performed in all patients. All patients underwent liver tests for measurement of the following laboratory parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), platelet count, hepatitis B serology (hepatitis B surface antigen, antibody to hepatitis B surface antigen, and antibody to hepatitis B core antigen), hepatitis C serology (antibody to hepatitis C virus and hepatitis C RNA polymerase chain reaction), and autoantibodies [antinuclear antibody (ANA), antismooth muscle antibody, and antimitochondrial antibody].

All liver biopsy specimens were examined using hematoxylin–eosin, Mallory, and silver reticulin staining. Fibrosis was scored using a 5-grade scale: F0, normal connective tissue; F1, perivenular or pericellular fibrosis in zone 3; F2, perivenular or pericellular fibrosis with focal or extensive portal/periportal fibrosis; F3, bridging or septal fibrosis; F4, cirrhosis [2, 24, 25]. Fibrosis was divided into mild (F0–2) and severe (F3–4) groups.

All control subjects were Japanese and matched for age and gender with NAFLD patients. Basically, control subjects consisted of hospital staff and medical students. To match age and gender to NAFLD patients, we recruited older volunteers from acquired relatives of hospital staff and patients. To exclude the possibility of latent fatty liver, control subjects with alcoholism or obesity (BMI < 25) were excluded. All control subjects were confirmed by a blood test to have normal liver function and no viral hepatitis infection. Informed consent was obtained from all patients and healthy controls before their entry into the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by our institution's research committee.

Genomic DNAs were obtained from peripheral blood leukocytes by standard phenol–chloroform extraction and amplified by the PCR method. Screening for adiponectin polymorphisms was determined with a fluorescent allele-specific DNA primer assay system, as described previously [26]. Adiponectin SNP sites were investigated at +45 of exon 2 and +276 of intron 2; both sites have been associated with diabetes or metabolic diseases [16].

Serum adiponectin levels were measured using enzyme-linked immunosorbent assays (human adiponectin ELISA kit, Otuka, Tokyo, Japan).

Statistical analysis

Analyses were performed with Dr SPSS (SPSS Institute, Tokyo, Japan). Data are presented as mean ± standard deviation (SD) or frequencies. Student's *t* test was used to compare the two groups with respect to normally distributed continuous variables, and the Mann–Whitney *U* test was used for skewed continuous variables. Normality was evaluated by the Shapiro–Wilks test. To evaluate the genotype between mild fibrosis (F0–2) and severe fibrosis (F3–4), univariable and multivariable logistic regression models were used. In subgroup analysis, the heterogeneity of the genotype prevalence was also evaluated in the model. Influences of profile, linearity, interaction, and collinearity in multivariate generalized linear models were examined using regression diagnostic analysis. Two-tailed *p* values less than 0.05 were considered to indicate a statistically significant difference.

Results

Table 1 shows the clinical data for NAFLD patients. There is no difference in age or gender between NAFLD patients and control subjects.

Table 2A shows data on the +276 SNP. The frequency of G/G tended to be higher in NAFLD patients (56.8%) than in the controls (51.3%), but the difference was not significant. In NASH patients (NAS ≥ 5) only, the G/G frequency was 56.8%, and there was no difference between NASH and other types of NAFLD (simple steatosis and borderline). Regarding the +45 SNP, the frequency of T/T tended to be lower (45.4%) in NAFLD patients than in the controls (51.3%), but the difference was not significant (Table 2B). In NASH patients only, the frequency of T/T homozygotes (47.7%) was not significantly different than in other types of NAFLD. BMI differs between NAFLD patients and control subjects. To exclude the influence of BMI, we compared non-obese control subjects and non-obese NAFLD patients (BMI < 25). In the 31 NAFLD patients without obesity, the frequency of G/G at +276 was 61.3% and the frequency of T/T at +45 was 48%. The tendency is almost the same, suggesting that BMI did not influence genotype data.

Table 1 Patient profiles

	NAFLD	Control
No.	119	115
Age (mean ± SD)	50.3 ± 17.8	47.5 ± 18.7
Gender (M/F)	65/54	58/57
Obesity (BMI > 25)	88 (73.9%)	0 (0%)
DM	53 (44.5%)	2 (1.7%)
HL	75 (63.0%)	12 (10.4%)
HT	29 (24.4%)	10 (8.7%)
BMI (kg/m^2)	28.3 ± 5.2	21.9 ± 2.0
AST	55.1 ± 31.7	18.5 ± 4.2
ALT	79.9 ± 58.9	16.2 ± 7.0
Plt	23.9 ± 28.8	ND
NASH (NAS ≥ 5)	88 (73.4%)	
F0–2	72 (60.5%)	
F3–4	47 (39.4%)	

ND not done

We compared the frequency of SNPs between females and males (Table 3A, B). Regarding the +276 SNP, in females only the frequency of the G allele (77.8%) was significantly higher than in female controls (64.9%). In contrast, in males only the frequency of the G allele did not differ. Regarding the +45 SNP, in females the frequency of the G allele (34.3%) tended to be higher than that of female controls (24.6%). In contrast, in males only, the frequency of the G allele was almost the same.

Tables 4A and B show the association between liver fibrosis and adiponectin SNPs. Regarding the +276 SNP, the G/G frequency of the severe-fibrosis group was 66.0%, indicating a higher frequency with fibrosis severity. Regarding the +45 SNP, the frequency of the G allele was significantly higher in the severe fibrosis group than in the mild fibrosis group (41.5 vs 27.1%, respectively; $p = 0.02$). Also, in the severe fibrosis group the frequency of G/G homozygotes (23.4%) was significantly higher than in

Table 2 Results of adiponectin gene SNPs

	Genotype			G allele frequency	OR	95% CI	*p* value
	G/G	G/T	T/T				
(A) +276 T/G SNP of intron 2							
Control ($n = 115$)	59	47	9	165/230	1		
	51.3%	40.9%	7.8%	71.7%			
NAFLD ($n = 118$)	67	47	4	181/236	1.30	0.85–1.97	NS
	56.8%	39.6%	3.4%	76.7%			
NASH ($n = 88$)	50	35	3	135/176	1.30	0.83–2.04	NS
	56.8%	39.8%	3.4%	76.7%			
	Genotype			G allele frequency	OR	95% CI	*p* value
	T/T	T/G	G/G				
(B) +45 T/G SNP of exon 2							
Control ($n = 115$)	59	44	12	66/230	1		
	51.3%	38.2%	10.4%	28.7%			
NAFLD ($n = 119$)	54	52	13	78/238	1.21	0.82–1.80	NS
	45.4%	43.7%	10.9%	32.8%			
NASH ($n = 88$)	42	36	10	56/176	1.16	0.76–1.78	NS
	47.7%	40.9%	11.4%	31.8%			

OR odds ratio, *CI* confidence interval, *NS* not significant

Table 3 Results of adiponectin gene SNPs in female and male patients

	Genotype			G allele frequency	OR	95% CI	*p* value
	G/G	G/T	T/T				
(A) +276 T/G SNP of intron 2							
Male control ($n = 58$)	36	19	3	91/116	1		
	62.0%	32.8%	5%	78.4%			
Male NAFLD ($n = 64$)	34	29	1	97/114	0.86	0.47–1.57	NS
	53.1%	45.3%	1.6%	75.8%			
Female control ($n = 57$)	23	28	6	74/114	1		
	40.4%	49.1%	10.5%	64.9%			
Female NAFLD ($n = 54$)	33[a]	18	3	84/108	1.89	1.04–3.43	0.03
	61.1%	33.3%	5.5%	77.8%			
	Genotype			G allele frequency	OR	95% CI	*p* value
	T/T	G/T	G/G				
(B) +45 T/G SNP of exon 2							
Male control ($n = 58$)	27	22	9	40/116			
	46.6%	37.9%	15.5%	34.5%	1		
Male NAFLD ($n = 65$)	31	27	7	41/130			
	47.7%	41.5%	10.8%	31.5%	0.88	0.51–1.49	NS
Female control ($n = 57$)	32	22	3	28/114	1		
	56.1%	38.6%	5.3%	24.6%			
Female NAFLD ($n = 54$)	23	25	6	37/108	1.60	0.89–2.87	NS
	42.6%	46.3%	11.1%	34.3%			

OR odds ratio, *CI* confidence interval, *NS* not significant

[a] The frequency of G/G homozygotes; female NAFLD vs female control, $p = 0.029$ by χ^2 test

the mild fibrosis group (2.8%) ($p < 0.01$). In addition, we compared the mild and severe fibrosis groups by multivariate analysis (Table 5). Gender, age, BMI, HOMA-IR, serum adiponectin level, +45 SNP, +276 SNP, DM, AST, ALT, hypertension, platelet, triglyceride, and total cholesterol were analyzed. In this multivariate analysis, G/G homozygotes of the +45 SNP proved to be an independent factor of severe fibrosis.

We next investigated the influence of SNPs on insulin resistance, as shown in Tables 6A and B. We measured HOMA-IR as an indicator of insulin resistance in non-diabetes patients, because in diabetes patients HOMA-IR does not represent correct insulin resistance. HOMA-IR tended to be higher in NAFLD patients with adiponectin +276 G/G than in those without it, but the difference was not significant (Table 6A). The frequency of HOMA-IR was significantly higher in NAFLD patients with adiponectin +45 G/G than in those without it (Table 6B; G/G, 5.67 ± 2.73; G/T or T/T, 3.40 ± 2.26, $p = 0.02$). Also, in patients with adiponectin +45 G/G, the frequency of HOMA > 4 was significantly increased by χ^2 test (frequency of HOMA > 4: 75% in G/G, 26% in G/T or T/T; $p < 0.01$).

We measured adiponectin levels of peripheral blood in 83 NAFLD patients. Tables 7A and B show the association between adiponectin SNPs and serum adiponectin levels. The level in NAFLD patients with both +45 G/G and BMI ≥25 tended to be lower than in patients with other genotypes, but we did not detect a significant difference between genotypes and serum adiponectin levels.

Among NAFLD patients, there was no significant association between genotypes and DM or obesity.

Regarding haplotypes, the frequencies of +45 G/G and +276 G/G were investigated. The frequencies of +45 G/G and +276 G/G (11.0%) were not particularly different from those of control subjects (10.4%). The frequencies of +45 G/G and +276 G/G in severe fibrosis (23.4%) are significantly higher than those in patients with mild fibrosis (2.8%).

Discussion

The present results suggested that adiponectin SNP is associated with the progression of liver fibrosis and insulin resistance.

Table 4 The association between liver fibrosis and adiponectin SNPs

Fibrosis grade	Genotype			G allele frequency	OR	95% CI	*p* value
	G/G	G/T	T/T				
(A) +276 T/G SNP of intron 2							
F0–2 ($n = 71$)	36	32	3	104/142	1		
	50.7%	45.1%	4.2%	73.2%			
F3–4 ($n = 47$)	31	15	1	77/94	1.65	0.87–3.15	NS
	66.0%	31.9%	2.1%	81.9%			
Fibrosis grade	Genotype			G allele frequency	OR	95% CI	*p* value
	T/T	T/G	G/G				
(B) +45 T/G SNP of exon 2							
F0–2 ($n = 72$)	35	35	2	39/144	1		
	48.6%	48.6%	2.8%	27.1%			
F3–4 ($n = 47$)	19	17	11[a]	39/94	1.91	1.10–3.31	0.02
	40.4%	36.2%	23.4%	41.5%			

OR odds ratio, *CI* confidence interval, *NS* not significant

[a] The frequency of G/G homozygotes; F3–4 vs F0–2, $p < 0.01$ by χ^2 test

Table 5 Multivariate analysis of clinical data and two adiponectin gene polymorphisms between mild fibrosis group and severe fibrosis group

	OR	95% CI	*p* value
Adiponectin +45 SNP (G/G)	71.7	3.67–1399	0.005
Age	1.16	1.07–1.26	0.001
Gender (male 0, female 1)	0.027	0.003–0.26	0.002
DM	8.12	1.49–44.2	0.015
BMI	1.28	1.06–1.55	0.011

Factors: age, gender, BMI, DM, hypertension, HOMA-IR, AST, ALT, platelets, triglyceride, total cholesterol, adiponectin +45 SNP(G/G), adiponectin +276 SNP(G/G), serum adiponectin level

Table 6 Association between HOMA-IR and adiponectin gene SNPs in patients without DM

Genotype	No.	HOMA-IR (mean ± SD)	*p* value
(A) +276 T/G SNP of intron 2			
G/G	28	4.06 ± 2.45	
G/T or T/T	20	3.25 ± 2.38	NS
(B) +45 T/G SNP of exon 2			
G/G	8	5.67 ± 2.73	
G/T or T/T	48	3.40 ± 2.26	0.02

NS not significant by Mann–Whitney test

Adiponectin is associated with insulin resistance and is an important factor in the pathogenesis of DM [7, 8]. In addition, adiponectin levels predict the severity of liver disease in NAFLD, even in the absence of diabetes and obesity [27, 28]. Hara et al. [16] have reported that in

Table 7 The association between serum adiponectin levels and adiponectin SNPs

Genotype	BMI > 25			BMI < 25		
	No.	Adiponectin	*p* value	No.	Adiponectin	*p* value
(A) +276 T/G SNP of intron 2						
G/G	34	6.89 ± 3.49		13	6.54 ± 4.17	
G/T	22	6.80 ± 3.10	NS	10	8.84 ± 5.12	NS
T/T	2	9.15 ± 1.48	NS	2	7.15 ± 2.76	NS
(B) +45 T/G SNP of exon 2						
G/G	7	5.70 ± 2.41		0		
G/T	25	6.72 ± 3.17	NS	11	6.64 ± 3.7	NS
T/T	27	7.53 ± 3.53	NS	13	8.15 ± 4.98	NS

Adiponectin was expressed as mean ± standard deviation (SD)
NS not significant

diabetes patients, the frequency of G/G at the +276 SNP is significantly higher (DM 58.3%, control 49.2%), and the frequency of T/T at the +45 SNP is significantly lower than in controls (DM 42.7%, control 52.3%). Almost the same percentages were observed in all NAFLD patients, but we did not observe a significant difference. To determine whether or not the difference between NAFLD and controls was significant, many more patients would be needed. In addition, this study did have a limitation with regard to the control subjects. Although none of the control subjects had abnormal liver function and none were obese, and although abdominal ultrasound examinations were performed in approximately 70% of the controls and no clear fatty liver disease was apparent, we cannot

completely rule out the possibility that the control group included those with mild steatosis, because we did not perform liver biopsies on the control subjects.

Sample size is a limitation of this study. However, we performed a liver biopsy in all NAFLD patients. Romeo et al. [29] performed an SNP study of many NAFLD patients but did not perform liver biopsies. On this point, our study provides important information.

We have already reported that significantly more male NAFLD patients overate and got less exercise than female patients, based on a questionnaire survey [30]. The frequencies of the adiponectin SNP sites differed significantly between NAFLD patients and controls only among females. Considering the survey data, it was speculated that genomic background might have more influence in female NAFLD and that lifestyle might have more influence in male NAFLD. Other papers have reported associations between adiponectin SNPs and insulin resistance, especially in women [31, 32]. Although only females showed significant differences, the numbers of female NAFLD and control subjects were small. In other reports, G/G frequencies at +276 among healthy women were 37.1–48.8% [31, 33], demonstrating that our female control data are reliable. When we compare the G/G frequency at +276 among female NAFLD patients to data on female control subjects in other papers, we find p values of 0.02–0.06. Therefore, these data suggest that our hypothesis is reasonable.

Recently, Musso et al. [34] reported that adiponectin SNPs at +45 and +276 modulate the acute adiponectin response to dietary fat and are associated with the presence of NAFLD in an Italian population. In addition, +45 TT and +276 GT/TT carriers had significantly increased prevalence and severity in NAFLD than in the other genotypes. Their results are not consistent with our own. But in Japan and other countries, many papers have reported that +276 GG and +45 GG are associated with low adiponectin, diabetes, insulin resistance, metabolic syndrome, and coronary artery diseases [19–21, 31, 33, 35]. This difference might be explained by ethnic differences. Even in NAFLD, the ethnic differences are well documented [36]. Differences similar to those found in the SNP study have frequently been observed. Regarding the TNF promoter SNPs in NASH, different results between Japanese and Italian populations have been reported [15, 37]. Adiponectin has been reported to be associated with insulin resistance and liver fibrosis [7, 12, 13]. In the present study, significant associations were found between adiponectin +45 SNP G/G and insulin resistance or liver fibrosis. Some papers have reported that insulin resistance is important for the pathogenesis of NASH [2, 3], but the relationship between insulin resistance and NAFLD has been controversial [4]. In our patients, about 16% did not have insulin resistance (HOMA < 2), suggesting that the etiologies of NAFLD were formed from several factors. Therefore, the difference in adiponectin SNPs between NAFLD and controls might not be so large. Anyway, our data indicate that adiponectin +45 SNP might play an important role in the pathogenesis and progression of NAFLD. Shimada et al. [38] reported that age and DM were proven to be risk factors for severe fibrosis. In the present study, it was important that adiponectin +45 SNP was added as an independent factor of severe fibrosis in NAFLD.

The serum adiponectin levels in NAFLD patients with both +45 G/G and BMI ≥25 tended to be lower than in those with other genotypes, but we did not detect a significant difference. Basically, adiponectin production was influenced by the volume of visceral fat. To clarify this association, we must measure serum adiponectin levels in patients who have almost equal BMI. However, it is difficult to compare subjects who have the same BMI. Another possibility is that adiponectin gene SNPs might link with the expression of other genes except adiponectin genes.

Recently, it was reported that adiponectin receptor SNPs might affect insulin sensitivity and liver fat [39]. The SNPs of both adiponectin and adiponectin receptor might play important roles. Finally, we hope that genomic analysis, including that of adiponectin genes, as well as lifestyle surveys will clarify the pathogenesis and progression of NAFLD, leading to therapy.

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