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Induction of ABCA1 by overexpression of hormone-sensitive lipase in macrophages

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ABSTRACT

Initial step toward the reverse-cholesterol transport is cholesterol efflux that is mediated by the ATP-binding cassette transporter A1 (ABCA1). However, it is unknown how the cholesteryl ester (CE) hydrolysis induces the expression of the ABCA1 gene. Overexpression of hormone-sensitive lipase (HSL) increased the hydrolysis of CE and stimulated the expression of ABCA1 gene at the transcriptional level in RAW 264.7 macrophages. The stimulatory effects of the HSL overexpression and cholesterol loading on the ABCA1 promoter activity were additive. Mutational analyses of the promoter of ABCA1 identified the responsible element as the direct repeat-4 (DR-4) that binds LXR/RXR heterodimers. In conclusion, stimulation of hydrolysis of CE in macrophages induces the expression of ABCA1 gene primarily via the LXR-dependent pathway and can be useful for the prevention of atherosclerosis.

Introduction

The initial step for the elimination of cholesterol from foam cells, a hallmark of atherosclerosis, is mediated by two pathways: passive efflux from the cell membrane to HDL and energy-dependent efflux mediated by apolipoproteins [1]. The latter pathway has been reported to be defective in Tangier disease or familial HDL deficiency [2] which is caused by inherited mutations in the gene for ATP-binding cassette (ABC) A1 [3–9], a member of ABC transporter superfamily whose members are characterized by two nucleotide binding folds (NBF) with conserved Walker A and B motifs and two transmembrane domains, each consisting of six membrane spanning helices [10]. The human ABCA1 gene on chromosome 9q31 [6] encodes a protein with 2231 amino acids and is ubiquitously expressed particularly in placenta, fetal tissues, liver, lung, and adrenal glands [11,12].

The mRNA expression of ABCA1 is induced by cholesterol loading of macrophages through incubation with acetylated LDL (acetyl-LDL) [11]. This sterol-dependent transactivation is mediated by nuclear hormone receptors of the liver X receptor (LXR) and retinoid X receptor (RXR) family [13–15]. The cytosolic CE is in turn hydrolyzed by neutral CE hydrolase (NCEH) to generate FC and fatty acids [16]. Several investigators [17–19] have attributed the NCEH activity to hormone-sensitive lipase (HSL), a cytosolic enzyme that hydrolyzes intracellular triacylglycerol (TG), diacyl glycerol (DG), CE, and retinyl ester in various organs including adipose tissue, adrenal gland and testes [20,21]. Previously, we have shown that the adenovirus-mediated overexpression of HSL in THP-1 macrophages stimulates the efflux of cholesterol from foam cell macrophages with a concomitant increase in ABCA1 mRNA, resulting in nearly complete elimination of the intracellular CE [22]. However, it is unclear how the increased hydrolysis of CE up-regulates the ABCA1 gene expression. Here, we show that the transactivation of human ABCA1 promoter by increased hydrolysis of CE depends on the binding of LXR/RXR to a DR4 element.

Materials and methods

General procedures. Immunoblot analysis and measurements of cellular lipids were performed essentially as described [22].

Lipoproteins. Ultracentrifugation was used to isolate β-migrating VLDL (β-VLDL) from plasma of Japanese white rabbits (3 kg male) that were fed a chow diet supplemented with 1% (w/w) cholesterol.

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Plasmid constructs. The HSL cDNA was subcloned into the polylinker region of pCI to obtain the plasmid vector expressing HSL cDNA under the promoter of CMV [23]. The promoter region of ABCA1 gene (from −928 to +101 bp) was amplified from human genomic DNA (CLONTECH) by PCR using Platinum Pfx DNA polymerase (Invitrogen Life Technologies, Inc.) and the following primers: forward primer, 5′-GATCGATCAGATCTTAAGTTGGAGGTCT GGAGTG-3′; reverse primer, 5′-GATCGATCAAGCTTGCTCTGTTGGT GCGCGGA-3′, which were designed based on the reported DNA sequence [24] and contained restriction sites for BglII and HindIII at the 5′ end of the forward and reverse primers, respectively. ABCA1 promoters with various deletion mutations were generated by PCR using TaKaRa LA TaqTM (TaKaRa) and the following primers: forward primer1 (−406 to +928), 5′-GATCGATCAGATCTAGCAGGA TTTAGAGGAAGCA-3′; forward primer2 (−106 to +928), 5′-GATCGA TCAGATCTCGGGCCCCGGCTCCACGTG-3′; forward primer3 (−100 to +928), 5′-GATCGATCAGATCTAGGGGGCGGGGAGGAGGGA-3′; forward primer4 (−36 to +928), 5′-GATCGATCAGATCTGAATCTAT AAAAGGAACTA-3′; reverse primer, 5′-TAGATCGCAGATCTCGAGCCC G-3′. Primers used for generation of the mutated LXR/DR4 element were as follows: forward primer, 5′-GATCGATCctgcaGATAGTAA CCTCTGCGCTCGG-3′; reverse primer, 5′-GATCGATCtgcagAAGCCTG TGCTGTGCTCTCCCTCCTC-3′ [13,14]. The amplified fragments were digested with BglII and HindIII and subcloned into the site of the polylinker region of pGL3-basic vector (Promega) by using DNA Ligation Kit Ver. 2 (TaKaRa).

Recombinant adenovirus. A fragment of ABCA1 promoter region (−926 to +101) and luciferase gene were subcloned into Gateway® entry vector pENTR4 (Invitrogen). pAd-ABCA1-luc was obtained by Vira Power Adenoviral Expression system (Invitrogen). Briefly, the plasmid was generated by performing an LR recombination reaction between pENTR-ABCA1 and pAd/PL-DEST. pAd-ABCA1-luc was transfected into 293A cells and the recombinant adenoviruses were produced according to the manufacturer's instructions. In our experiments, 1 multiplicity of infection (m.o.i.) corresponded to 183 particles of adenovirus of ABCA-luc (Ad-ABCA1-luc) and 25 particles of adenovirus LacZ (Ad-LacZ) used as a control per cell. Cells were infected at m.o.i. indicated in figures.

Cell culture. RAW264.7, THP-1 cells and mouse peritoneal macrophages (MPM) prepared from 8- to 9-month-old C57BL/6J mice were cultured as described [22,23].

NCEH assay. Protein for the NCEH assay was prepared as described previously [22,23].

Northern blot analysis. Total RNA was prepared from RAW264.7 cells with TRIzol reagent (Invitrogen Life Technologies, Inc.). RNA (30 μg) was electrophoresed through formalin-denatured agarose gels and transferred to Hybond-N+ membranes (GE Healthcare). Probes for ABCA1 were constructed from cDNA fragments amplified by RT-PCR using cDNA obtained from mouse liver as a template. Primer sequences forward primer: 5′-TAGGCTTGTTGGCCT CAGCT-3′, reverse primer: 5′-TTGCGCATGTCCTTCATGCT-3′. Probes were labeled with [α-^{32}P]dCTP (~6000 Ci/mmol; Perkin Elmer) using Megaprime DNA Labeling System (GE Healthcare). Membranes were hybridized with the radiolabeled probes in Rapid-hyb Buffer (GE Healthcare), washed in 0.1× SSC, 0.1% SDS at 65 °C, and exposed to BAS2000 phosphoimager (Fuji Film, Tokyo).

Luciferase reporter assay. Twenty-four hours after culturing of 4.0×10^4 cells/well in 96-well plates, the cells were transfected with 0.5 μg of total DNA mixture of pGL3-ABCA1 promoter with or without pCI-HSL by Superfect® Transfection Reagent (QIAGEN). After incubation in DMEM containing 5 mg/ml BSA for 12 h, the cells were incubated in DMEM containing 5 mg/ml BSA and various concentrations of β-VLDL for 12 h. Luciferase activities were measured by Steady-Glo Luciferase Assay System (Promega) and TR717 Microplate Luminometer (Applied Biosystems).

Electrophoretic mobility shift assay. A fragment containing His(HHHHHH)-HA(YPYDVPDYA)-FLAG(DYKDDDDK) was subcloned into HindIII/BamHI of the polylinker region of pcDNA3.1(+) (Invitrogen) to obtain pcDNA3.1/HisHAFLAG/N. Mouse cDNA for LXRα or RXRα amplified by RT-PCR was then ligated into BamHI/XhoI site in the pcDNA3.1/HisHAFLAG/N to obtain pcDNA3.1/HisHAFLAG/N-mLXRα or pcDNA3.1/HisHAFLAG/N-mRXRα, respectively. A fragment containing cDNA for mouse LXRβ, which was obtained from pCMV7-mLXRβ (a gift from Dr. David Russell) by digestion with SalI and NotI, was blunt ended with Klenow fragment and subcloned into EcoRV site of pcDNA3.1/myc-HisB to get pcDNA3.1/myc-HisB-mLXRβ.

mLXRα, mLXRβ and mRXRα proteins were translated by a TNT Quick Coupled Transcription/Translation System (Promega) using pcDNA3.1/HisHAFLAG/N-mLXRα, pcDNA3.1/myc-HisB-mLXRβ or pcDNA3.1/HisHAFLAG/N-mRXRα as templates.

Oligonucleotide containing LXR/DR4 (5′-TTTGACCGGTAGTAG TAACCCCGGCGC-3′) was labeled with [γ^{32}P]ATP (Perkin Elmer) by T4 polynucleotide kinase (TaKaRa), and was mixed with the proteins synthesized by IVTT and incubated in binding buffer containing 10 mM Hepes, 50 mM KCl, 1 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 0.4 μg of poly(dI–dC), and 10 μg of BSA on ice for 30 min and reacted with or without antibodies against LXRα, LXRβ, or RXRα for 30 min, and subjected to electrophoresis in 4% polyacrylamide gel. The binding was competed by the unlabeled oligonucleotides containing LXR/DR4 or mutated LXR/DR4 (5′-TTCTGCAGGTAGTAACCCCGGCG C-3′). The gels was dried and exposed to BAS2000 phosphoimager (Fuji Film, Tokyo).

Statistical analyses. All values are stated as means ± SD, and differences between groups were evaluated with ANOVA, unless otherwise stated. All calculations were performed with STAT view version 5.0 for Macintosh (SAS Institute).

Results and discussion

Transfection of pCI-HSL resulted in a dose-dependent increase in the expression of HSL protein, whose molecular weight is identical to that expressed in white adipose tissue (WAT), in RAW264.7 cells (Fig. 1A). In parallel, it increased the NCEH activity by 1.8-fold compared to that expressed endogenously in RAW264.7 cells (Fig. 1B). The levels of expression were much lower than those which we reported in our previous study, in which we used adenovirus-mediated gene transfer in THP-1 cells [22].

Incubation with β-VLDL increased the cellular contents of CE by 9-fold at the concentration of 10 μg/ml. The overexpression of HSL significantly decreased the cellular CE contents by 11%, while it increased the FC contents by 50% at the concentration of 10 μg/ml of β-VLDL (data not shown).

To examine whether the overexpression of HSL affects the mRNA expression of endogenous ABCA1gene, we performed Northern blot analyses (Fig. 1C). Incubation with β-VLDL or transfection with pCI-HSL alone did not affect the mRNA expression of ABCA1. However, the overexpression of HSL in the presence of β-VLDL increased the mRNA expression of ABCA1 gene by 2-fold.

To investigate the mechanism behind the increased expression of ABCA1 gene in RAW264.7 cells, we have generated a firefly luciferase reporter gene construct containing nucleotides −928 to +101 of the ABCA1 gene (pGL3-ABCA1) and co-transfected both pCI-HSL and pGL3-ABCA1 to RAW264.7 cells which were subsequently incubated with increasing concentrations of β-VLDL (Fig. 1D). Incubation with 2.5 μg/ml of β-VLDL per se stimulated the ABCA1 promoter activities by 1.8-fold. Further increases in the concentration of β-VLDL did not cause additional increase in the ABCA1 promoter activity. At every concentration of β-VLDL, the overexpression of HSL further stimulated the ABCA1 promoter activities

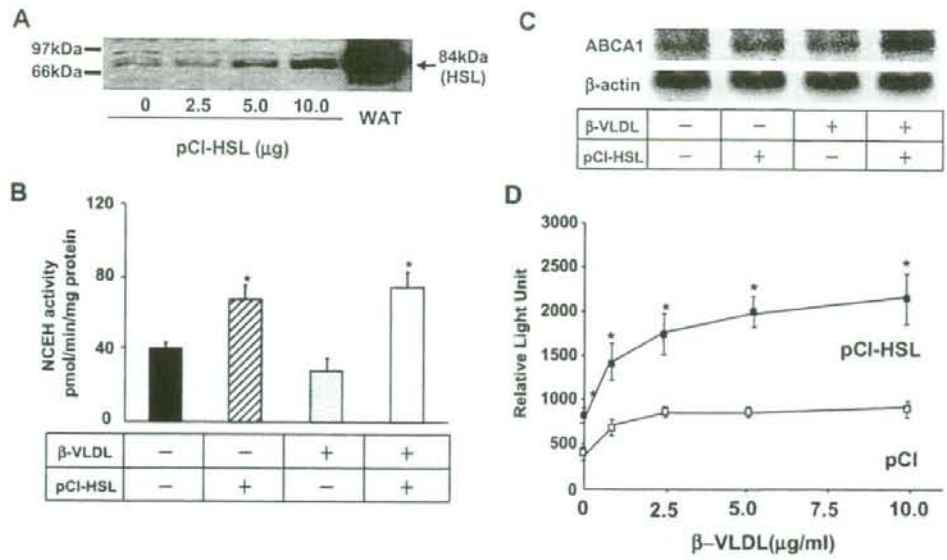

Fig. 1. HSL protein expression (A) and NCEH activities (B) in RAW 264.7 cells overexpressing HSL. Stimulation of mRNA expression (C) and promoter activities (D) of ABCA1 gene by the overexpression of HSL. RAW 264.7 cells were plated in 10-cm dishes and transiently transfected with the indicated amounts of pCI-HSL and total cell lysates were subjected to Western blot analysis using an epididymal fat pad as a control. White adipose tissue, WAT (A). RAW 264.7 cells were grown in 12-well (B) or 6-well plates (C) and transiently transfected with 0.75 or 1.0 μg of pCI-HSL or pCI, respectively, followed by incubation with or without 2.5 μg/ml of β-VLDL. Cell lysates were used for the measurements of NCEH activities (B) and total RNA was subjected to Northern blot analyses for ABCA1 or β-actin (C). RAW 264.7 cells were grown in 96-well plates and transiently transfected with 0.25 μg of pCI-HSL (closed square) or pCI (open square) and 0.25 μg of pGL3-ABCA1-luc in RAW 264.7 cells, and incubated with the indicated concentrations of β-VLDL (D). Bars indicate means ± S.D. $^{*}P < 0.001$, HSL(−)β-VLDL(−) vs HSL(+)β-VLDL(−); HSL(−)β-VLDL(+) vs HSL(+)β-VLDL(+) (B). pCI-HSL vs pCI (D).

by ~2.6-fold. Similar results were obtained in an independent experiment using the fixed concentration of β-VLDL (data not shown).

It is known that activation of peroxisome proliferator-activated receptor (PPAR)α or PPARγ up-regulates ABCA1 gene expression via increasing the expression of LXRα [25,26]. Furthermore, hydrolysis of CE by the overexpressed HSL may provide fatty acids, which may function as a ligand for PPARs, by hydrolyzing CE, TG and retinyl ester. Therefore, it is important to know whether agonists for PPARs transactivate the expression of ABCA1gene in various macrophage-like cell lines. To compare the stimulatory activities of ligands for PPARs with that of ligands for LXRs, we treated three different macrophage-like cells with bezafibrate, a PPARα agonist, pioglitazone, a PPARγ agonist, T0901317, 22(R)-hydroxycholesterol and 24(*S*),25-epoxycholesterol, LXR agonists, and 9-*cis*-retinoic acid, a RXRα agonist (Supplementary figure). With regards to LXR agonists, all of the three significantly transactivated the ABCA1 gene in all of the three lines of cells. 9-cis-retinoic acid transactivated the ABCA1 gene in THP-1 and RAW264.7 cells, but not in MPM. However, neither pioglitazone nor bezafibrate significantly transactivated the ABCA1 gene at least in RAW264.7 cells. In THP-1 cells and MPM, pioglitazone transactivated the gene marginally, but bezafibrate did not. Thus, liberation of cholesterol for conversion to oxysterols might be the most plausible explanation at present.

To define the element in the promoter of ABCA1 that mediated the stimulation of the transcription of the gene, we performed electrophoretic mobility shift assays using recombinant LXRα, LXRβ and RXRα (Fig. 2A). When the ABCA1 wild-type DR4 element was used alone, a single major shift in activity was detected for both LXRα/RXRα and LXRβ/RXRα (Fig. 2A, lanes 2 and 8), which disappeared with excess of cold wild-type competitor (Fig. 2A, lanes 3 and 9) but not with excess of cold mutant competitor (Fig. 2A, lanes 4 and 10). Inclusion of an antibody against LXRα or LXRβ in the binding reaction resulted in a supershift in the LXRα/RXRα or LXRβ/RXRα, respectively (Fig. 2A, lanes 5 and 11, while inclusion of an antibody against RXRα resulted in a supershift in both reactions (Fig. 2A, lanes 6 and 12).

To map the response element which mediates the HSL-stimulated ABCA1 transcription, we generated several promoter constructs with various length of deletion (Fig. 2B). The response was clearly preserved when the promoter insert was sequentially deleted down to position −100, and subsequently lost when sequences −100 to −36 were removed. However the latter deletion mutant lacked promoter activity, presumably because it no longer contained the element for basic transcription factors such as Sp1. The relative induction by β-VLDL was the lowest in the construct with the sequences from −160 to +101 of the promoter, while it was the highest in the construct with the sequences from −100 to +101 of the promoter. Similarly, the relative induction by the overexpression of HSL was the lowest in the construct with the sequences from −160 to +101 of the promoter, while it was the highest in the construct with the sequences from −100 to +101 of the promoter. These results indicate that a negative regulatory element is present between −160 and −100 bp of the promoter. It is of note that the promoter region between −100 and −36 bp is required for the induction of the transcription both by β-VLDL and by the overexpression of HSL. Since this region contains DR4 element, an established binding site for LXR/RXR, we presume that this element mediates the HSL-induced stimulation of the transcription of the ABCA1 gene. To test this hypothesis, we have mutated the DR4 element in the promoter sequences from −928 to +101 bp (Fig. 2C). The promoter with the mutated DR4 element had negligible basal transcription activity. Furthermore, it did not respond either to β-VLDL or to the overexpression of HSL, supporting the critical role of this element in the stimulation of the transcription of ABCA1 gene by the increased hydrolysis of CE. This pattern of

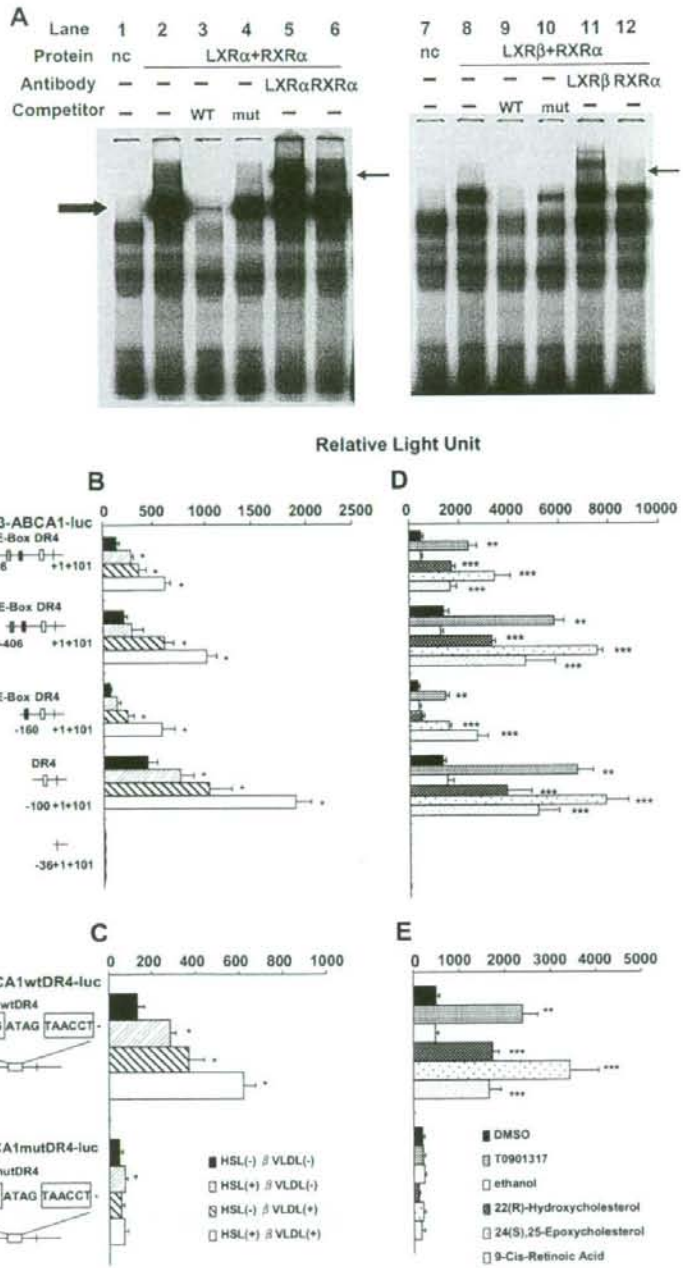

Fig. 2. Electrophoretic mobility shift assay of human ABCA1 promoter fragment (A) and mutational analysis of ABCA1 promoter (B, C, D, and E). Oligonucleotides containing the DR4 element were ^{32}P-radiolabeled and incubated with recombinant LXRα/RXRα (lanes 1–6) or LXRβ/RXRα without or with cold wild-type (lanes 3 and 9) or mutated (lanes 4 and 10) competitor oligonucleotides. Antibody against LXRα (lane 5), LXRβ (lane 11) or RXRα (lanes 6 and 12) was included in the reaction mixture. Position of bands corresponding to the ABCA1 promoter fragment that was bound to LXRα/RXRα or LXRβ/RXRα is indicated by a big arrow. Position of supershift bands is indicated by a small arrow (A). RAW 264.7 cells were grown in 96-well plates and transiently transfected with 0.25 μg of the plasmids containing the indicated hABCA1 promoter with deletions (B,D) or mutated DR4 (C,E). (B,C) The cells were co-transfected with 0.25 μg of pCI-HSL or pCI followed by incubation with or without 2.5 μg/ml of β-VLDL. (D,E) The cells were incubated with 10^{-5} M of various compounds indicated in (E). Bars indicate means ± SD. $^{*}P < 0.05$, HSL(−) β-VLDL(−) vs HSL(−) β-VLDL(−); HSL(−) β-VLDL(−) vs HSL(−) β-VLDL(+); HSL(−) β-VLDL(+) vs HSL(+) β-VLDL(+); $^{**}P < 0.01$ vs DMSO; $^{***}P < 0.01$ vs ethanol.

transactivation of ABCA1 gene was recapitulated by agonists for LXR/RXRα, T0901317, 22(*R*)-hydroxycholesterol, 24(*S*),25-epoxyxholesterol and 9-*cis*-retionoic acid as shown in Fig. 2D and E.

In conclusion, overexpression of HSL increases the supply of free cholesterol as a ligand for LXR, thereby stimulates the expression of ABCA1 gene in macrophages. This pathway can be used as a therapeutic target for the treatment of atherosclerosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.08.101.

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Original Article

Pitavastatin Decreases Plasma Preβ1-HDL Concentration and Might Promote its Disappearance Rate in Hypercholesterolemic Patients

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Aim: Preβ1-HDL is involved in the initial step of cholesterol efflux from peripheral cells and plays an important role in reverse cholesterol transport. We studied the effect of pitavastatin on the HDL subfraction profile, preβ1-HDL concentration and its disappearance rate.
Methods: Twenty-nine hypercholesterolemic patients were treated with pitavastatin at 2 mg/day for 4 weeks, and plasma levels of total cholesterol (TC), triglyceride, HDL-cholesterol (C), HDL_2-C, HDL_3-C, preβ1-HDL, LCAT activity, and CETP mass were assayed. The preβ1-HDL disappearance rate was determined as the difference in preβ1-HDL concentration before and after incubation at 37 ℃ for 90 min divided by the pre-incubation preβ1-HDL concentration.
Results: Pitavastatin led to significant decreases in TC by 26.9% and LDL-C by 39.8%. HDL-C and HDL_2-C increased significantly by 6.0% and 9.0%, respectively, but there was no significant change in HDL_3-C. Preβ1-HDL concentration significantly decreased (−8.7%; $p<0.05$); however, its disappearance rate significantly increased (13.0%; $p<0.05$). There were significant decreases in both LCAT activity and CETP mass.
Conclusion: Although pitavastatin decreased plasma preβ1-HDL concentration, it increased the preβ1-HDL disappearance rate. These data suggest that pitavastatin might promote the early step of reverse cholesterol transport.

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Key words; Statin, HDL subfraction, preβ1-HDL, LCAT

Introduction

High-density lipoprotein (HDL) is the only anti-atherosclerotic lipoprotein in plasma and its clinical significance has attracted attention[1]. One important mechanism of the anti-atherosclerotic effect of HDL is its role in reverse cholesterol transport (RCT)[2]. This effect is supposed to be prominent in patients with high plasma levels of HDL-cholesterol (HDL-C). Many epidemiological studies have demonstrated that HDL-C is a negative risk factor for coronary artery disease[3-5]; however, there are also reports that the plasma HDL-C concentration does not always reflect the magnitude of its anti-atherosclerotic effect, and this issue remains the subject of debate[6].

Preβ1-HDL, a subclass of HDL migrating to the preβ position on agarose gel electrophoresis, plays an important role in the initial step of RCT[7]. In one pathway, called the specific pathway, preβ1-HDL interacts with cell membranes and takes up cholesterol via ATP-binding cassette (ABC) A1 expressed on the cell surface. Preβ1-HDL conversion to α-HDL is one of the critical steps in this specific RCT and is mediated by lecithin: cholesterol acyltransferase (LCAT)[8]. Although the clinical significance of plasma preβ1-HDL concentration and its conversion reaction is not completely clear, accumulating evidence suggests that impaired conversion of preβ1-HDL, resulting in high plasma preβ1-HDL concentration, is involved in the initiation and progression of atherosclerosis.

HMG-CoA reductase inhibitors (statins) are

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widely used to treat hypercholesterolemia and have proved the usefulness of primary and secondary prevention for cardiovascular disease. The anti-atherosclerotic effect of statins on plasma lipoproteins can be explained largely by the reduction of LDL-C levels[9, 10]; the effects are thought to involve the elevation in HDL-C[11]. The effects of statins on plasma lipoproteins in Japanese hyperlipidemic patients have been described[12, 13]; however, the mechanisms are not completely clear. Although there are several reports of the effects of statins on the HDL subfraction profile and plasma preβ1-HDL concentration[14, 15], there have been no studies of their effects on the disappearance of preβ1-HDL.

In this study, we assessed the effects of pitavastatin on the HDL subfraction profile and the disappearance rate of preβ1-HDL in patients with hypercholesterolemia. We also assessed changes in LCAT activity and the cholesteryl ester transfer protein (CETP) mass, which play important roles in HDL metabolism.

Materials and Methods

The institutional ethics committee of Jichi Medical University approved the conduct of this study from January to December, 2004. The principal investigator or sub-investigator provided adequate explanation to the subjects concerning this study, and the subjects consented to participate and gave written consent.

Twenty-nine subjects (15 men, 14 women; mean age: 57.31 ± 11.0 years (29 – 74)) with hypercholesterolemia were recruited. Eleven subjects had type 2 diabetes (7 men, 4 women). The inclusion criteria were: patients whose physician had determined that pitavastatin was needed, patients with type IIa or IIb hyperlipidemia who were adult men or post-menopausal women with total cholesterol (TC) of 220 mg/dL or greater, triglyceride (TG) of 400 mg/dL or less, and patients not receiving other HMG-CoA reductase inhibitors. The exclusions were: patients with serious hepatic or renal dysfunction, poorly controlled diabetes or severe hypertension, hypothyroidism, patients on cyclosporin, patients with recent myocardial infarction or cerebrovascular accident (within 3 months of the event), patients with heart failure, patients with drug or alcohol abuse, patients with a history of drug hypersensitivity or serious adverse drug reactions.

The subjects were treated with pitavastatin 2 mg/day for 4 weeks. During the study, co-administration of drugs that may affect serum lipids (drugs for hyperlipidemia other than the study drug, drugs for insulin resistance, various hormonal agents, anti-psychotic agents, tricyclic antidepressants, cholagogue drugs, anti-obesity drugs or appetite-stimulating drugs, levothyroxin, immunosuppressive agents) was prohibited. Concurrent administration of other drugs was permitted. Whenever possible, the type and dose were not changed during the study. The subjects were instructed to maintain their normal daily activities and diet during the study without any change. On the day prior to blood drawing, excessive eating and drinking, including alcohol after 9 pm, were prohibited. On the day of blood drawing, the subjects fasted prior to the blood drawing, although water was permitted. At week 0 and 4, a fasting blood sample was collected into an EDTA containing a glass tube precooled with ice-water. Plasma was separated by centrifugation at 0℃ and further analysis was performed immediately.

Lipoprotein Assay

TC, TG, and HDL-C were measured by automated enzymatic assays, LDL-C was calculated by the Friedewald formula (LDL-C = (TC – HDL-C) – TG/5)[16]. HDL_2-C and HDL_3-C were measured by automated enzymatic assay after ultracentrifugation. Preβ1-HDL was measured by a sandwich enzyme immunoassay using an anti-preβ1-HDL antibody, reported by Miyazaki *et al.* (Mab55201, Daiichi Pure Chemicals Co., Ltd., Tokyo)[17].

Determination of Preβ1-HDL Disappearance Rate

To determine the disappearance rate of preβ1-HDL, we incubated plasma at 37℃ for 90 min and measured the preβ1-HDL concentration before and after incubation. The disappearance rate was defined as the difference in preβ1-HDL concentration before and after incubation divided by the pre-incubation preβ1-HDL concentration: {disappearance rate = [(preβ1-HDL concentration before incubation) – (preβ1-HDL concentration after incubation at 37℃ for 90 min.)] / (preβ1-HDL concentration before incubation)}, reported by Fielding *et al.*[18].

Enzyme Activity Assays

LCAT activity was determined by the method of Nagasaki *et al.*[19] using dipalmitoyl lecithin as the substrate, and CETP mass was determined by an ELISA assay (CETP ELIZA-DAIICHI, Daiichi Pure Chemicals Co., Ltd., Tokyo).

Statistical Analysis

Each parameter was analyzed using the one-sample *t*-test. The correlation between variables was assessed using Pearson's correlation coefficient test. The significance level was 5% (two-tailed), and data are expressed as the mean ± standard deviation.

Table 1. Effects of pitavastatin on plasma lipids in 29 hypercholesterolemic patients

	before treatment	after treatment	% change
TC (mg/dL)	277 ± 63	200 ± 38***	−26.9
TG (mg/dL)	136 ± 78	126 ± 74	−2.7
LDL-C (mg/dL)	195 ± 62	117 ± 39***	−39.8
HDL-C (mg/dL)	54.3 ± 14.6	57.4 ± 16.1**	+6.0

***$p<0.001$, **$p<0.01$

Table 2. Effects of pitavastatin on HDL subfraction, disappearance rate of preβ1-HDL, LCAT activity and CETP mass

	normal range	before treatment	after treatment	% change
HDL_2-C (mg/dL)	16.0-73.0	35.4 ± 13.7	38.6 ± 16.1***	+9.0
HDL_3-C (mg/dL)	13.0-25.0	19.5 ± 3.0	19.5 ± 3.0	+0.9
Preβ1HDL (μg/dL)	15.0-30.0[23]	14.9 ± 9.1	12.3 ± 6.0*	−8.7
Disappearance rate of Preβ1-HDL (%)		66.8 ± 16.0	71.9 ± 10.1*	+13.0
LCAT activity (nmol/mL/hr/37℃)	53.3-108.2	125 ± 24	103 ± 21***	−16.9
CETP mass (μg/mL)	0.8-3.0	2.0 ± 0.3	1.8 ± 0.4**	−7.6

***$p<0.001$, **$p<0.01$, *$p<0.05$

Results

Changes in Plasma Lipids and Lipoproteins

After treatment with pitavastatin of 2 mg/day for 4 weeks, TC decreased by −26.9% ($p<0.001$) and LDL-C by −39.8% ($p<0.001$). HDL-C increased significantly by +6.0% ($p<0.01$). On the other hand, TG changed by −2.7%, which was not significant (**Table 1**).

Changes in HDL Subfractions, Preβ1-HDL Concentrations and its Conversion Rate, LCAT Activity, and CETP Mass

HDL_2-C increased significantly by +9.0% ($p<0.001$), while HDL_3-C increased by +0.9%, which was not significant (**Table 2**). Preβ1-HDL concentration decreased significantly by −8.7% ($p<0.05$), while the preβ1-HDL conversion rate increased significantly by +13.0% ($p<0.05$) (**Table 2**). LCAT activity decreased significantly by −16.9% ($p<0.05$). CETP decreased significantly by −7.6% ($p<0.01$). The changes in LCAT activity showed a significant positive correlation with the changes in LDL-C ($r=0.44$; $p<0.05$) (**Fig. 1**).

Correlation between LCAT Activity and Preβ1-HDL Disappearance Rate

LCAT activity did not correlate with the preβ1-HDL concentration (data not shown), although there was a significant positive correlation between LCAT activity and the preβ1-HDL disappearance rate. There was no change in the positive correlation with the same slope before or after treatment, but the preβ1-HDL disappearance rate was higher after pitavastatin treatment (**Fig. 2**).

Discussion

To assess the effects of pitavastatin on the RCT, plasma lipids, HDL subfractions, the disappearance rate of preβ1-HDL, LCAT activity, and CETP mass were assayed. The results indicate that pitavastatin decreases the plasma preβ1-HDL concentration and might promote the disappearance of preβ1-HDL.

In this study, pitavastatin significantly increased HDL-C by 6.0%. In the HDL subfractions, there was a significant increase in HDL_2-C by 9.0% but essentially no change in HDL_3-C. The mechanism of the pitavastatin-induced increase of HDL-C has been reported in *in vitro* studies to be the promotion of apoprotein-AI production from HepG2 cells mediated via the activation of peroxisome proliferator-activated receptor α (PPARα)[20, 21]. Many studies, but not all, have shown that HDL with a larger particle size (HDL_2) has relatively potent anti-atherosclerotic effects[22, 23]. Increased HDL_2 might promote non-specific cholesterol efflux from the cells[24].

In regard to the clinical significance of the plasma preβ1-HDL concentration and its disappearance, supposing conversion to α-HDL, accumulating evidence suggests that impaired conversion of preβ1-HDL resulting in high plasma preβ1-HDL concen-

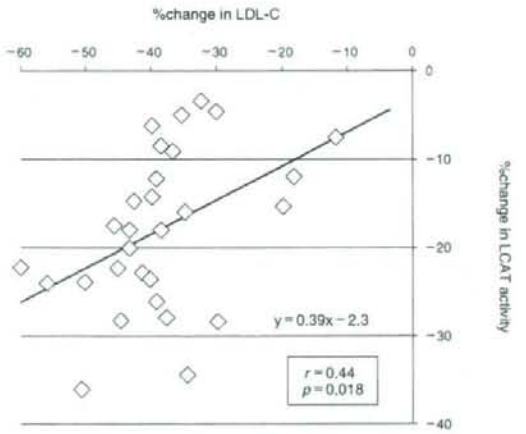

Fig. 1. The relation between the changes in LDL-C and in LCAT activity after 4 weeks of treatment in 29 hyperlipidemia patients was investigated by Pearson's correlation coefficient test.

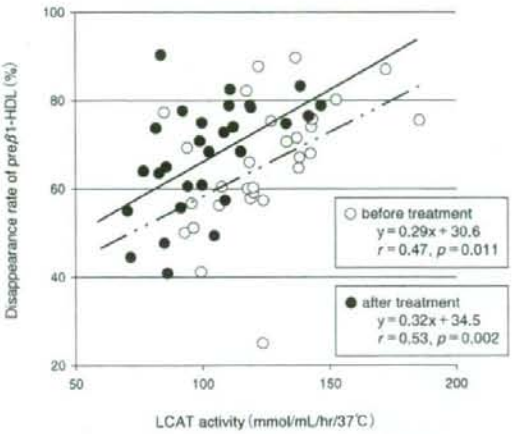

Fig. 2. The relation between LCAT activity and conversion rate of preβ1-HDL to α-HDL before (open circles) and after (closed circles) pitavastatin treatment was investigated by Pearson's correlation coefficient test.

tration is involved in the initiation and progression of atherosclerosis[25-27]. In this study, pitavastatin also significantly decreased plasma preβ1-HDL concentration, but significantly increased the disappearance rate of preβ1-HDL. Miida *et al.*[25] have reported that the preβ1-HDL conversion rate is a major determinant of plasma preβ1-HDL concentration. In fact, patients who have an impaired conversion rate of preβ1-HDL have high plasma preβ1-HDL concentration[25, 27]. Decreased plasma preβ1-HDL concentration by pitavastatin in this study may be thought to be primarily due to the increased disappearance rate. Based on these observations, pitavastatin is proposed to act as an antiatherosclerotic.

Asztalos *et al.*[14] have reported the effects of five different statins, excluding pitavastatin, on HDL subpopulation profiles analyzed by 2-dimensional electrophoresis. Preβ1-HDL concentrations after 4-week treatment with each statin were consistently increased, different from our study, although the extent varied. These differences from our study be a result of the HDL-subpopulation analytical method, subjects who were all coronary heart disease patients, or the dosage of statins, although the precise causes are not clear. To further confirm this effect of pitavastatin, future work is needed using a larger sample size with a comparative study design.

LCAT is an important enzyme involved in the maturation of preβ1-HDL to α-HDL[28]. Cell-derived free cholesterol on preβ1-HDL is converted to cholesterol ester by LCAT and stored in the core of HDL particles[8], and simultaneously smaller discoidal preβ1-HDL converts to larger spherical α-HDL. In this study, pitavastatin decreased LCAT activity and plasma preβ1-HDL concentration, but increased its disappearance rate, in contrast to previous reports; however, LCAT exists in two forms, α-LCAT present in the high-density fraction ($1.121 < d. < 1.25$) and β-LCAT present in the low-density fraction ($1.019 < d. < 1.063$), and it is known that α-LCAT is important for the maturation of preβ1-HDL to α-HDL[29, 30]. The LCAT activity in this study was the sum of α- and β-LCAT activities[19], because it is impossible to measure α- or β-LCAT activity individually. There was a positive correlation between the decrease in LDL-C and the decrease in LCAT activity in this study (**Fig. 1**). It is supposed that the decrease in LCAT activity by pitavastatin might be due to a decrease in β-LCAT activity, not α-LCAT activity.

This study also demonstrated a significant positive correlation between LCAT activity and the preβ1-HDL disappearance rate. Although there was no change in the positive correlation with the same slope before or after treatment, the preβ1-HDL disappearance rate was higher after pitavastatin treatment (**Fig. 2**). The cause is still unclear and needs to be studied in the future, but it is suggested that pitavastatin might promote preβ1-HDL disappearance by a mechanism independent of LCAT activity.

There have been several reports that statins de-

crease CETP[31], and our data also showed a decrease in the CETP mass. CETP is involved in the production of preβ1-HDL from α-HDL[32], and a positive correlation has been reported between CETP and preβ1-HDL[32, 33], thus, our data are in agreement. Since the magnitude of the CETP decrease is small, it is unclear whether the pitavastatin-induced decrease in CETP is truly involved in the decrease in preβ1-HDL.

It has been demonstrated that the formation and metabolism of preβ1-HDL was influenced by hepatic lipase (HL) activity and phospholipid transfer protein (PLTP) activity[34]. As a limitation of our study, we did not evaluate the effects of these factors on preβ1-HDL concentration and its disappearance rate, which were included in the conversion pathway of preβ1-HDL to α-HDL and other metabolic pathways of preβ1-HDL.

In conclusion, our data showed that the HMG-CoA reductase inhibitor pitavastatin: 1) markedly decreased TC and LDL-C and significantly increased HDL-C and HDL_2-C, 2) decreased plasma preβ1-HDL concentration but promoted its disappearance, 3) and decreased plasma LCAT activity, which correlated positively with the decrease in LDL-C. These data indicate that pitavastatin, in addition to decreasing LDL-C, might also exert a potent anti-atherosclerotic effect by improving the HDL subfraction profile and promoting the early step of RCT; however, further work is required.

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ORIGINAL ARTICLE

Variations in the *FTO* gene are associated with severe obesity in the Japanese

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Abstract Variations in the fat-mass and obesity-associated gene (*FTO*) are associated with the obesity phenotype in many Caucasian populations. This association with the obesity phenotype is not clear in the Japanese. To investigate the relationship between the *FTO* gene and obesity in the Japanese, we genotyped single nucleotide polymorphisms (SNPs) in the *FTO* genes from severely obese subjects [$n = 927$, body mass index (BMI) ≥ 30 kg/m^2] and normal-weight control subjects ($n = 1{,}527$, BMI < 25 kg/m^2). A case-control association analysis revealed that 15 SNPs, including rs9939609 and rs1121980, in a linkage disequilibrium (LD) block of approximately 50 kb demonstrated significant associations with obesity; rs1558902 was most significantly associated with obesity. P value in additive mode was 0.0000041, and odds ratio (OR) adjusted for age and gender was 1.41 [95% confidential interval (CI) = 1.22–1.62]. Obesity-associated phenotypes, which include the level of plasma glucose, hemoglobin A1c, total cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol, and blood pressure were not associated with the

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rs1558902 genotype. Thus, the SNPs in the *FTO* gene were found to be associated with obesity, i.e., severe obesity, in the Japanese.

Keywords Fat-mass and obesity-associated gene · Obesity · Japanese population · Association · SNP

Introduction

Obesity is the most common nutritional disorder in developed countries, and it is a major risk factor for hypertension, cardiovascular disease, and type 2 diabetes (Kopelman 2000; Wilson et al. 2003). Genetic and environmental factors contribute to obesity development (Maes et al. 1997; Barsh et al. 2000; Rankinen et al. 2006). Recent progress in single nucleotide polymorphism (SNP) genotyping techniques has enabled genome-wide association studies on common diseases (Herbert et al. 2006; Frayling et al. 2007; Scuteri et al. 2007; The Wellcome Trust Case Control Consortium 2007; Hinney et al. 2007). Using a large-scale case-control association study, we found that secretogranin III (SCG3) (Tanabe et al. 2007) and myotubularin-related protein 9 (MTMR9) (Yanagiya et al. 2007) are involved in susceptibility to the obesity phenotype. Genome-wide association studies have shown that the fat-mass and obesity-associated gene (*FTO*) is also associated with the obesity phenotype (Frayling et al. 2007; Scuteri et al. 2007; Hinney et al. 2007). This association was also found in many Caucasian and Hispanic American populations (Frayling et al. 2007; Scuteri et al. 2007; Dina et al. 2007; Field et al. 2007; Andreasen et al. 2008; Wåhlén et al. 2008; Peeters et al. 2008), whereas it was not found in the Chinese Han population (Li et al. 2008). Among Japanese, body mass index (BMI) was higher in subjects who had the A allele of rs9939609, similar to that observed in Caucasians; however, this finding was not significant (Horikoshi et al. 2007). Another group reported that rs9939609 was associated with BMI in the Japanese (Omori et al. 2008). Thus, the association of SNPs in the *FTO* gene with obesity in the Japanese remains controversial.

To investigate the relationship between the *FTO* gene and obesity in the Japanese, we performed a case-control association study using patients with severe adult obesity (BMI $\geq$ 30 kg/m^2) and normal-weight controls (BMI $<$ 25 kg/m^2); we found that SNPs in intron 1 of the *FTO* gene were associated with severe adult obesity.

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Materials and methods

Study subjects

The sample size for severely obese Japanese subjects (BMI $\geq$ 30 kg/m^2) was 927 (male:female ratio 419:508, age 48.7 $\pm$ 14.2 years, BMI 34.2 $\pm$ 5.4 kg/m^2), whereas that for Japanese normal weight controls (BMI $<$ 25 kg/m^2) was 1,527 (male:female ratio 685:842, age 48.1 $\pm$ 16.5 years, BMI 21.7 $\pm$ 2.1 kg/m^2). The severely obese subjects were recruited from among outpatients of medical institutes. Patients with secondary obesity and obesity-related hereditary disorders were not included, and neither were patients with medication-induced obesity. The normal-weight controls were recruited from among subjects who had undergone a medical examination for screening of common diseases. Clinical features of the subjects are illustrated in Table 1. Additionally, 1,604 subjects were recruited (male:female ratio 803:801, age 48.7 $\pm$ 16.9 years, BMI 22.66 $\pm$ 3.16 kg/m^2) from the Japanese general population. Each subject provided written informed consent, and the protocol was approved by the ethics committee of each institution and that of RIKEN.

DNA preparation and SNP genotyping

Genomic DNA was prepared from the blood sample of each subject by using the Genomix (Talent Srl, Trieste, Italy). We searched for dbSNPs with minor allele frequencies (MAF) $>$ 0.10 in the *FTO* gene of Japanese people. We selected 90 SNPs and were able to construct Invader probes (Third Wave Technologies, Madison, WI)

Table 1 Clinical characterization of obese and control subjects

	Obese	Control	*P* value
Gender (M/F)	419/508	658/842	
Age (year)	49.1 ± 14.2	48.2 ± 16.5	0.049
Body mass index (kg/m^2)	34.50 ± 5.39	21.65 ± 2.08	<0.000001
Glucose (mg/dl)	129.2 ± 49.6	97.7 ± 23.9	<0.000001
HbA1c (%)	6.5 ± 1.8	5.1 ± 0.6	<0.000001
Total cholesterol (mg/dl)	209.9 ± 37.9	201.2 ± 36.4	<0.000001
Triglycerides (mg/dl)	153.2 ± 99.5	104.0 ± 73.2	<0.000001
High-density lipoprotein cholesterol (mg/dl)	53.1 ± 18.9	65.1 ± 15.7	<0.000001
Systolic blood pressure (mmHg)	136.4 ± 18.1	123.4 ± 17.8	<0.000001
Diastolic blood pressure (mmHg)	83.8 ± 12.0	76.0 ± 11.1	<0.000001

P values were analyzed using Mann–Whitney *U* test. Data are mean ± standard deviation

for them (Supplementary Table 1). SNPs were genotyped using Invader assays as described previously (Ohnishi et al. 2001; Takei et al. 2002). Nine SNPs (rs9937053, rs9939973, rs9940128, rs7193144, rs8043757, rs9923233, rs9926289, rs9939609, and rs9930506) reported in a previous genome-wide association study (Scuteri et al. 2007) were genotyped using TaqMan probes (C__29910458_10, C__11776771_10, C__29621384_10, C__29387650_10, C__29387665_10, C__29693738_10, C__30270568_10, C__30090620_10, and C__29819994_10; Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Genotype or allele frequencies were compared between cases and controls in three different modes. In the first mode, i.e., the additive mode, χ^2 test was performed according to Sladek et al. (Sladek et al. 2007). In the second mode, i.e., the minor allele recessive mode, frequencies of the homozygous genotype for the minor allele were compared using a 2 × 2 contingency table. In the third mode, i.e., the minor allele dominant mode, frequencies of the homozygous genotype for the major allele were compared using a 2 × 2 contingency table. A test of independence was performed using Pearson's χ^2 method. *P* values were corrected by Bonferroni adjustment and $P < 0.00017$ [0.05/99 (total SNP number)/3 (number of modes)] was considered significant. The odds ratio (OR) and 95% confidence interval (CI) were calculated by Woolf's method. We coded genotypes as 0, 1, and 2, depending on the number of copies of the risk alleles. OR adjusted for age and gender was calculated using multiple logistic regression with genotypes, age, and gender as independent variables. Hardy–Weinberg equilibrium was assessed using the χ^2 test (Nielsen et al. 1998). Haplotype blocks were determined using Haploview (Barrett et al. 2005). Simple comparison of the clinical data among the different genotypes was performed using one-way analysis of variance (ANOVA). Simple comparison of the clinical data between case and control groups was analyzed using Mann–Whitney *U* test. Difference in BMI between genotypes was analyzed using a multiple linear regression, with BMI as the dependent variable and genotype as the independent variable, and with gender and age as covariates for BMI. Statistical analyses were performed using StatView 5.0 (SAS Institute, Cary, NC, USA). Power was calculated by the Monte Carlo method.

Results

Case-control association studies

We searched for dbSNPs with MAF > 0.10 in the *FTO* gene. By using Invader and TaqMan assay, we successfully genotyped 99 SNPs spanning the *FTO* gene (Supplementary Table 1). Using these SNPs, we performed tests of independence between the phenotype and genotypes of obesity at each SNP by using severely obese subjects (BMI ≥ 30 kg/m^2) and normal weight controls (BMI < 25 kg/m^2). For each SNP, the lowest *P* value among the three different modes was selected as the minimum *P* value. All SNPs, including rs1421084, were in Hardy–Weinberg equilibrium ($P > 0.01$) (Supplementary Table 1).

The power of the test was calculated by Monte Carlo method with different MAFs and different effect sizes. Effect of the risk allele on penetrance was assumed to be multiplicative; i.e., the penetrances for three genotypes were assumed to be a, ar, and ar^2, respectively, where a and r denote the lowest penetrance and genotype relative risk, respectively. Supplementary Table 2 shows the calculated values of the power of the test with different MAFs and different genotype relative risks (r). The lowest penetrance (a) was calculated for each gender by assuming the affection rates of 2.3% for men and 3.4% for women (Yoshiike et al. 2002). Genotype relative risk (r) was assumed to be

the same for both genders. Supplementary Table 2 shows that the test has significant power at relative high risk allele frequency when genotype relative risk is >1.7.

As shown in Fig. 1 and Supplementary Table 1, 15 SNPs demonstrated significant associations with the obesity phenotype; the threshold of significance using Bonfferoni correction was $P < 0.00017$. These SNPs included rs9939609 (Frayling et al. 2007) and rs1121980 (Hinney et al. 2007) that were reported to be significantly associated with the obesity phenotype in the Caucasian population, as determined by genome-wide association studies; rs9930506 (Scuteri et al. 2007) showed marginal association with obesity in the Japanese. Linkage disequilibrium (LD) analysis revealed that these 15 SNPs were in almost complete LD ($D' > 0.98$, $r^2 > 0.80$) and were located within the same LD block of approximately 50 kb (Fig. 1). The most significant association was observed for rs1558902 [additive mode, $P = 0.0000041$ and allele-specific OR (95% CI) adjusted for age and gender was 1.41 (1.22–1.62)]. The minor alleles of rs9939609 (MAF = 0.24) and rs1121980 (MAF = 0.26) were significantly more frequent in the obese group than in the normal-weight control group (additive mode, $P = 0.000012$ and $P = 0.000051$, respectively), and ORs were 1.38 (95% CI = 1.20–1.59) and 1.33 (95% CI = 1.16–1.52), respectively (Table 2, Supplementary Table 1). The MAF of both SNPs in the control group was 0.18; this was consistent with data obtained from the haplotype map of the human genome (HapMap) (Supplementary Table 1). Our data indicated that the SNPs in the *FTO* gene were associated with severe obesity in the Japanese.

Analysis of various quantitative phenotypes with rs1558902

To investigate whether the genotypes of SNP rs1558902 are associated with the phenotypes of metabolic disorders, we compared the following among the different genotypes in the cases, controls, and combined groups: ANOVA results, BMI, levels of fasting plasma glucose, hemoglobin A1c (HbA1c), total cholesterol, triglycerides, HDL cholesterol, and blood pressure. As rs1558902 showed the most significant association with obesity and its call rate was the highest, we analyzed various quantitative phenotypes by using this SNP. The quantitative phenotypes regarding BMI and the levels of fasting plasma glucose, HbA1c, total cholesterol, triglycerides, HDL cholesterol, and blood pressure were not found to be significantly associated with the genotypes at rs1558902 in either the case or control group (Table 3). Although there was no significant difference in BMI values among genotypes in either the control or case group, the direction of the difference (AA > AT > TT) was in accordance with the association between the qualitative obesity phenotype and the genotype shown.

Finally, we examined the BMI distribution of rs1558902 in the Japanese general population and found that rs1558902 genotype was significantly associated with BMI

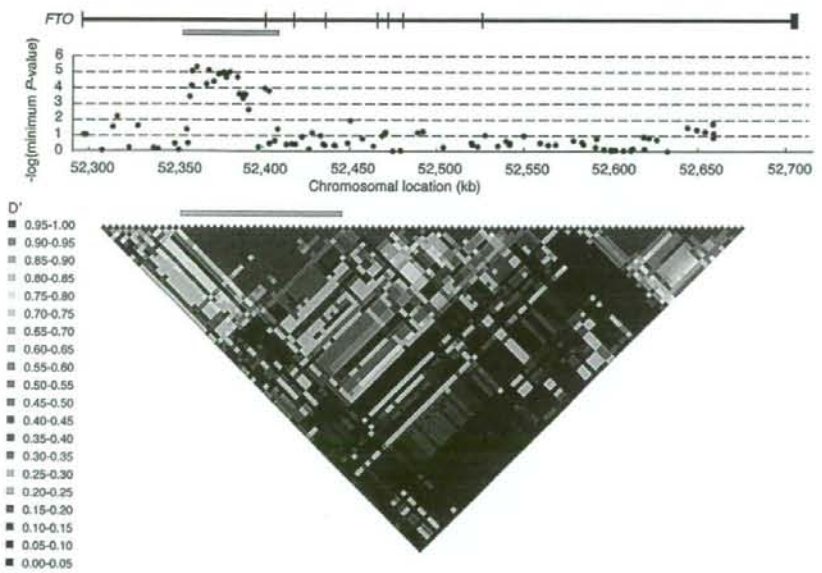

Fig. 1 Linkage disequilibrium (LD) mapping, polymorphisms, and *P* values obtained in the test of independence between the phenotype and genotypes of obesity at various single nucleotide polymorphisms (SNPs) in the fat-mass and obesity-associated gene (*FTO*) gene. *P* values are expressed as negative logarithm of the minimum *P* values obtained in the three models (additive, minor allele dominant, and minor allele recessive modes). LD coefficients (*D'*) between each pair of SNPs were calculated and are displayed as a strand in the LD blocks. Minor allele frequencies of all SNPs used in this analysis are ≥10%. The genomic structure is shown in the *upper*. The *gray bar* marks the LD block associated with obesity

Table 2 Associations of single nucleotide polymorphisms (SNPs) in the fat-mass and obesity-associated gene (*FTO*) gene with obesity existing in the 50-kb linkage disequilibrium (LD) block

dbSNP ID	Allele	Genotype								Additive mode			Recessive mode			Dominant mode		
		Case				Control												
	1/2	11	12	22	Sum	11	12	22	Sum	OR (95% CI)	$\chi 2$	*P* value	$\chi 2$	*P* value	OR (95% CI)	$\chi 2$	*P* value	OR (95% CI)
rs9937053	A/G	59	360	494	913	63	414	773	1250	1.31 (1.13–1.51)	12.3	0.00047	2.0	0.16	1.30 (0.90–1.88)	13.0	0.00031	1.37 (1.16–1.63)
rs9939973	A/G	61	367	496	924	75	504	941	1520	1.32 (1.15–1.51)	15.7	0.000077[a]	3.0	0.081	1.36 (0.96–1.93)	16.1	0.000061[a]	1.40 (1.19–1.66)
rs9940128	A/G	60	366	498	924	75	500	941	1516	1.31 (1.15–1.50)	15.2	0.00010[a]	2.6	0.11	1.33 (0.94–1.89)	15.9	0.000068[a]	1.40 (1.19–1.65)
rs1421085	C/T	49	338	537	924	57	443	1019	1519	1.38 (1.20–1.59)	19.6	0.000011[a]	3.3	0.068	1.44 (0.97–2.12)	20.0	0.0000078[a]	1.47 (1.24–1.74)
rs1558902	A/T	48	341	536	925	52	449	1021	1522	1.41 (1.22 -1.62)	21.2	0.0000041[a]	4.6	0.032	1.55 (1.04–2.31)	20.8	0.0000052[a]	1.48 (1.25–1.75)
rs1121980	A/G	61	367	499	927	73	504	947	1524	1.33 (1.16–1.52)	16.5	0.000051[a]	3.6	0.059	1.40 (0.99–1.99)	16.5	0.000050[a]	1.41 (1.19–1.66)
rs7193144	C/T	49	339	532	920	55	447	1014	1516	1.39 (1.21–1.61)	20.4	0.0000067[a]	4.0	0.044	1.49 (1.01–2.22)	20.3	0.0000067[a]	1.47 (1.24–1.74)
rs8043757	T/A	48	319	541	908	54	436	1027	1517	1.36 (1.18–1.57)	17.4	0.000037[a]	4.2	0.040	1.51 (1.02–2.25)	16.4	0.000052[a]	1.42 (1.20–1.69)
rs8050136	A/C	51	336	538	925	56	450	1018	1524	1.38 (1.20–1.59)	19.4	0.000012[a]	4.7	0.031	1.53 (1.04–2.26)	18.5	0.000017[a]	1.45 (1.22–1.71)
rs3751812	T/G	51	340	534	925	55	458	1013	1526	1.38 (1.20–1.59)	19.6	0.0000098[a]	5.1	0.024	1.56 (1.06–2.31)	18.5	0.000017[a]	1.45 (1.22–1.71)
rs9923233	C/G	51	335	533	919	55	449	1010	1514	1.38 (1.20–1.60)	19.8	0.0000093[a]	5.0	0.025	1.56 (1.06–2.30)	18.7	0.000015[a]	1.45 (1.23–1.72)
rs9926289	A/G	50	323	531	904	56	425	993	1474	1.37 (1.19 -1.58)	18.7	0.000020[a]	3.9	0.047	1.48 (1.00–2.19)	18.1	0.000021[a]	1.45 (1.22–1.72)
rs9939609	A/T	51	334	534	919	56	443	1005	1504	1.38 (1.20–1.59)	19.5	0.000012[a]	4.5	0.034	1.52 (1.03–2.24)	18.7	0.000015[a]	1.45 (1.23–1.72)
rs7185735	G/A	51	340	536	927	55	455	1014	1524	1.38 (1.20–1.59)	19.9	0.0000089[a]	5.0	0.025	1.55 (1.05–2.30)	18.8	0.000014[a]	1.45 (1.23–1.72)
rs9931494	G/C	64	363	494	921	71	504	942	1517	1.35 (1.18–1.55)	18.4	0.000018[a]	5.6	0.018	1.52 (1.07–2.15)	16.9	0.000039[a]	1.42 (1.20–1.67)
rs17817964	T/C	62	361	500	923	68	524	930	1522	1.30 (1.14–1.49)	13.5	0.00022	5.8	0.016	1.54 (1.08–2.19)	11.4	0.00075	1.33 (1.13–1.57)
rs9930506	G/A	67	365	488	920	82	521	913	1516	1.28 (1.12–1.46)	12.8	0.00038	3.5	0.061	1.37 (0.98–1.92)	12.1	0.00051	1.34 (1.14–1.58)
rs9932754	C/T	66	368	491	925	78	525	919	1522	1.29 (1.13–1.48)	13.6	0.00023	4.2	0.040	1.42 (1.01–2.00)	12.6	0.00040	1.35 (1.14–1.59)
rs9922619	T/G	66	368	489	923	78	529	919	1526	1.29 (1.13–1.48)	13.5	0.00024	4.3	0.038	1.43 (1.02–2.01)	12.3	0.00044	1.34 (1.14–1.58)
rs7204609	C/T	134	418	373	925	273	717	529	1519	0.83 (0.73–0.93)	9.68	0.0022	5.0	0.025	0.77 (0.62–0.97)	7.5	0.0063	0.79 (0.67–0.94)
rs12149832	A/G	53	349	525	927	62	480	982	1524	1.33 (1.15–1.53)	15.2	0.000098[a]	3.5	0.061	1.43 (0.98–2.08)	14.8	0.00012[a]	1.39 (1.17–1.64)

The odds ratio (OR) for each SNP was adjusted simultaneously for age and gender using additive model

CI confidence interval, χ^2 chi-square

[a] Significant *P* value ($P < 0.00017$)

Table 3 Comparison of various quantitative phenotypes among different genotypes at single nucleotide polymorphism (SNP) rs1558902 in obese and control subjects

	Obese			Control		
	AA ($n = 48$)	AT ($n = 341$)	TT ($n = 536$)	AA ($n = 52$)	AT ($n = 448$)	TT ($n = 1022$)
Age (year)	49.8 ± 15.3	49.6 ± 14.3	48.8 ± 14.1	46.9 ± 15.4	46.9 ± 16.7	48.8 ± 16.5
P value		0.64			0.098	
BMI (kg/m^2)	35.16 ± 5.70	34.61 ± 5.43	34.39 ± 5.33	21.94 ± 2.23	21.62 ± 2.10	21.65 ± 2.06
P value		0.58			0.56	
Glucose (mg/dl)	142.8 ± 54.8	125.4 ± 43.2	130.8 ± 53.3	101.7 ± 44.1	96.3 ± 18.1	98.2 ± 24.7
P value		0.054			0.34	
HbA1c (%)	6.9 ± 2.1	6.4 ± 1.7	6.5 ± 1.8	5.1 ± 1.2	5.0 ± 0.5	5.1 ± 0.7
P value		0.19			0.15	
Total cholesterol (mg/dl)	215.1 ± 46.7	211.3 ± 38.8	208.6 ± 36.6	195.6 ± 38.8	201.4 ± 37.8	201.4 ± 35.6
P value		0.37			0.53	
Triglycerides (mg/dl)	171.7 ± 119.5	151.3 ± 102.1	153.2 ± 96.0	111.7 ± 70.6	102.0 ± 71.4	104.4 ± 74.2
P value		0.42			0.63	
HDL cholesterol (mg/dl)	53.2 ± 13.8	54.8 ± 24.0	52.0 ± 15.4	62.1 ± 14.2	65.1 ± 15.9	65.3 ± 15.6
P value		0.14			0.53	
SBP (mmHg)	134.2 ± 20.4	137.0 ± 17.8	136.2 ± 18.2	122.7 ± 17.3	123.2 ± 18.8	123.5 ± 17.5
P value		0.61			0.91	
DBP (mmHg)	80.3 ± 11.7	84.1 ± 12.0	83.9 ± 12.0	75.5 ± 11.1	75.2 ± 11.7	76.3 ± 10.9
P value		0.14			0.22	

Data of each quantitative phenotype were compared among different genotypes at the rs1558902 in obese and control subjects. *P* values were analyzed using analysis of variance in each group of obese and control subjects. Data are mean ± standard deviation.

HDL high-density lipoprotein, *SBP* systolic blood pressure, *DBP* diastolic blood pressure.

Table 4 Association of body mass index (BMI) with rs1558902 genotypes in the Japanese general population

	AA	AT	TT	*P* value (additive model)[a]
BMI (kg/m^2) (*n*)	23.17 ± 3.20 (59)	22.79 ± 3.26 (482)	22.57 ± 3.11 (1063)	0.041

[a] The difference in BMI according to genotypes was analyzed using a multiple linear regression, with BMI as the dependent variable and genotype as the independent variable and with gender and age as covariates for BMI. Data are represented as mean ± standard deviation

(Table 4). This result would confirm the association of rs1558902 with obesity.

Discussion

Recent genome-wide association studies have shown that the *FTO* gene is associated with obesity (Frayling et al. 2007; Scuteri et al. 2007; Hinney et al. 2007). The associations between variations in the *FTO* gene and the obesity phenotype have been observed in many Caucasian subjects (Frayling et al. 2007; Scuteri et al. 2007; Dina et al. 2007; Field et al. 2007; Andreasen et al. 2008; Wåhlén et al. 2008; Peeters et al. 2008). However, these associations were controversial with regard to Asian subjects (Horikoshi et al. 2007; Li et al. 2008; Omori et al. 2008). BMI values did not significantly differ among the genotypes in the general population of Chinese and Japanese (Horikoshi et al. 2007; Li et al. 2008). We performed a case-control association study with regard to severe obesity and found that the SNPs in the *FTO* gene were significantly associated with severe obesity. Although the SNPs demonstrated the most significant association in the Japanese, which was different from that in Caucasians, the significantly associated SNPs existed in a similar block as that in Caucasians. Therefore, the *FTO* gene could also contribute to the development of severe obesity in the Japanese.

BMI was modestly different among rs1558902 genotypes in the general population in this study; rs9939609 was not significantly associated with BMI in the general population (AA 23.22 ± 3.14 vs AT 22.79 ± 3.25 vs TT 22.58 ± 3.13, $P = 0.063$). In the Japanese population, rs1558902 may be more tightly associated with BMI than rs9939609. The National Nutrition Survey of Japan reported that the prevalence of subjects with a BMI of

$\geq$30 kg/m^2 is only 2.3% in men and 3.4% in women aged 20 years and older (Yoshiike et al. 2002), and the mean BMI was approximately 23 kg/m^2 for ages 15–84 years (Yoshiike et al. 1998). Inconsistency in the results of effects of variations in the *FTO* gene on BMI between Japanese and Europians may be due to the relatively small mean and variance of BMI in the former than the latter.

The significant SNPs were located in intron 1 of the *FTO* gene. The rs1558902 and other significant SNPs, for example, rs9939609 and rs1121980, would affect transcriptional activity of the *FTO* gene, although further investigation is necessary. The precise mechanism by which the *FTO* gene leads to obesity development is unclear (Gerken et al. 2007; Sanchez-Pulido et al. 2007). However, the *FTO* gene is expressed in the hypothalamus and regulated by fasting and leptin (Frayling et al. 2007; Gerken et al. 2007). Using large-scale case-control association studies, we determined that the *SCG3* (Tanabe et al. 2007) and *MTMR9* (Yanagiya et al. 2007) genes are involved in susceptibility to the obesity phenotype. These two genes are expressed in the hypothalamus. Genetic studies in mice have suggested that mutations in several genes, such as those encoding leptin, proopiomelanocortin, and melanocortin-4 receptor, are implicated in a monogenic form of inherited obesity (Barsh et al. 2000; Rankinen et al. 2006). Such mutations have also been reported in obese humans. As most such genes are expressed in the hypothalamus and have been indicated to play important roles in the regulation of food intake, genes expressed in the hypothalamus are likely to be good candidates for susceptibility to obesity.

In summary, we have identified the genetic variations in the *FTO* gene that may influence the risk of severe obesity in the Japanese.

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Original Article: Complications

Stratified analyses for selecting appropriate target patients with diabetic peripheral neuropathy for long-term treatment with an aldose reductase inhibitor, epalrestat

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Abstract

Aims The long-term efficacy of epalrestat, an aldose reductase inhibitor, in improving subjective symptoms and nerve function was comprehensively assessed to identify patients with diabetic peripheral neuropathy who responded to epalrestat treatment.

Methods Stratified analyses were conducted on data from patients in the Aldose Reductase Inhibitor—Diabetes Complications Trial (ADCT). The ADCT included patients with diabetic peripheral neuropathy, median motor nerve conduction velocity ≥ 40 m/s and with glycated haemoglobin (HbA_{1c}) ≤ 9.0%. Longitudinal data on HbA_{1c} and subjective symptoms of the patients for 3 years were analysed (epalrestat $n = 231$, control subjects $n = 273$). Stratified analyses based on background variables (glycaemic control, grades of retinopathy or proteinuria) were performed to examine the relationship between subjective symptoms and nerve function. Multiple logistic regression analyses were conducted.

Results Stratified subgroup analyses revealed significantly better efficacy of epalrestat in patients with good glycaemic control and less severe diabetic complications. In the control group, no improvement in nerve function was seen regardless of whether symptomatic benefit was obtained. In the epalrestat group, nerve function deteriorated less or improved in patients whose symptoms improved. The odds ratio of the efficacy of epalrestat vs. control subjects was approximately 2 : 1 (4 : 1 in patients with $HbA_{1c} \leq 7.0\%$).

Conclusion Our results suggest that epalrestat, an aldose reductase inhibitor, will provide a clinically significant means of preventing and treating diabetic neuropathy if used in appropriate patients.

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Keywords aldose reductase inhibitor, diabetic peripheral neuropathy, good condition of blood glucose level, polyol pathway

Abbreviations ADCT, Aldose Reductase Inhibitor—Diabetes Complications Trial; AEs, adverse events; ARI, aldose reductase inhibitor; HbA_{1c}, glycated haemoglobin; MFWL, minimum F-wave latency; MNCV, motor nerve conduction velocity; VAS, visual analogue scale; VPT, vibration perception threshold

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