ANGPTL3 Decreases Very Low Density Lipoprotein Triglyceride Clearance by Inhibition of Lipoprotein Lipase*

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KK/San is a mutant mouse strain established in our laboratory from KK obese mice. KK/San mice show low plasma lipid levels compared with wild-type KK mice, despite showing signs of hyperglycemia and hyperinsulinemia. Recently, we identified a mutation in the gene encoding angiopoietin-like protein 3 (Angptl3) in KK/San mice, and injection of adenoviruses encoding Angptl3 or recombinant ANGPTL3 protein to mutant KK/San mice raised plasma lipid levels. To elucidate the regulatory mechanism of ANGPTL3 on lipid metabolism, we focused on the metabolic pathways of triglyceride in the present study. Overexpression of Angptl3 in KK/San mice resulted in a marked increase in triglyceride-enriched very low density lipoprotein (VLDL). In vivo studies using Triton WR1339 revealed that there is no significant difference between mutant and wild-type KK mice in the hepatic VLDL triglyceride secretion rate. However, turnover studies using radiolabeled VLDL revealed that the clearance of 2H-triglyceride-labeled VLDL was significantly enhanced in KK/San mice, whereas the clearance of 125I-labeled VLDL was only slightly enhanced. In vitro analysis of recombinant protein revealed that ANGPTL3 directly inhibits LPL activity. These data strongly support the hypothesis that ANGPTL3 is a new class of lipid metabolism modulator, which regulates VLDL triglyceride levels through the inhibition of LPL activity.

Hyperlipidemia is a major risk factor of coronary heart disease. Variations in human plasma lipid levels result from both genetic and environmental factors. Genetic factors account for more than 50% of the variation in plasma lipid levels in the human population (1–4). Naturally occurring mutations that affect lipid metabolism in mice have also been reported (5–7). In most cases, the mutated genes have not yet been identified, but elucidating the mutations could lead to the identification of the relevant genes.

KK obese mice have a multigenic syndrome of moderate obesity and a diabetic phenotype that resembles human hereditary type 2 diabetes; they show signs of hyperinsulinemia, hyperglycemia, and hyperlipidemia (8–10). We have found that KK mice in our laboratory (KK/San) have significantly low plasma lipid levels despite showing signs of hyperinsulinemia and hyperglycemia (11). Genetic analysis shows that the mutant phenotype of KK/San mice is inherited recessively as a Mendelian trait. We therefore named this locus hypl (for hypolipidemia). We observed the autosomal recessive hypl phenotype in the progeny of the KK/San strain and mapped the locus to the middle of chromosome 4. We identified a mutation in the gene encoding angiopoietin-like protein 3 (Angptl3) as the cause of the hypl trait (12). The mRNA of Angptl3 is predominately localized in the liver. The expression of Angptl3 in KK/San mice was found to be 1/30 to 1/40 that of wild-type mice. Overexpression of Angptl3 using adenoviruses or by an intravenous injection of the recombinant protein in KK/San mice elicited a marked increase in circulating plasma total cholesterol, non-esterified fatty acids (NEFAs), and especially triglyceride levels (12).

Angptl3 is ~7 kb long and is composed of seven exons, the last four of which located at the carboxyl terminus end of the protein and encode the fibrinogen-like domain. The amino terminus contains a putative signal sequence and a coiled-coil domain. COS-1 and CHO-K1 cells transfected with SRα promoter-driven mammalian cell expression vectors containing ANGPTL3 cDNA secreted a major ANGPTL3 protein of ~70 kDa. Deglycosylation reduced the apparent molecular mass of the recombinant ANGPTL3 to 53 kDa as predicted by sequence analysis.

Angiopoietins are members of the vascular endothelial growth factor family (13, 14). They have fibrinogen-like domains that are conserved and are predicted to come into direct contact with receptors (15, 16). Within the fibrinogen-like domain, angiopoietins have a cystein-based motif (15). In contrast with other members of the angiopoietin family, however, ANGPTL3 lacks this motif, and preliminary data obtained from a BLAcore assay indicate that ANGPTL3 does not bind Tie2, an angiopoietin receptor. ANGPTL3 is involved primarily in the regulation of lipid metabolism rather than in mediating the growth of vascular epithelium.

Although ANGPTL3 regulates lipid metabolism, the direct effect of ANGPTL3 on VLDL triglyceride metabolism has not yet been investigated. Therefore, in the present study, the effect of a mutation in a gene encoding Angptl3 on VLDL metabolism was investigated by performing VLDL turnover studies in KK/San and wild-type KK mice. We found that KK/San mice showed enhanced VLDL clearance compared with wild-type KK mice due to enhanced lipolysis of VLDL triglyceride.

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1 The abbreviations used are: NEFA, non-esterified fatty acid; ANGPTL3, angiopoietin-like protein 3; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; VLDL, very low density lipoprotein; LPL, lipoprotein lipase; HL, hepatic lipase; HPLC, high performance liquid chromatography.
erides, whereas VLDL triglyceride production was not affected. We also found that recombinant ANGPTL3 protein directly inhibited LPL activity. Our results suggest that ANGPTL3 affects VLDL triglyceride clearance by interfering with LPL activity.

**EXPERIMENTAL PROCEDURES**

**Animals**—KK mice were obtained from Nagoya University and BALB/c mice from Charles River. The KK mice used were males between 3 and 5 months old, which were housed in a room under controlled temperature (23 ± 1 °C) with free access to water and mouse chow ( Oriental Yeast).

**Plasma Lipid and Lipoprotein Analysis**—Total plasma cholesterol and triglyceride levels were measured enzymatically using assay kits from Wako Pure Chemical Industries. The distribution of cholesterol within the plasma lipoproteins was determined by high performance liquid chromatography (HPLC) (17). Samples of 40 μl of the diluted plasma were applied onto two columns of TSKgel Lipopropak XL (Tosoh). The elution was performed at a flow rate of 0.7 ml/min for the TSK eluent LP-2 (Tosoh) and 0.35 ml/min for the enzyme solution (Determiner LTC, Kyowa Medex Co., Tokyo). The detection of cholesterol in the eluted fractions was carried out at a wavelength of 550 nm following the enzymatic reaction using a computerized system.

**Recombinant Adenovirus Construction and Animal Studies**—We isolated cDNA length mouse Angptl3 cDNA from KK mouse liver cDNA library constructed using the ZAP Express cDNA Gigapack III Gold cloning kit (Stratagene). We used a 200-bp PCR-amplified fragment of Angptl3 cDNA (nucleotides 62–260 from the GenBank™ library) as a probe. We obtained full-length human ANGPTL3 cDNA from a human liver cDNA library (CLONTECH) by plaque hybridization. We digested this cDNA with EcoRI and XbaI and subcloned the resulting fragment into the EcoRI/XbaI sites of pME18S (pMEH55-1). We determined the nucleotide sequences of these cloned fragments for both strands using the ABI BigDye Terminator kit (Applied Biosystems). We generated recombinant adenoviruses as previously described (18) using an Adenovirus Expression Vector Kit (Takara Shuzo). We prepared AdAdoC and AdAngptl3 by inserting the β-galactosidase and Angptl3 cDNAs, respectively, into the Ad E1-deleted region under the control of the CAG promoter. Recombinant adenoviruses were propagated and purified as described previously (19). We injected 2 × 10^9 plaque-forming units of each recombinant adenovirus intravenously to KK/San mice via the tail vein. We obtained blood from the retro-orbital plexus 3 days after injection.

**In Vivo Hepatic VLDL Triglyceride Production**—Fasted KK/San and wild-type KK mice were injected intravenously with 500,000 dpm of [3H]triglyceride-labeled VLDL. KK/San and wild-type KK mice were injected intravenously with 1 mCi/ml. BALB/c mice were injected intravenously via the 0.9% NaCl containing 2 mg/ml bovine serum albumin to a final concentration of 1 M. To study the retro-orbital plexus 3 days after injection.

**Labeling and Removal of 125I-labeled VLDL in Vivo**—Blood was collected from 16 BALB/c mice. Plasma samples were pooled, and VLDL (d < 1.006 g/ml) was obtained by ultracentrifugation. VLDL was labeled with 125I by the ICl method (22). The specific activity of 125I-VLDL was ~300 cpm/μg of protein. Thereafter, the VLDL samples were dialyzed extensively against a buffer containing 0.15 M NaCl and 0.3 mM EDTA, pH 7.4. KK/San and wild-type KK mice were injected into the tail vein with 125I-labeled VLDL (10 μl of tracer in 200 μl of 0.9% NaCl containing 2 mg/ml of bovine serum albumin). Blood samples of 70 μl were collected from the retro-orbital plexus at the specified time points after the injection. The plasma content of VLDL was determined by measuring the 125I content in the pellet obtained after prepan-2-ol precipitation (23, 24).

**Recombinant ANGPTL3 Protein Preparation**—We digested full-length human ANGPTL3 cDNA with EcoRI and XbaI and subcloned the resulting fragment into the EcoRI/XbaI sites of pME18S (pMEH55-1). We expressed and purified the expression vector pMEH55-1 containing CHO-K1 cells using FuGENE 6 (Roche Molecular Biochemicals). The serum-free culture medium of CHO-K1 cells transfected with the pMEH55-1 expression plasmid DNA was concentrated from 10 to 1 liters. We applied it to a Sephadex 25 column (Amersham Biosciences) and eluted it with 20 mM Tris-HCl, pH 7.5, buffer. We then applied the sample to a Q Sepharose fast flow column (Amersham Biosciences) and eluted it with 0.5 M NaCl in 20 mM Tris-HCl, pH 7.5, buffer. We next applied the sample to a Cibacron 3G column (Bio-Rad) and eluted it with 1 M NaCl in 20 mM Tris-HCl, pH 7.5, buffer. Finally, we applied the sample to a Poros Q column (Perspective Biosystems) and eluted it with 0.5 M NaCl in 20 mM Tris-HCl, pH 7.5, buffer.

**Source of LPL**—Purified bovine milk LPL was obtained from Sigma. The low-heparin medium containing rat LPL was obtained from rat dipocytes. Rat white adipose precursor cells were purchased from Hokudo. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum, 17 mM pantothenic acid, 33 μM (±)-biotin, 100 mM ascorbic acid, 1 mM octanoic acid, 50 mM triiodothyronine, 100 units/ml penicillin, and 100 μg/ml streptomycin (this medium is subsequently referred to as complete DMEM). After the cells had reached confluence, the medium was replaced with the adipocyte induc- tion medium, which contained DMEM containing 0.1% bovine serum albumin, 0.3 mM EDTA, pH 7.5, and 0.3 mM methionine, and also that a low level expression of ANGPTL3 caused hypolipidemia. To investigate the mechanism responsible for the decreased plasma triglyceride levels compared with wild-type KK mice and also that a low level expression of Angptl3 causes hypolipidemia. To investigate the mechanism responsible for the decreased plasma triglyceride levels compared with wild-type KK mice, we first analyzed lipoprotein profiles in detail. As shown in Table I, plasma triglyceride and total cholesterol levels in KK/San mice were lower than in wild-type KK mice at the age of 5 months. No significant differences in body weights were observed between the wild-type KK and KK/San mice. To investigate the lipopro-
tein profile among lipoprotein subclasses, plasma samples of wild-type KK mice and KK/San mice were subjected to HPLC analysis (Fig. 1). The lower levels of triglycerides and total cholesterol in KK/San mice were mainly due to a decrease in the fractions of VLDL.

Table II shows the plasma lipid levels after the adenovirus-mediated gene transfer of Angptl3 in KK/San mice. Overexpression of Angptl3 in KK/San mice resulted in a marked increase in plasma triglyceride levels as compared with Ad/lacZ-injected KK/San mice. Although cholesterol levels were only slightly elevated, Ad/Angptl3-injected KK/San mice showed a marked increase in the VLDL fraction (Fig. 1).

We also compared the apoB levels of KK/San and wild-type KK mice. No significant differences were observed between the KK/San and wild-type KK mice for plasma apoB100 and apoB48 levels (Fig. 2).

In Vivo VLDL Metabolism—The predominant decrease in plasma triglycerides rather than cholesterol levels in KK/San mice suggests that ANGPTL3 may interfere with triglyceride metabolism. ANGPTL3 may influence either the VLDL triglyceride secretion or VLDL triglyceride clearance.

First, to determine whether the low plasma triglyceride levels in KK/San mice were due to decreased VLDL triglyceride production, we measured the VLDL triglyceride secretion rate by the Triton WR1339 method. As shown in Fig. 3, the increase in plasma triglycerides was equal for wild-type KK and KK/San mice. The VLDL triglyceride secretion rate, calculated based on the experimental data, was not found to be significantly different between KK/San and wild-type KK mice.

We next investigated whether the low plasma triglyceride levels in KK/San mice were due to enhanced triglyceride clearance. To study triglyceride clearance, KK/San and wild-type KK mice were injected with [3H]triglyceride-labeled VLDL. As shown in Fig. 4a, triglycerides were more rapidly cleared from

Table I

| Strain   | Age (months) | Body weight (g) | Triglyceride (mg/dl) | Cholesterol (mg/dl) |
|----------|--------------|-----------------|----------------------|---------------------|
| KK/San   | 5            | 36.8 ± 0.9      | 25.8 ± 4.3"         | 60.9 ± 3.7"        |
| Wild-type KK | 5          | 36.6 ± 1.2      | 273.9 ± 38.5        | 114.4 ± 4.6        |

*p < 0.001; significantly different from wild-type KK mice, using Student’s t test.

Fig. 1. Lipoprotein profiles by HPLC assay. Lipoprotein profiles obtained by HPLC of KK/San mice (a), wild-type KK mice (b), KK/San mice injected with Ad/lacZ (c), and KK/San mice injected with Ad/Angptl3 (d). Plasma samples were collected 5 h after fasting.
the circulation in KK/San mice. To examine whether this enhanced clearance of triglycerides in KK/San mice was due to enhanced lipolysis or hepatic particle uptake, VLDL apoB turnover studies were performed as a marker for whole particle clearance. After the injection of 125I-labeled VLDL, 125I-labeled VLDL apoB was seen to be cleared at a slightly faster rate in KK/San mice (Fig. 4b). Thus, the low plasma triglyceride levels in KK/San mice compared with wild-type KK mice were primarily due to enhanced lipolysis rather than to enhanced whole particle uptake.

Effect of ANGPTL3 on LPL and HL Activities in Vitro—We next examined whether the effect of ANGPTL3 on VLDL triglyceride clearance resulted from a direct effect of ANGPTL3 on the activities of LPL and HL. Recombinant ANGPTL3 protein was added to purified bovine milk LPL or to a post-heparin medium of rat adipocytes, and the inhibitory effect on LPL activity was measured. As shown in Fig. 5, ANGPTL3 inhibited not only crude rat adipocyte LPL but also purified bovine LPL. The percent inhibition of rat adipocyte LPL by ANGPTL3 was ~70% at a concentration of 10 µg/ml. HL activity was also measured using plasma from mice because mouse HL is found in free circulation. As shown in Fig. 6, the inhibition of HL by ANGPTL3 was very weak compared with that of LPL. ANGPTL3 inhibited HL by only 16% even at a concentration of 10 µg/ml.

DISCUSSION

In the present study, we have demonstrated that ANGPTL3 is involved in the metabolism of VLDL. This is illustrated by the fact that adenovirus-mediated overexpression of ANGPTL3 in KK/San mice increased plasma total cholesterol, NEFAs, and especially plasma triglycerides. This increase in plasma lipids was mainly confined to the VLDL fraction. Plasma VLDL triglyceride levels are thought to be regulated by the balance between its secretion and clearance. As shown in Fig. 3, the hepatic VLDL triglyceride secretion rate was not significantly different between KK/San and wild-type KK mice. Previously, using another lot of KK/San mice, we observed a slight decrease (15%) in the hepatic VLDL triglyceride secretion rate compared with wild-type KK mice (11). As the difference between the two mice lots was very small, the low triglyceride levels in KK/San mice is not due to a lower secretion rate. Therefore, we concluded that ANGPTL3 does not affect VLDL triglyceride secretion.

Next, we analyzed VLDL triglyceride clearance, using two different types of labeled VLDL (3H]-triglyceride-labeled VLDL and 125I-labeled VLDL) to distinguish VLDL triglyceride clearance and VLDL whole particle uptake by the liver. Based on

| Strain | Adenovirus | n  | Immediately after adenovirus injection | Day 3 after adenovirus injection |
|--------|------------|----|---------------------------------------|-------------------------------|
|        |            |    | Triglyceride | Cholesterol                  | Triglyceride | Cholesterol |
|        |            |    | mg/dl       | ND                          | mg/dl         | mg/dl        |
| KK/San | Ad/lacZ    | 1  | 56.0        | ND                          | 65.6          | 48.0         |
| KK/San | Ad/Angptl3 | 3  | 49.5 ± 4.9  | ND                          | 700.5 ± 94.5  | 90.7 ± 24.4  |

Next, we examined the relationship between ANGPTL3 and apoCII, a coactivator of LPL. Purified human apoCII was used as a coactivator instead of fetal bovine serum, and the addition of 0.07 µM apoCII was enough to elicit the maximal activation of purified LPL. As shown in Fig. 7a, ANGPTL3 inhibited purified LPL in a similar manner both in the presence and absence of apoCII. However, the LPL inhibition by ANGPTL3 was attenuated at higher concentrations of apoCII in a dose-dependent manner (Fig. 7b).

Fig. 3. Hepatic VLDL triglyceride secretion in KK/San and wild-type KK mice. Triton WR1339 (400 mg/kg body weight) was injected into fasted KK/San (closed circles) and wild-type KK mice (open circles). Plasma triglyceride levels were determined at the indicated time points and corrected for the triglyceride level at the time of Triton injection (0 min). The values represent means ± S.D. of five mice/group.

Table II

| Strain   | Adenovirus | n   | Immediately after adenovirus injection | Day 3 after adenovirus injection |
|----------|------------|-----|---------------------------------------|-------------------------------|
|          |            |     | Triglyceride | Cholesterol | Triglyceride | Cholesterol |
|          |            |     | mg/dl       | ND          | mg/dl         | mg/dl        |
| KK/San   | Ad/lacZ    | 1   | 56.0        | ND          | 65.6          | 48.0         |
| KK/San   | Ad/Angptl3 | 3   | 49.5 ± 4.9  | ND          | 700.5 ± 94.5  | 90.7 ± 24.4  |

Fig. 2. Immunoblot analysis of plasma apoB. Plasma was obtained from fasted KK/San and wild-type KK mice. Plasma samples (1 µl/lane) were separated on 2–15% gradient gels and immunoblotted with the polyclonal antibody against mouse apoB. The intensity of the bands was estimated using an imaging analyzer.
the rapid clearance of [3H]triglyceride-labeled VLDL and only a slight enhancement of the particle clearance rate in KK/San mice, it is strongly suggested that the low plasma triglyceride levels in KK/San mice were primarily due to enhanced lipolysis of VLDL triglycerides rather than to enhanced whole particle uptake. This notion is also supported by the fact that plasma apoB100 and apoB48 levels of KK/San mice were similar to those of wild-type KK mice.

Maeda et al. (26) have previously reported that apoCIII deficiency reduces plasma VLDL cholesterol and triglyceride levels. These effects on VLDL metabolism in apoCIII-deficient mice appear to be due to increased triglyceride hydrolysis in the circulation, whereas intestinal lipid absorption and hepatic VLDL triglyceride secretion were not affected. Thus, apoCIII deficiency leads to a very similar phenotype in KK/San mice with respect to VLDL metabolism. ApoCIII is thought to be one of the physiological modulators of VLDL triglyceride metabolism through the inhibition of both LPL- and HL-mediated hydrolysis of VLDL triglycerides (27–29). Our in vitro studies revealed that ANGPTL3 inhibited LPL activity in a dose-dependent manner. Therefore, ANGPTL3 probably regulates VLDL metabolism via the inhibition of lipases in a manner similar to apoCIII. This notion is also supported by the rapid increase (within 1–3 h) in plasma triglyceride levels after administration of recombinant ANGPTL3 (12, 30, 31). The determination of plasma concentrations of ANGPTL3 is very important in clarifying the relationship between the in vivo effects and in vitro inhibition of ANGPTL3. We detected the ANGPTL3 protein in the circulating blood of Ad/ANGPTL3-injected KK/San mice by Western blot analysis. A rough estimate of plasma ANGPTL3 levels in the mice was ~100–500 ng/ml (data not shown). As ANGPTL3 inhibited LPL activity at doses of 1–10 μg/ml, we believe that lipase inhibition is the main mechanism by which ANGPTL3 regulates VLDL in vivo.

To further understand the mechanism of LPL inhibition by ANGPTL3, we have examined the relationship between a co-factor (apoCII) and ANGPTL3. ANGPTL3 seems to inhibit LPL directly because purified LPL was inhibited by ANGPTL3 even without a co-factor.

Fig. 4. In vivo metabolism of [3H]triglyceride- and 125I-labeled VLDL in mice. KK/San (closed circles) and wild-type KK (open circles) mice were injected with [3H]triglyceride-labeled VLDL (d < 1.006) (a) or 125I-labeled VLDL (d < 1.006) (b). The plasma decay of the respective labels was determined at the indicated time points. The values represent means ± S.D. of five mice/group. TG, triglyceride.

Fig. 5. Effect of ANGPTL3 on LPL enzyme activity in vitro. LPL activity was determined in the presence of recombinant ANGPTL3 at the indicated doses. Purified bovine milk LPL (a) or a post-heparin medium of rat adipocytes (b) was used as the enzyme source. Fetal bovine serum (5%) was used as a cofactor for LPL activation. The values represent means ± S.D. expressed as a percentage of the control activity determined in the absence of recombinant ANGPTL3.

Fig. 6. Effect of ANGPTL3 on HL enzyme activity in vitro. HL activity in mouse plasma was determined in the presence of recombinant ANGPTL3 at the indicated doses. The values represent means ± S.D. expressed as a percentage of the control activity determined in the absence of recombinant ANGPTL3.
in the absence of apoCII (Fig. 7a). As both apoCII-inactivated and -activated LPL showed very similar inhibitory profiles by ANGPTL3 (Fig. 7a), ANGPTL3 probably binds to a different site on the LPL molecule from the apoCII-binding site and inhibits LPL activity independently of apoCII activation. However, ANGPTL3 may have some interactions with apoCII or may bind to a site on LPL near the apoCII-binding site, because high concentrations of apoCII decreased the inhibition by ANGPTL3 (Fig. 7b). Further investigations are needed to clarify the precise mechanism of the inhibition by ANGPTL3.

We also found a slight increase in total cholesterol and NEFA levels in the presence of recombinant ANGPTL3. However, ANGPTL3 may have some interactions with apoCII or may be one of the LPL inhibitory factors reported previously. Further investigation is needed to elucidate the pathophysiological significance of ANGPTL3.

In this paper, we propose that ANGPTL3 is a new class of lipid metabolism modulator that regulates VLDL triglyceride levels through the inhibition of LPL activity both physiologically and pathologically. Overproduction of VLDL, or a decrease in VLDL catabolism, is the apparent reason for the triglyceride elevation in mild type IV hyperlipoproteinemia and the absence of LPL for the increased triglyceride levels in type I hyperlipoproteinemia. These findings raise the possibility that ANGPTL3 affects the triglyceride levels in some types of hyperlipoproteinemia. Thus, ANGPTL3 may be a useful target in the development of new treatments for atherosclerosis and other human diseases involving hyperlipidemia.

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