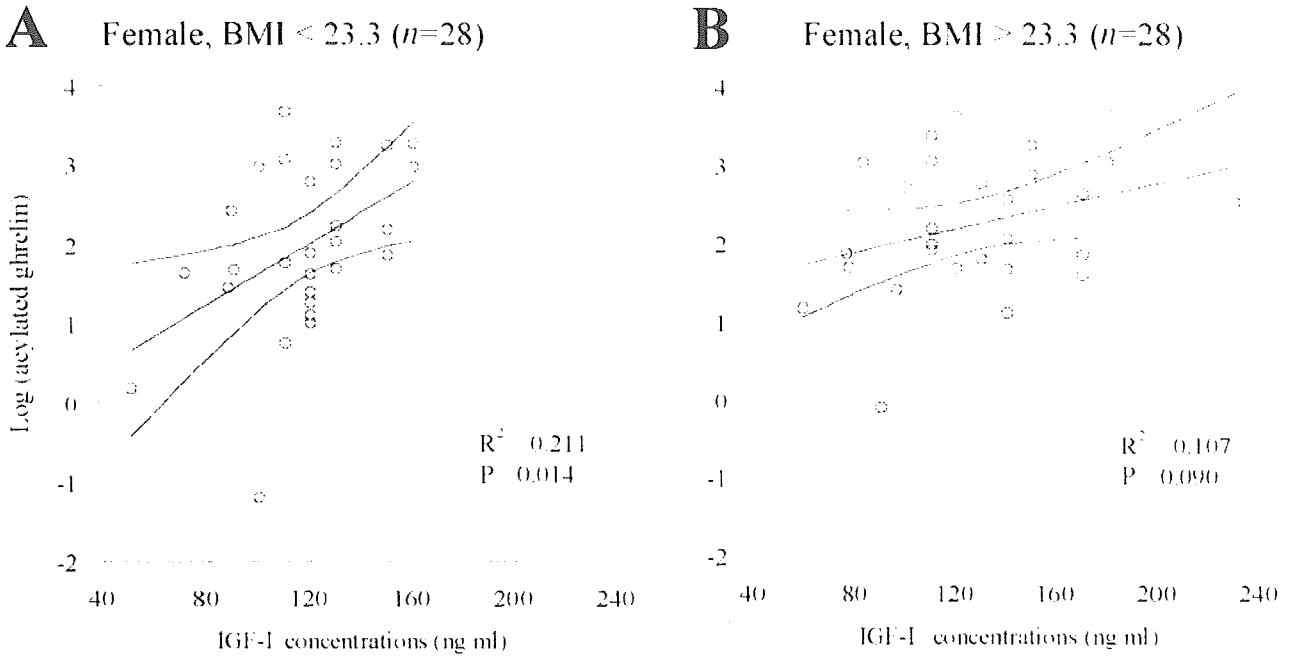

Figure 2 Linear regression analysis of the relationship between IGF-I and plasma acylated ghrelin levels in elderly female subjects of differing BMI levels. (A) Subjects with lower BMI (<23·3, $n=28$); (B) those with higher BMI (>23·3, $n=28$).

significant correlations between either acylated ghrelin and GH levels or GH and IGF-I levels in the group with a lower BMI. Although statistically not significant, plasma levels of acylated and desacyl ghrelin in elderly female subjects tended to be positively associated with BMI, while those in elderly men tended to be negatively associated (Table 2 and Fig. 3).

In the multivariate model, acylated ghrelin levels in women, but not in men, correlated with systolic BP levels, independently of site, age, BMI, sleeping duration,

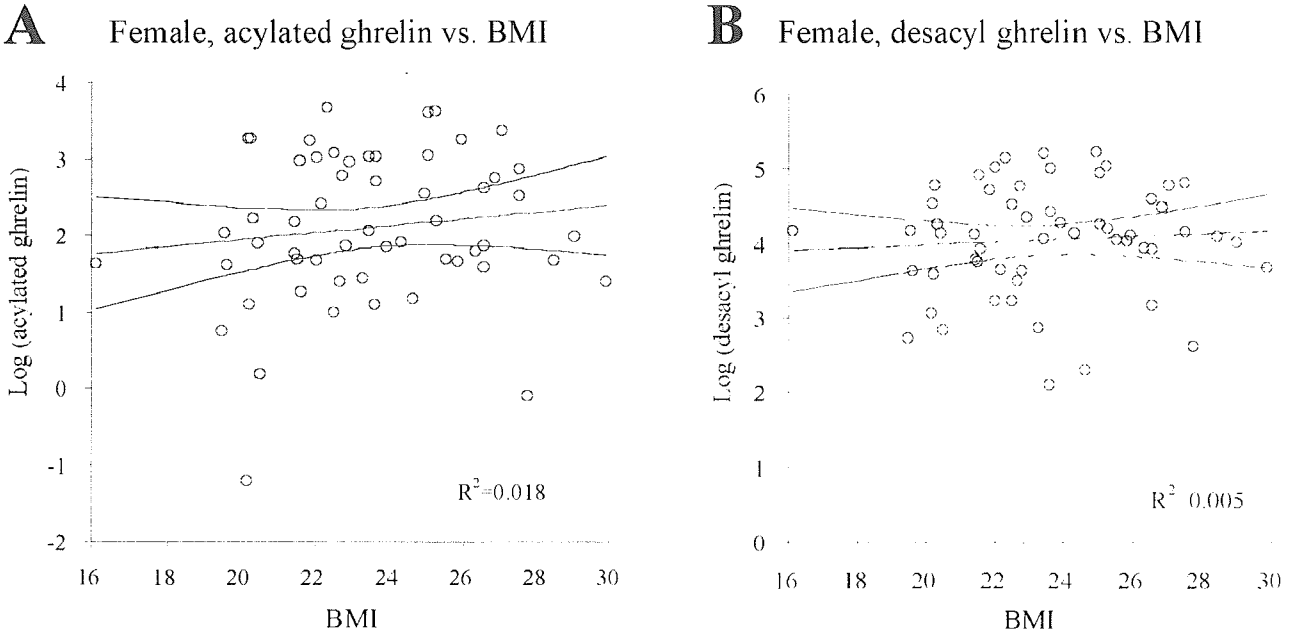

Figure 3 Linear regression analysis of the relationship between BMI and plasma levels of acylated (A) or desacyl (B) ghrelin in elderly female subjects.

Table 3 Relationship between plasma ghrelin concentrations and blood pressure in healthy elderly subjects

	Acylated ghrelin		Desacyl ghrelin		A/D ratio	
	β	*P**	β	*P**	β	*P**
Male						
Systolic blood pressure	−0·003	0·789	0·009	0·467	−0·012	0·050
Diastolic blood pressure	0·012	0·616	−0·016	0·525	0·028	0·027
Female						
Systolic blood pressure	−0·022	**0·039**	−0·016	0·074	−0·006	0·291
Diastolic blood pressure	−0·007	0·719	−0·001	0·969	−0·007	0·565

*Adjusted by recruitment site, age, BMI, sleeping duration, blood pressure (mutually) and blood levels of GH, IGF I, insulin, glucose and leptin.
Bold values: $P<0·05$.

diastolic BP levels and blood levels of GH, IGF-I, insulin, glucose and leptin ($P=0·039$) (Table 3). Finally, acylated ghrelin levels and A/D ratio in women, but not in men, also correlated significantly with frequencies of bowel movement ($P=0·014$ and $P=0·008$ respectively) (Table 4). In men, desacyl ghrelin levels correlated with this parameter ($P=0·037$). There were no significant correlations between ghrelin levels and smoking habits in any subject groups. Gender difference was not an independent determinant of plasma ghrelin levels; β and P values for sex were $\beta=0·366$ and $P=0·138$ (acylated), and $\beta=0·293$ and $P=0·175$ (desacyl) respectively.

Other hormone levels

The correlations between other hormone levels and physiologic parameters in healthy elderly subjects are summarized in Table 5. Significantly, in both sexes, serum GH and IGF-I levels correlated negatively with BMI and age respectively, while serum leptin levels correlated positively with BMI. Plasma glucose levels positively correlated with both serum IGF-I levels and age. Serum IGF-I levels in females correlated positively with plasma concentrations of acylated ghrelin and negatively with serum GH levels, while serum leptin levels in men were significantly associated with age.

Discussion

Although two studies have demonstrated that mean plasma concentrations of total ghrelin in elderly, normal-weight subjects were 36% (Rigamonti *et al.* 2002) and 20% (Sturm *et al.* 2003) lower than those seen in younger, normal-weight subjects, these studies used a small number of subjects. In addition, only total ghrelin levels were examined, and no attempt was made to investigate gender differences. In this study, we demonstrated that the

Table 4 Relationship between plasma ghrelin concentrations and bowel movement in healthy elderly subjects

		Acylated ghrelin		Desacyl ghrelin		A/D ratiio	
	Number	Mean	S.D.	Mean	S.D.	Mean	S.D.
Bowel movement (male)							
≥2/day	15	10·8	10·1	62·9	42·6	15·4	6·0
1/day	29	9·4	13·1	52·7	41·4	17·8	10·3
<1/day	5	4·0	3·1	27·7	34·2	27·0	21·5
P value* (≥1/day vs <1/day)		0·144		**0·037**		0·249	
Bowel movement (female)							
≥2/day	11	15·0	9·8	91·1	43·0	15·6	4·8
1/day	38	12·3	10·2	71·3	47·8	17·3	9·0
<1/day	7	5·5	4·4	45·2	28·6	11·8	6·1
P value* (≥1/day vs <1/day)		**0·014**		0·188		**0·008**	

*Adjusted by recruitment site, age, BMI, sleeping duration and blood levels of GH, IGF-1, insulin, glucose and leptin.
Bold values: $P<0·05$.

Table 5 Mulitiple regression analysis between other hormone levels and various parameters in healthy elderly subjects*

	Male			Female		
	Parameters	β	*P*	Parameters	β	*P*
Hormones						
GH						
	BMI	−0·971	0·001	BMI	−0·411	0·001
	Sleeping time	1·347	0·007			
	Leptin	0·610	0·048			
IGF-I						
	Age	−3·262	0·001	Leptin	3·177	0·001
	Glucose	1·540	0·003	Age	−2·317	0·002
	Leptin	4·466	0·028	BMI	−5·987	0·004
				Acylated ghrelin	0·900	0·012
				GH	−3·739	0·013
Insulin						
	BMI	0·602	0·001	Leptin	0·438	0·001
Glucose						
	IGF-I	0·098	0·009	IGF-I	0·128	0·002
	Age	0·528	0·012	Age	0·552	0·040
	BMI	1·006	0·048			
Leptin						
	BMI	0·513	0·001	BMI	1·410	0·001
	Age	0·106	0·017	Insulin	0·506	0·001
				IGF-I	0·044	0·010

β: regression coefficient.
*Multiple regression analysis with backward elimination procedure was performed after adjustment for the effect of recruitment site; candidate independent parameters were age, BMI, sleeping duration and blood levels of GH, IGF-I, insulin, glucose and leptin.

plasma levels of acylated ghrelin in elderly male and female subjects were respectively 20% and 40% lower than those seen in younger subjects. In contrast, plasma concentrations of desacyl ghelin in elderly subjects of both sexes did not differ from those observed in younger subjects in both sexes. As a result, the A/D ratios in elderly female subjects were significantly lower than those in younger female subjects. In addition, plasma acylated ghrelin levels did not show significant gender difference, while plasma desacyl ghrelin levels in elderly female subjects were significantly higher than those in elderly male subjects, although gender difference was not an independent determinant for them. The reductions in acylated ghrelin levels observed in elderly female subjects may be partially related to a higher BMI than that seen in younger women ($23{\cdot}5 \pm 2{\cdot}9$ vs $20{\cdot}3 \pm 1{\cdot}9$; $P<0{\cdot}001$), as plasma levels of acylated ghrelin in all females were not correlated with age group in the multiple regression analyses. Other modifying factors, especially menopause, should be considered as possibly affecting plasma acylated ghrelin levels in women. In support of this hypothesis, Kellokoski *et al.* (2005) recently reported that estrogen replacement therapy increases plasma levels of acylated ghrelin. Further studies will be necessary to delineate the mechanisms by which estrogen affects the production and/or secretion of acylated ghrelin.

ELISAs used for the measurement of plasma ghrelin levels in this study were two-site sandwich assays with two monoclonal antibodies. One monoclonal antibody recognizes the octanoyl-modified (Active Ghrelin kit) and the other the nonmodified N-terminal portion of ghrelin (Desacyl-Ghrelin kit) (Akamizu *et al.* 2005). The ratio of acylated to (acylated plus desacyl) ghrelin (A/(A+D) ratio) determined by ELISAs was lower than that of acylated to total ghrelin previously determined by RIA, which measures total ghrelin with an antiserum against the C-terminal region of ghrelin. This finding suggests that a fragmented form of ghrelin lacking the N-terminal region may naturally exist in human plasma or may be artificially produced during the RIA procedure. If so, then approximately 40–60% of the total ghrelin measured by RIA is probably fragmented. As a fragmented form of ghrelin is not measured in these two assays, its existence and physiologic implications should be considered and investigated in the future. A limitation of the study was that the measurements of ghrelin concentrations were not undertaken by inclusion of samples from both young and elderly subjects in the same assays. This increases the risk that interassay variation or drift may have reduced our ability to compare concentrations between the two age groups. To mitigate this risk, we used the same kind of assay kit.

A negative correlation between BMI and plasma levels of total or acylated ghrelin was reported by many investigators, including us (Ariyasu *et al.* 2001, Tschop *et al.* 2001, Akamizu *et al.* 2005). On the contrary, plasma levels of both acylated and desacyl ghrelin in elderly female subjects tended to be associated positively with BMI, while those in elderly men tended to be negatively associated (Table 2 and Fig. 3). Particularly, the relationship between plasma acylated ghrelin levels and BMI should be noted, although statistically not significant (β=0·149, P=0·065). These findings suggest that the regulation of ghrelin secretion and/or production in elderly female subjects is altered in comparison to that seen in younger subjects. This altered regulation might be related to the anorexia and undernutrition associated with aging. For example, plasma ghrelin levels may not rise sufficiently when elderly subjects lose weight, resulting in poor appetite and a state of negative energy balance.

In women, acylated ghrelin concentrations correlated positively with IGF-I independently of recruitment site, age, BMI, sleeping duration or blood levels of GH, IGF-I, insulin, glucose and leptin. While a negative correlation between ghrelin and IGF-I levels was reported in children and adolescents (Bellone *et al.* 2002, Whatmore *et al.* 2003), such a correlation has not been observed in adult subjects (Dall *et al.* 2002, Malik *et al.* 2004). Recently, Poykkyo *et al.* (2005) reported a negative correlation between plasma ghrelin and IGF-I in adult subjects with obesity, insulin resistance and type 2 diabetes. The association was particularly strong in both men and subjects in the higher BMI tertiles (maximum: 29·2 or less). In women, the correlation disappeared in the lowest BMI tertile (minimum: 26·5 or more). In agreement with this report, we did not observe a significant correlation between plasma ghrelin and IGF-I levels in the higher BMI population (>23·3). Acylated ghrelin levels, however, correlated positively with IGF-I levels with lower BMI values (<23·3). The positive correlation of ghrelin and IGF-I observed in elderly subjects implicates the dysregulation of ghrelin secretion and/or production during aging, suggesting that the negative feedback regulation of IGF-I may be lost. The IGF-I levels observed in the lower BMI group, 117·4 ± 25·1 ng/ml, may be too low to inhibit ghrelin secretion. In this group, the positive correlation suggests that ghrelin regulates IGF-I production by affecting GH secretion. Although we could not identify significant correlations between either acylated ghrelin and GH levels or GH and IGF-I levels, such associations between plasma ghrelin levels and serum GH levels have been observed in previous studies (Yoshimoto *et al.* 2002, Akamizu *et al.* 2005). Thus, the regulation of ghrelin/GH/IGF-I axis in elderly women with low IGF-I levels may be different from that seen in the younger subjects with normal IGF-I levels.

Acylated ghrelin levels in elderly women correlated negatively with systolic BP. The inverse relationship between total ghrelin levels and BP has previously been reported in pregnant women (Makino *et al.* 2002) and patients with hypertension (Poykko *et al.* 2003). Ghrelin, which exerts vasorelaxant or vasodilatory effects *in vitro* (Okumura *et al.* 2002, Shimizu *et al.* 2003), decreases BP (Nagaya *et al.* 2001). Our study also demonstrated the novel correlation of ghrelin levels with frequency of bowel movement in elderly subjects. It should be noted that the smaller number of men than women might have resulted in the borderline correlation between male acylated ghrelin level and the frequency of bowel movement. Ghrelin administration in humans stimulates peristalsis (Takaya *et al.* 2000, Nagaya *et al.* 2001, Akamizu *et al.* 2004) and enhances gastric and intestinal motilities in rats (Masuda *et al.* 2000, Trudel *et al.* 2002, Fujino *et al.* 2003). These findings suggest that ghrelin might play a role in the regulation of bowel motility.

In this study, we confirmed that both blood IGF-I and glucose levels were significantly correlated with age (Davidson 1979, Corpas *et al.* 1993, Muller *et al.* 1999). Serum leptin levels, adjusted for various parameters including BMI, exhibited a significant positive association with age in men (Table 5), but a nonsignificant negative association with age in women (data not shown). This finding corresponds to a report by Baumgartner *et al.* (1999) suggesting that the differences among men and the changes with age in serum leptin levels are associated with differing circulating levels of testosterone. Although plasma glucose levels correlate positively with serum IGF-I levels, few investigators, as far as we know, have reported this positive correlation. As several regulatory factors affect both blood glucose and IGF-I levels, further investigations will be necessary to clarify the mechanisms underlying this correlation. Finally, we confirmed previously observed correlations between BMI and serum GH or leptin levels in elderly subjects (Baumgartner *et al.* 1999, Iranmanesh *et al.* 1991, Chapman 2004).

In summary, we measured plasma levels of acylated and desacyl ghrelin in healthy elderly subjects. The levels of acylated ghrelin in women correlated positively with IGF-I levels, suggesting that the negative feedback mechanism does not function properly in nonobese elderly subjects. These results suggest, however, that ghrelin may regulate IGF-I levels through control of GH. Acylated ghrelin concentrations in women correlated with both systolic BP and the frequency of bowel movements. These findings strongly suggest that, in elderly women, acylated ghrelin may play a role in the regulation of the GH/IGF-I axis, BP and bowel movements. The obvious next step is to explore and confirm these physiologic effects of ghrelin experimentally. In addition, analysis of 24-h acylated and desacyl ghrelin secretion is extremely important to determine the physiologic control of ghrelin secretion during the lifespan. Finally, understanding the relationship between plasma ghrelin levels and these clinical parameters in the elderly may provide therapeutic opportunities to target ghrelin in disorders related to aging.

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ORIGINAL ARTICLE

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Association of nucleotide variations in the apolipoprotein B48 receptor gene (*APOB48R*) with hypercholesterolemia

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Abstract Factors predisposing to the phenotypic features of high total cholesterol (T-Cho) in human plasma have not been clearly defined. Here we report an association between two variations in the apolipoprotein B48 receptor gene (*APOB48R*) and plasma T-Cho levels among 352 adult individuals in Japan. By analyzing phenotypic associations between age- and gender-adjusted levels of plasma T-Cho, low-density lipoprotein (LDL) cholesterol (LDL-C), and high-density lipoprotein (HDL) cholesterol (HDL-C), we detected a significant correlation between genotypes of the A419P variation and adjusted T-Cho levels. Among homozygous G-allele carriers ($n=265$), heterozygous carriers ($n=78$), and homozygous minor C-allele carriers ($n=9$), T-Cho levels were 2.43 ± 0.21 mg/cm^3, 2.48 ± 0.24 mg/cm^3, and 2.63 ± 0.21 mg/cm^3, respectively, indicating a codominant T-Cho-elevating effect of the minor C-allele ($r=0.15$, $P=0.007$). A similar effect was detected for c.934-960/del ($r=0.13$, $P=0.015$). Linkage disequilibrium (LD) analysis detected significant LD among eight variant sites that included neighboring loci. Our results indicate that variations in *APOB48R* and nearby genes are among the many factors involved in hypercholesterolemia. The etiological studies should now include consideration of this novel aspect of the mechanism(s) leading to hypercholesterolemic disease.

Keywords Nucleotide variations · *APOB48R* · Total cholesterol · Association study · Quantitative trait

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Introduction

Hyperlipidemia is a very common multifactorial disease, the onset and progression of which are determined by genetic risk as well as by environmental factors (Hegele 2001; Cullen et al. 1997). Clarification of genetic risk is essential for prevention and effective early treatment of hyperlipidemia because accumulating evidence from clinical, epidemiological, and experimental studies suggests that lipid and lipoprotein concentrations in plasma are strongly influenced by genetic factors (Zannis and Breslow 1985), not only in some types of familial hyperlipoproteinemia but in nonfamilial primary hyperlipidemias as well.

Plasma lipids are regulated by multiple metabolizing and transport systems in which the lipoprotein system is the critical gateway, as many of the known causes of dyslipidemia depend on the functions of apolipoproteins or their receptors. Rare mutations in certain genes are known to be responsible for abnormalities of plasma lipoproteins: examples are the low-density lipoprotein (LDL) receptor gene (*LDLR*) in familial hypercholesterolemia, the apolipoprotein B gene (*APOB*) in familial defective apoB100, and the lipoprotein lipase gene (*LPL*) in hyperchylomicronemia (Goldstein et al. 1995). However, common variations within apolipoprotein genes may also affect plasma lipoprotein levels, exemplified by a known association of variations of apolipoprotein E (apoE) with dysbetalipoproteinemia (de Knijff et al. 1994). By analogy, we assume that common

variations in genes encoding receptor molecules might also affect lipoprotein metabolism.

A gene encoding a specific receptor for apoB48, a short isoform of apoB, was recently identified on chromosome 16p11 (Brown et al. 2000). ApoB48 protein is a major constituent of triglyceride-rich lipoproteins such as chylomicrons (CM), very low-density lipoproteins (VLDL), and LDLs. In contrast to full-length apoB100 in the lipoprotein moiety derived from liver (VLDL, VLDL remnants, and LDL), intestine-derived apoB48 lacks an LDL receptor (LDLR)-binding domain in its C-terminal half (Yang et al. 1986) and thus requires specific receptor(s). Several common variations have been reported in the *APOB48R* gene involving codons for the repetitive motif in the extracellular domain of apoB48R: these include a 9-amino-acid deletion (27 nucleotides), and some of them would cause significant alteration in the amino-acid sequence of the translated protein. Although the influence of those variations on physiological function has not been investigated thoroughly, it is reasonable to assume that such differences could influence lipid levels in plasma.

To test that hypothesis, we examined the potential involvement of *APOB48R* and neighboring genes in the pathogenesis of hypercholesterolemia by investigating several single-nucleotide polymorphisms (SNPs) and analyzing linkage disequilibrium (LD) among them. Multiple regression analyses were carried out to examine possible associations of genotypes or haplotypes with plasma lipoprotein levels among 352 Japanese.

Materials and methods

Subjects

The subjects for our work were participants in a cohort study carried out concurrently with health-check screening in east-central Japan, as described previously in detail (Fujita et al. 2003). From among the 22,228 participants screened initially, we selected 352 individuals whose LDL-cholesterol (LDL-C) levels were higher than 1.40 mg/cm^3 (LDL-C > 140 mg/dl). None of the selected participants had medical complications or were undergoing treatment for conditions known to affect plasma lipoprotein levels, such as pituitary disease, hypo- or hyperthyroidism, diabetes mellitus, liver disease, or renal disease. None were receiving antihyperlipidemic therapy. The selected participants visited lipid clinics for detailed examination of their lipoprotein profile and other clinical parameters. In addition to these study subjects, 383 individuals were recruited from eastern Japan as a reference cohort for the genotyping results. All gave written informed consent prior to the study, which was approved by the Institutional Review Boards of the Research Consortium.

Physical and clinical profiles of these subjects (age, gender, body-mass index, and plasma lipoprotein and lipid levels) were obtained from records of the special outpatient clinic for dyslipidemic patients at the University of Chiba. These records had been carefully collected during detailed examinations at the first visit. Because initial screening was designed for the health-check assessment, some differences from the general Japanese population were found, representing less than 0.5 times SD differences of mean values. None of the selected participants had medical complications or were undergoing treatment for conditions known to affect plasma lipoprotein levels, such as pituitary disease, hypo- or hyperthyroidism, diabetes mellitus, liver disease, or renal disease. None were receiving antihyperlipidemic therapy. Genomic DNA was extracted from peripheral blood samples, as previously described (Fujita et al. 2003).

Measurement of lipoproteins

Lipid and lipoprotein concentrations in plasma were measured in blood samples collected from each participant after 12–16 h of fasting, according to procedures described previously (Hattori et al. 2002). In brief, plasma cholesterol and triglyceride concentrations were assayed enzymatically, and concentrations of HDL-cholesterol (HDL-C) were determined by the $MgCl_2$-dextran precipitation method. LDL-C concentration was calculated by subtracting the HDL-C value from the fraction containing both LDL-C and HDL-C, as described elsewhere (Ishii et al. 2002).

SNP selection and genotyping

We extracted ten polymorphic variations of the *APOB48R* gene from the dbSNP of the NCBI (http://www.ncbi.nlm.nih.gov/SNP/) and denoted them -1820C/T, -1682C/T, -688TAGT/-, -343C/G, G343R (c.1027G/A), A419P (c.1255G/C), A776V (c.2327C/T), c.934-960/del, c.1034-1062/del, and 3'+119G/A according to their positions (Table 1). Variant sites in two neighboring genes, CLN3 IVS1-229T/C and LOC390688 S44N (c.131G/A), were selected as well. Among these, four variations (-1682A/G, -688TAGT/-, -343C/G, and A776V) appeared to be monogenic in our test of 24 subjects and thus were excluded from the analysis on the entire group of subjects.

Genotypes of five variations (-1820C/T, G343R, c.934-960/del, c.1036-1062/del, and CLN3 IVS1-229T/C) were determined by cycle sequencing using BigDye terminator v1.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 377 DNA Sequencing System (Applied Biosystems). Primer sequences were as follows:

For -1820C/T, 5'-TGGTCTAAGCACCACCTTTG-3' (forward) and 5'-GGCGCATTCCTGTAGTCAC-AGG-3' (reverse); for G343R, c.934-960/del and c.1036-1062/del, 5'-AGGAAGCCCGGACAATCTCA-G-3' (forward) and 5'-GGGACTGCTCCATAT-

Table 1 Summary of polymorphisms analyzed at the APOB48R and neighboring loci

No.	Gene symbol	Name of variations		Nt.	Location	dbSNP-ID[a]
1	LOC390688	S44N	(c.131G A)	aGc aAc	Exon3	rs2269782
2	APOB48R	-1820C T		C T	Promoter	rs27741
3	APOB48R	-1682A G[b]		A G	Promoter	rs27743
4	APOB48R	-688TAGT -[b]		TAGT -	Promoter	rs3834558
5	APOB48R	-343C G[b]		C G	Promoter	rs151234
6	APOB48R	c.934-960 del	(9aa-delA)	-	Exon2	rs3833079
7	APOB48R	G343R	(c.1027G A)	Ggg Agg	Exon2	rs3743960
8	APOB48R	c.1036-1062 del	(9aa-delB)	-	Exon2	rs3833080
9	APOB48R	A419P	(c.1255G C)	Gct Cct	Exon2	rs180743
10	APOB48R	A776V[b]	(c.2327C T)	gCg gTg	Exon2	rs40832
11	APOB48R	3F' + 119G A		G A	3'-Flanking	rs40834
12	CLN3	IVS1-229T C		T C	Intron1	rs34835

[a]Number from dbSNP database of NCBI (http://www.ncbi.nlm.nih.gov/SNP/)
[b]We excluded these variations from the analysis by the exclusion criterion that the minor allele frequency among the 24 test subjects was less than 0.05

TCTGTCT-3' (reverse); and for CLN3 IVS1-229T/C, 5'-AGTTTCTGAGTCCTTTCTGTCTGC-3' (forward), and 5'-GTCCCTTCATGGAGAGTGGC-3' (reverse). A419P and LOC390688 S44N genotypes were determined by Invader assay (Third Wave Technologies, Madison, WI, USA) according to the manufacturer's protocol (Mein et al. 2000; Lyamichev et al. 1999). The manufacturer provided designed probe sets along with the required reagents. The PCR primer sets were (for A419P) 5'-CTGCCTGTACCAGCTCTTCC-3' (forward) and 5'-CAACCTCAGGCAAAGAGGAG-3' (reverse); and (for S44N) 5'-CACATATTGTTGGATGTCTGGC-3' (forward) and 5'-CCAACACCAGGTTGAACTGC-3' (reverse). APOB48R 3'+119G/A genotypes were determined by TaqMan Assays, using specifically designed probes, primers, and reagents of TaqMan Assays-on-Demand (Applied Biosystems). Genotypes were determined according to the manufacturer's protocol, using the ABI prism 7900HT (Applied Biosystems) (Livak 1999).

Haplotyping, LD, and statistical analysis

Plasma levels of lipoproteins were adjusted according to gender and age of each subject using standard data for the general Japanese population obtained from 11,994 individuals in a 2001 cohort study (Kita 2001). Haplotype frequencies among the 704 alleles investigated were calculated by Arlequin software (Genetics and Biometry Laboratory, Geneva, Switzerland). LD for all possible two-way combinations among the variations was tested with D' and r^2 (Millar et al. 2000; Thompson et al. 1988). The diplotype of each individual was estimated by an EM algorithm using SNPAlyze v3.1 (DYNA-COM, Chiba, Japan).

Quantitative associations between genotypes and adjusted plasma-lipid levels (mg/dl) were analyzed by analysis of variance (ANOVA), with regression analysis as a post-hoc test. The three genotypic categories of each SNP (e.g., C/C, C/T, and T/T for rs27741) were converted into incremental values 0, 1, and 2, respectively. These values corresponded to the number of chromosomes possessing a minor allele of the variation. When correlation was examined among 352 subjects, we defined significant association when the given P value of the ANOVA F test was less than 5% ($P < 0.05$). Chi-square tests were used to ascertain Hardy-Weinberg equilibrium among genotypes ($P > 0.05$). Multiple regression analysis was applied for defining the most significant combination(s) of variations that correlated with the adjusted plasma-lipid levels using GLM multivariate ANOVA on SPSS software Advanced Models 11.0 (SPSS Inc., Chicago, IL, USA).

Results

To analyze potential correlation between variants of *APOB48R* and neighboring genes with lipid phenotypes, we first clarified the allelic frequencies and heterozygosities of eight polymorphic sites in these loci by genotyping 352 subjects (Table 2). The subjects then fell into three genotypically subdivided groups (i.e., homozygous major-allele carriers, heterozygous individuals, and homozygous minor-allele carriers) for each of the variations, with no deviation of genotypic frequencies from Hardy-Weinberg equilibrium ($P = 0.54$, χ^2 test). The maximum-likelihood haplotype frequencies estimated by an expectation-maximization algorithm indicated that 12 major haplotypes covered 92% of the chromosomes in our test population, suggesting significant LD within the region (Fig. 1a). Analysis of D' and r^2 also indicated the existence of LD block that covers the *APOB48R* and the neighboring *CLN3* locus (Fig. 1b, c).

By analyzing distribution of the adjusted total cholesterol (T-Cho), LDL-C, and HDL-C concentrations among the genotypic groups for each of the eight variations, we detected a significant difference in T-Cho levels associated with allelic status for A419P (Table 3). The number of the individuals genotypically categorized for homozygous G-allele carriers, heterozygous indi-

Table 2 Summary of correlation analysis

No.	Gene symbol	Variation	Allele frequency (heterozygosity)	N^a	Correlation coefficient (r^2)	P value[b]
1	LOC390688	S44N	0.80:0.20 (34%)	340	0.08	ND
2	APOB48R	-1820C>T	0.78:0.22 (32%)	339	0.07	NS
3	APOB48R	c.934-960 del	0.93:0.07 (16%)	348	0.02	NS
4	APOB48R	G343R	0.85:0.15 (15%)	350	0.08	NS
5	APOB48R	c.1036-1062 del	0.87:0.13 (22%)	348	0.13	0.015
6	APOB48R	A419P	0.86:0.14 (22%)	352	0.14	0.007
7	APOB48R	3'+119G A	0.73:0.27 (38%)	350	0.09	NS
8	CLN3	IVS1-229T C	0.77:0.23 (34%)	347	0.07	ND

[a]Number of the subjects whose genotype was determined
[b]P value was calculated for regression analysis with ANOVA F-test. *NS* not significant, *ND* not determined

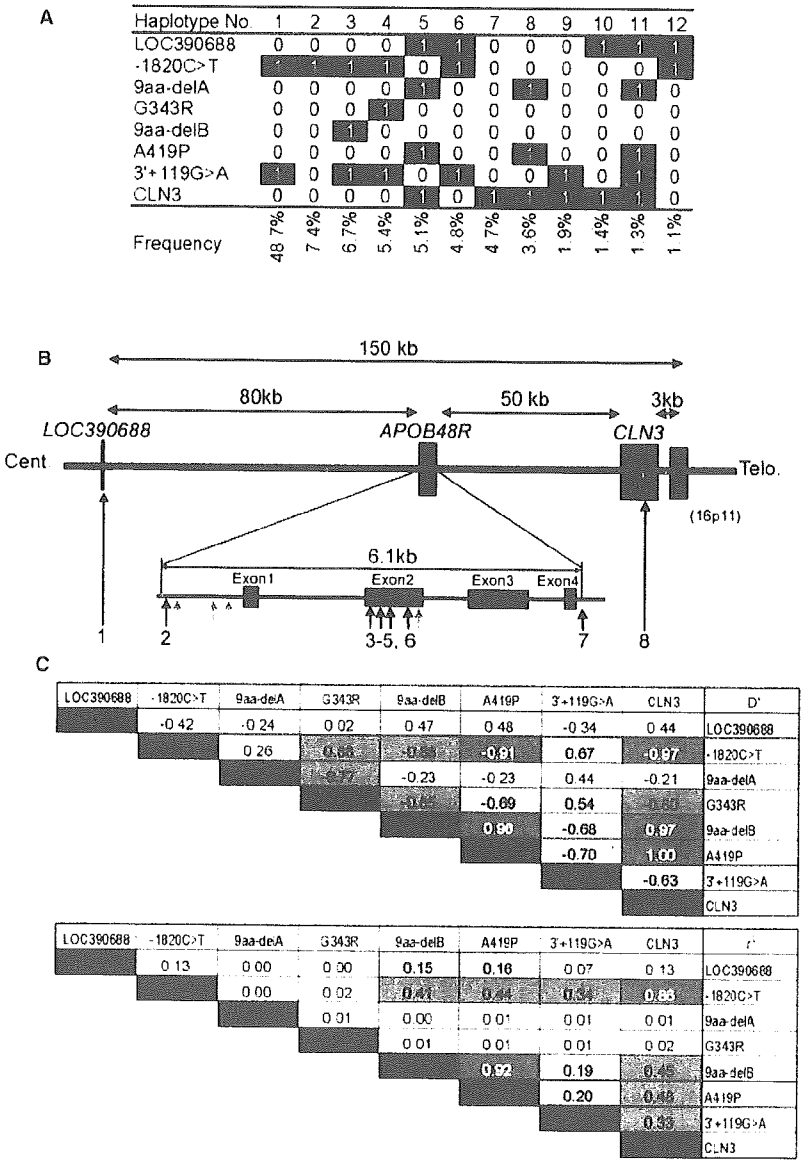

Fig. 1 Analysis of haplotypes and linkage disequilibrium (LD) for eight variations in the *CLN3*, *APOB48R*, and *LOC390688* loci. **a** Haplotype analysis on this locus revealed that 12 major haplotypes cover 93% of the chromosomes among the individuals in this cohort. Each haplotype was designated by the number of the order in estimated frequencies, as indicated on the *top*. Frequencies are indicated at the *bottom*. In the table, *number 1* indicates the minor allele of each variation. *Number 0* indicates the major alleles. **b** Schematic diagram of the genomic structure containing the *APOB48R* and neighboring genes. *Upper line* indicates genomic structure of the *APOB48R* and the neighboring loci. *Lower line* indicates the exon–intron structure of the *APOB48R* (*boxes* indicate the four exons). Locations of the 12 tested variations are indicated by *upward arrows*; *black arrows* indicate the eight variations analyzed, and *arrows in gray* halftone indicate the four excluded variations. **c** Index of LD (D') calculated for every possible pair among the eight variations, shown in tabular form. Values greater than 0.5 are highlighted with a gradient of *gray halftones*. In the table, variations were designated in the short form; i.e., *LOC390688* stands for LOC390688 S44N, *9aa-delA* for c.934-960 del, *9aa-delB* for c.1034-1062 del, and *CLN3* for CLN3 IVS1-229T C. **c** Index of LD (r^2) calculated for every possible pair. Values greater than 0.1 are highlighted with *gray halftones*

viduals, and homozygous minor allele carriers were 265, 75, and nine, respectively, which was not largely different from the frequencies obtained from 383 independent subjects as a reference cohort (309, 67, and seven). Among our study subjects, codominant T-Cho-elevating effect of the minor C-allele ($r=0.15$, $P=0.007$) was

Table 3 Physical and clinical profiles of the 352 subjects

	Whole	A419P			*P* value[a]
		Ala Ala	Ala Pro	Pro Pro	
Number	352	265	78	9	
Gender (M F)	142 210	106 159	31 47	5 4	NS[b]
Ages (years)	60.4 ± 9.2	60.3 ± 9.2	59.9 ± 8.8	67.3 ± 11.6	NS
BMI (kg m^2)	23.9 ± 3.7	23.9 ± 3.9	23.8 ± 3.0	24.0 ± 2.4	NS
TC (mg cm^3)	2.45 ± 0.22	2.43 ± 0.21	2.48 ± 0.24	2.63 ± 0.21	0.007
LDL-C (mg cm^3)	1.64 ± 0.19	1.63 ± 0.18	1.66 ± 0.20	1.70 ± 0.23	NS
HDL-C (mg cm^3)	0.51 ± 0.12	0.50 ± 0.11	0.51 ± 0.12	0.57 ± 0.17	NS
TG (mg cm^3)	1.50 ± 0.79	1.50 ± 0.75	1.46 ± 0.91	1.80 ± 0.84	NS

[a] *P* value was calculated for differences among genotypically categorized subjects by χ^2-test (for gender) or linear regression by ANOVA *F*-test (others)
[b] *NS* not significant, values are expressed in means ± SD

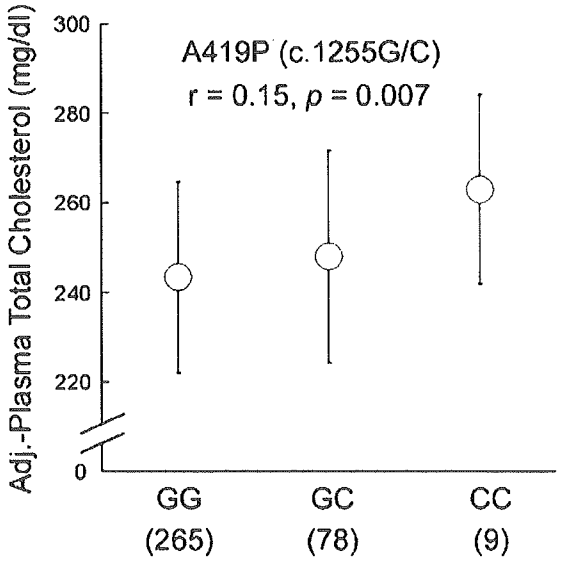

Fig. 2 Association of the A419P genotype with adjusted plasma T-Cho concentrations among 352 subjects. *Open circles* indicate mean values, and *error bars* indicate standard deviations. The correlation between numbers of minor alleles and the adjusted plasma T-Cho (mg/dl) was tested by linear regression analysis (A419P, $r = 0.15$, $P = 0.007$)

indicated because a stepwise distribution of the plasma T-Cho levels was detected in this order (2.43 ± 0.21 mg/cm^3, 2.48 ± 0.24 mg/cm^3, and 2.63 ± 0.21 mg/cm^3 respectively; Fig. 2). Codominant effect was supported by analyzing the differences between two genotypically divided groups based on dominant model (C-allele carriers: $n = 87$, T-Cho $= 243.4 \pm 21.4$ and noncarriers: $n = 265$, T-Cho $= 249 \pm 23.8$, $P = 0.026$) and recessive model (G-allele carriers: $n = 343$, T-Cho $= 244 \pm 22.0$ and noncarriers: $n = 9$, T-Cho $= 263.1 \pm 21.1$, $P = 0.012$).

In addition to A419P, a similar effect was detected for c.934-960/del ($r = 0.13$, $P = 0.015$), but this appeared to be due to strong LD between these two polymorphisms. This notion was supported by haplotype analysis of the APOB48R locus (Table 4), by which contribution of the third haplotype (1-1-0-0-1-1: -1820T -c.934-960/del -343G- c.1034-1062-419P-3'+119A) to higher T-Cho level was suggested (Table 5). No other individual variation or haplotype showed statistically significant correlation to T-Cho or levels of any lipoprotein.

The possibility of combined effects was tested by multiple regression analysis. Fitting an equation explained about 4% of the plasma T-Cho variances ($r^2 = 0.038$, $P = 0.02$) when all five variations in *APOB48R* were included. Selection of two dependent variables (A419P and -1820C/T) allowed us to predict individual T-Cho levels more efficiently ($r^2 = 0.033$, $P = 0.003$). However, when the stepwise method was introduced for variable selection, only one variable A419P was selected, suggesting that there is no statistical significant contribution of other variations with interactive effect.

Discussion

The work reported here detected significant association of *APOB48R* polymorphisms with adjusted levels of T-Cho in human plasma, allowing us to propose this gene as a novel candidate for susceptibility to hyperlipidemia. Multiple variations in *APOB48R* and neighboring genes revealed this association among hypercholesterolemic individuals. Among the variations examined, two in the *APOB48R* gene itself (c.934-960/del and A419P) were potentially functional and thus might be involved in determining plasma T-Cho levels for hypercholesterolemic individuals.

APOB48R was a rationally selected candidate gene because its product is distributed among the triglyceride-rich lipoprotein moieties in plasma. Reticuloendothelial systems should maintain balance between the uptake of intestine-derived lipoproteins (CM and CM remnants) and uptake of liver-derived lipoproteins (VLDL, VLDL remnants, and LDL). Changes in the ability to take up intestine-derived lipoproteins could affect the entire balance of serum lipoproteins. Significant association was detected only for T-Cho levels in our test subjects. Lack of statistical power is the likely reason why no

Table 4 Frequencies of 13 major haplotypes estimated by EM algorithm at the *APOB48R* and neighboring loci

Name of the polymorphisms[a]	Haplotype no.[b]												
	1	2	3	4	5	6	7	8	9	10	11	12	13
-1820C>T	0	0	1	0	1	0	1	1	0	1	0	0	0
c.934-960 del	0	0	1	0	0	0	0	1	1	1	0	1	1
G343R	0	0	0	0	0	1	0	0	0	0	1	0	0
c.1036-1062 del	0	0	0	1	0	0	0	0	0	1	0	0	0
A419P	0	0	1	0	0	0	0	1	1	1	0	0	1
3'+119G>A	0	1	1	0	1	0	0	0	0	1	1	0	1
Frequency (%)	52.8	9.3	8.6	8.1	6.7	6.5	2.9	2.1	0.5	0.5	0.4	0.3	0.3
Sum (%)													98.9

[a]Major and minor alleles for each polymorphism were designated as 0 and 1
[b]Haplotype numbers were designated in the order of greater frequencies

Table 5 Physical and clinical profiles of the 330 diplotype estimated subjects

	Whole	Diplotypes			*P* value[a]
		No Hap#3	1 Hap#3	2 Hap#3	
Number	330	275	50	5	–
Gender (M/F)	134/193	111/161	20/30	3/2	NS[b]
Ages (years)	60.1 ± 10.9	60.1 ± 11.2	59.5 ± 9.3	70.0 ± 10.6	NS
BMI (kg/m^2)	23.7 ± 2.9	23.7 ± 3.0	23.9 ± 2.7	24.4 ± 1.8	NS
TC (mg/cm^3)	2.44 ± 0.23	2.42 ± 0.23	2.46 ± 0.22	2.63 ± 0.26	0.05
LDL-C (mg/cm^3)	1.62 ± 0.19	1.62 ± 0.19	1.64 ± 0.18	1.63 ± 0.19	NS
HDL-C (mg/cm^3)	0.51 ± 0.12	0.50 ± 0.12	0.51 ± 0.11	0.57 ± 0.22	NS
TG (mg/cm^3)	1.49 ± 0.74	1.48 ± 0.73	1.48 ± 0.76	2.06 ± 1.06	NS

[a]*P* value was calculated for differences among genotypically categorized subjects by χ^2 test (for gender) or linear regression by ANOVA *F*-test (others)
[b]*NS* not significant, values are expressed in means ± SD

association was detected for the LDL-C, for which we detected some tendency ($r = 0.09$). Reproducible association should be examined in the larger cohort by examining factors including VLDL, VLDL remnants, LDL-C, and HDL-C levels.

Variation-exerted changes in plasma cholesterol levels may be related to ligand-binding affinities. Specific binding of apoB48 to this receptor (apoB48R) is evident although the ligand-binding domain has not been clearly defined. A previous study suggested that a series of 9-amino-acid repeats in the extracellular domain, an element of unknown function, might contribute to binding (Brown et al. 2000). In that respect, our result for the variation c.934-960/del was interesting since it deletes one of the eight repeating elements of this region. Another SNP, A419P, is also interesting because it localizes within the same region of the extracellular domain. Structural alterations of the extracellular domain of the receptor due to these variations might alter binding affinity for its specific ligand, apoB48, and affect uptake of CM and CM remnants. When we analyzed the predictive changes in protein function by missense coding SNPs using the "Sorting Intolerant From Tolerant" (SIFT) program (http://blocks.fhcrc.org/sift/SIFT.html; Ng and Henikoff 2002), a deleterious effect of 343-R (score $= 0.04 < 0.05$) was indicated; however, 419-P may have only a mild effect (score $= 0.11$). This notion could be tested by means of ligand-blotting or lipoprotein-uptake assays (Brown et al. 2000; Daniel et al. 1983). Another possibility that a variation in the 5'-upstream region of this gene, -1820C > T, may have additional effect on serum cholesterol should be functionally tested by examining the binding for nuclear factors HNF-3/fork-head homolog-2, cHox-cad or chorion factor CF2, the binding motif of which was indicated close to this polymorphism by TFSEARCH program (Searching Transcription Factor Binding Sites: http://www.rwcp.or.jp/papia/).

We observed significant LD within the *APOB48R* locus extending to neighboring loci (*CLN3*). The region with significant LD extended about 60 kb was within the standard range of LD reported at other loci (Reich et al. 2001); however, the internal pattern of the LD indices was somehow complicated. One possible reason for this complexity could be related to the existence of a highly homologous nearby gene (*LOC400514*: 99% identical to *APOB48R* in nucleotide sequence); i.e., some of the variations analyzed here might actually belong to the *LOC400514* gene (Fredman et al. 2004). This must be clarified by extensive challenge for direct sequencing effort to distinguish *APOB48R* from *LOC400514* localized in this long range of highly homologous and repetitive chromosomal region.

In conclusion, we identified multiple variations in a novel hyperlipidemia-susceptibility gene, *APOB48R*, that may affect plasma T-Cho levels among hyperlipidemic individuals. Further investigations of these variations should help to clarify the complex mechanism(s) determining hypercholesterolemia and lead to a better understanding of the pathogenesis of hyperlipidemia. Such analyses might present a novel point of view for establishing suitable treatment designs and plans for prevention of the disease.

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Roles of degree of fat deposition and its localization on VEGF expression in adipocytes

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Miyazawa-Hoshimoto, Saori, Kazuo Takahashi, Hideaki Bujo, Naotake Hashimoto, Kazuo Yagui, and Yasushi Saito. Roles of degree of fat deposition and its localization on VEGF expression in adipocytes. *Am J Physiol Endocrinol Metab* 288: E1128–E1136, 2005. First published December 21, 2004; doi:10.1152/ajpendo.00003.2004.—Vascular endothelial growth factor (VEGF) is an important angiogenic factor and is expressed in wide variety of cell types. In this study, we investigated the mechanism of VEGF production in adipocytes in three sets of experiments. First, to clarify the relation between plasma VEGF concentrations and their expressions in adipose tissues, we investigated the genetically obese *db/db* and KK-A^y mice. Plasma VEGF concentrations in obese mice were significantly higher than in control and were related to adiposity. VEGF expressions in visceral fat were enhanced during growth and were related to fat deposition. Next, to demonstrate the relation between VEGF production and lipid accumulation in adipocytes, we analyzed VEGF mRNA expression and its protein secretion in 3T3-L1 cells. VEGF production was enhanced during lipid accumulation in 3T3-L1 cells after adipocyte conversion. Next, to clarify the role of anatomic localization on VEGF expression in adipocytes, we implanted 3T3-L1 cells into visceral or subcutaneous fat in athymic mice. 3T3-L1 cells implanted into the mesenteric area expressed more VEGF mRNA than that into the subcutaneous area. Plasma VEGF concentration in the mice implanted in visceral fat was higher than in controls. These results suggest that both the anatomic localization and the lipid accumulation are important for the VEGF production in adipocytes.

vascular endothelial growth factor; fat distribution; cytokine; gene expression; 3T3-L1 cells

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) is a very potent angiogenic factor that induces migration and proliferation of vascular endothelial cells (9). VEGF also enhances vascular permeability and modulates thrombogenicity (18). It has therefore been implicated in normal blood vessel development as well as in pathological vessel formation (6). Pathogenic neovascularization plays a major role in the development of atherosclerosis (20), tumor growth (18), rheumatoid arthritis (9, 16), and various retinopathies (2, 3). VEGF mRNA expression has been identified in various cell types, including endothelial, epithelial, and mesenchymal cells (9, 18). It has also been reported that VEGF mRNA is expressed in 3T3-F442A cells, an established preadipocyte cell line (4).

VEGF is encoded by a single gene; however, four isoforms of 205, 188, 164, and 120 amino acids long are produced as a result of alternative splicing. The 164-amino acid-long isoform is the most abundant. We have reported that serum concentration of the 164-amino acid-long isoform of VEGF in human obese subjects is dependent on the intra-abdominal fat accumulation determined using computed tomography scan at the umbilical level (15). Furthermore, the elevated VEGF level or the accumulated visceral fat in the obese subjects was decreased after body weight reduction (15). These observations revealed that the VEGF secretion from adipose tissues, particularly from visceral adipose tissues, might regulate its serum concentration.

Adipose tissues have been reported to express and release various secretary molecules, such as leptin (10, 11), tumor necrosis factor-α (TNF-α) (7), plasminogen activator inhibitor-1 (PAI-1) (20), and IL-6 (22). Especially, the expression levels of TNF-α and PAI-1 in adipocytes are shown to be directly related to the degree of differentiation from preadipocytes and to be dependent on their anatomic location (12, 14, 20, 21).

Therefore, it is very important to clarify the mechanism of VEGF production in adipocytes from the point of view of the adipocyte differentiation process and the site of fat accumulation. In this study, we examined VEGF production from intrinsic adipocytes in *db/db* and KK-A^y mice during growth, from 3T3-L1 cells depending on the differentiation in culture, and from 3T3-L1 cells that were implanted into the visceral or subcutaneous fat area.

MATERIALS AND METHODS

Materials. MCDB131, FBS, and trypsin were purchased from Invitrogen (Carlsbad, CA). PBS was from Nissui Pharmaceuticals (Tokyo, Japan). DMEM, human insulin, dexamethasone, and 3-isobutyl-1-methylxanthine were obtained from Sigma-Aldrich (St. Louis, MO). Collagenase-S1 was from Nitta Gelatin (Osaka, Japan). Growth Factor Reduced BD Matrigel matrix was from Nippon Becton-Dickinson (Tokyo, Japan). ISOGEN reagent was from NIPPON GENE (Tokyo, Japan). RNeasy Mini Kits and QIAGEN OneStep RT-PCR Kit were from Qiagen (Tokyo, Japan). 3T3-L1 cells, an established preadipocyte cell line, was obtained from the American Type Culture Collection (Manassas, VA). Human umbilical vein endothelial cells (HUVECs) and EBM-2 medium were from BioWhittaker (Walkersville, MD).

Obese mice. BKS.Cg-+ Lepr^{db}/+ Lepr^{db}/Jcl (*db/db*) and KK-A^y/TaJcl (KK-A^y) mice, and control littermates BKS.Cg-m +/+ Lepr^{db}/Jcl (*db/+*) and C57BL/6JJcl (C57BL/6), respectively, were obtained from CLEA Japan (Tokyo, Japan) at 5 wk of age. The mice were maintained in a temperature-, humidity-, and light-controlled room (12:12-h light-dark cycle) with free access to water and standard

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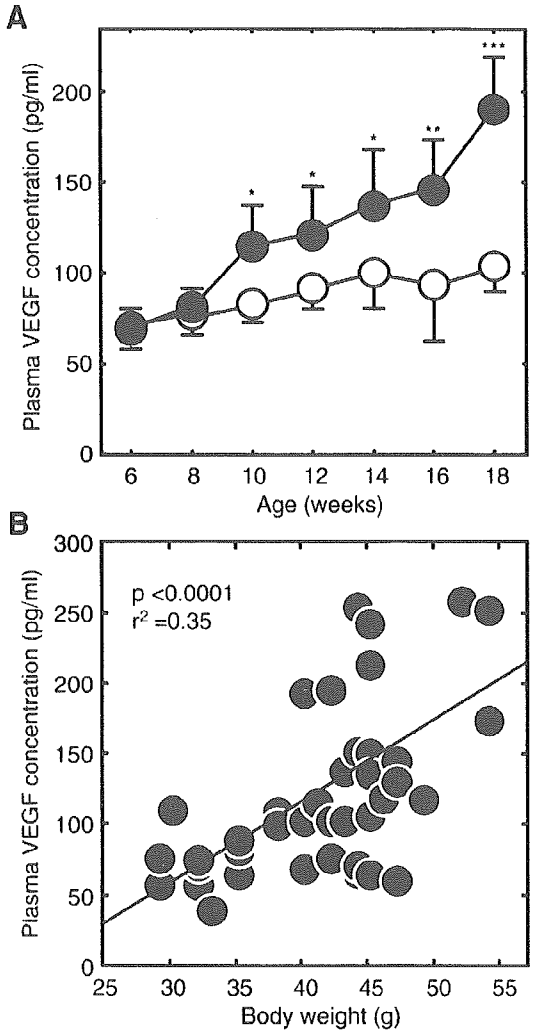

Fig. 1. Growth-dependent change of plasma vascular endothelial growth factor (VEGF) concentration in *db/db* mice. *A*: time course changes of plasma VEGF concentrations in *db/db* (●) and *db/+* (○) mice. Plasma VEGF concentrations were measured every 2 wk from 6 to 18 wk of age in *db/db* and *db/+* mice. Results are represented as means and SD. After 10 wk of age, plasma VEGF concentrations in *db/db* mice were significantly higher than in *db/+* mice. *B*: correlation between body weight and plasma VEGF concentrations in *db/db* mice. Plasma VEGF concentrations were significantly correlated with body weight in *db/db* mice. $^{*}P < 0.05$ vs. *db/+* mice; $^{**}P < 0.01$ vs. *db/+* mice; $^{***}P < 0.001$ vs. *db/+* mice.

rodent chow (352 kcal/100 g, CE-2; CLEA Japan). Male mice were used in the studies reported here. Animal care and procedures were approved by the Animal Care Committee of Chiba University School of Medicine.

Body weight and adiposity. Body weights of *db/db* and *db/+* mice were measured at every 2 wk from the time they were 6 wk old throughout the study. Blood samples were also obtained from the retroorbital venous plexus of the mice fasted more than 16 h. The *db/db* and *db/+* mice were killed at 6, 10, 14, and 18 wk of age by cervical dislocation before white adipose tissues were collected. Mesenteric adipose tissues were used as visceral fat, and inguinal subcutaneous adipose tissues were used as subcutaneous fat in the studies reported here. The white adipose tissues were weighed on an analytic balance and processed for cell counts as described previously (13). Briefly, minced adipose tissues were incubated with PBS containing collagenase S-1. The tissue fragments were removed by passage through a 250-μm nylon screen. The isolated cells were then stained with methylene blue, and aliquots were placed on a Neubauer hemocytometer. Total cell counts were measured using a light microscope.

Total RNA and protein extraction from adipose tissues in obese mice. Mesenteric and subcutaneous adipose tissues of *db/db* and *db/+* mice were processed for total RNA isolation using ISOGEN reagents according to the manufacture's instructions. In another set of experiments, the adipose tissues were homogenized in an ice-cold buffer containing 50 mM Tris·HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 130 mM NaCl, 1% NP-40, 10 μM 4-amidinophenylmethanesulfonyl fluoride, and 5 μM leupeptin. Insoluble materials in the tissue were removed by centrifugation at 12,000 *g* at 4°C for 20 min. After centrifugation, tissue extracts were collected. Moreover, total RNA in the isolated adipocytes of mesenteric fat was also prepared. The mesenteric adipose tissue was digested with collagenase S-1 and passed through a 250-μm nylon screen to remove tissue debris. Then, the isolated cells, containing adipocytes and vascular-stromal cells, were separated by centrifugation. After the adipocytes were allowed to float, the vascular-stromal cells were removed from the bottom layer. The floating layer, as adipocyte fraction, was washed three times with PBS. Finally, the isolated adipocytes were collected and processed for cell counts, using a Neubauer chamber as described above. To compare directly the cellular expression of VEGF in adipocyte, 2×10^4 cells were processed for total RNA isolation using ISOGEN reagent. The KK-A^y and C57BL/6 mice were killed at 16 wk of age by cervical dislocation before mesenteric and inguinal subcutaneous fat was collected for total RNA isolation.

3T3-L1 cells culture and differentiation. 3T3-L1 preadipocytes were cultured with DMEM containing 10% FBS at 37°C in a 5% CO_2 incubator. Adipocyte differentiation was carried out by changing to a differentiation medium containing 10 μg/ml insulin, 0.25 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine. After 48 h, the medium was replaced with a maturation medium containing 5 μg/ml insulin, and cells were maintained in this medium until use. Every week after differentiation, the cells were washed with PBS and then cultured in fresh DMEM medium alone. After incubation for 24 h, the conditioned media were collected. In another set of experiments, the cells were processed for total RNA isolation using an RNeasy Mini Kit.

In vitro endothelial tube formation assay. HUVECs were grown in EBM-2 medium containing 10% FBS. Formation of capillary tube-like structures by HUVECs was assessed in a Matrigel-based assay as previously described (8). Briefly, HUVECs were incubated with MCDB131 containing 2% FBS for 48 h prior to tube formation assay. Cells (7×10^4) were plated onto 300 μl of Growth Factor Reduced BD Matrigel matrix (7 mg/ml protein), pregelled at 37°C in 24-well culture plates. Then, the cells were incubated for 13 h at 37°C with 150 μl of MCDB131 and 150 μl of the conditioned medium derived from pre- or postdifferentiated 3T3-L1 cells in the presence or absence of anti-mouse VEGF-neutralizing antibody. Three different phase-contrast microscopic low-power fields (×100) per well were photographed. The total length of capillary tubes in each photograph was measured using a scale ruler.

Preadipocyte transplantation. 3T3-L1 cells were implanted into athymic mice as described previously (19). Briefly, 3T3-L1 preadipocytes were grown to near confluence, trypsinized, and suspended in DMEM with 10% FBS. After centrifugation, cell pellets were resuspended in PBS and injected 1×10^7 cells (500 μl) through 22-gauge needles into the mesenteric area near the small intestine or the subcutaneous fat area of athymic mice of the BALB/C strain under

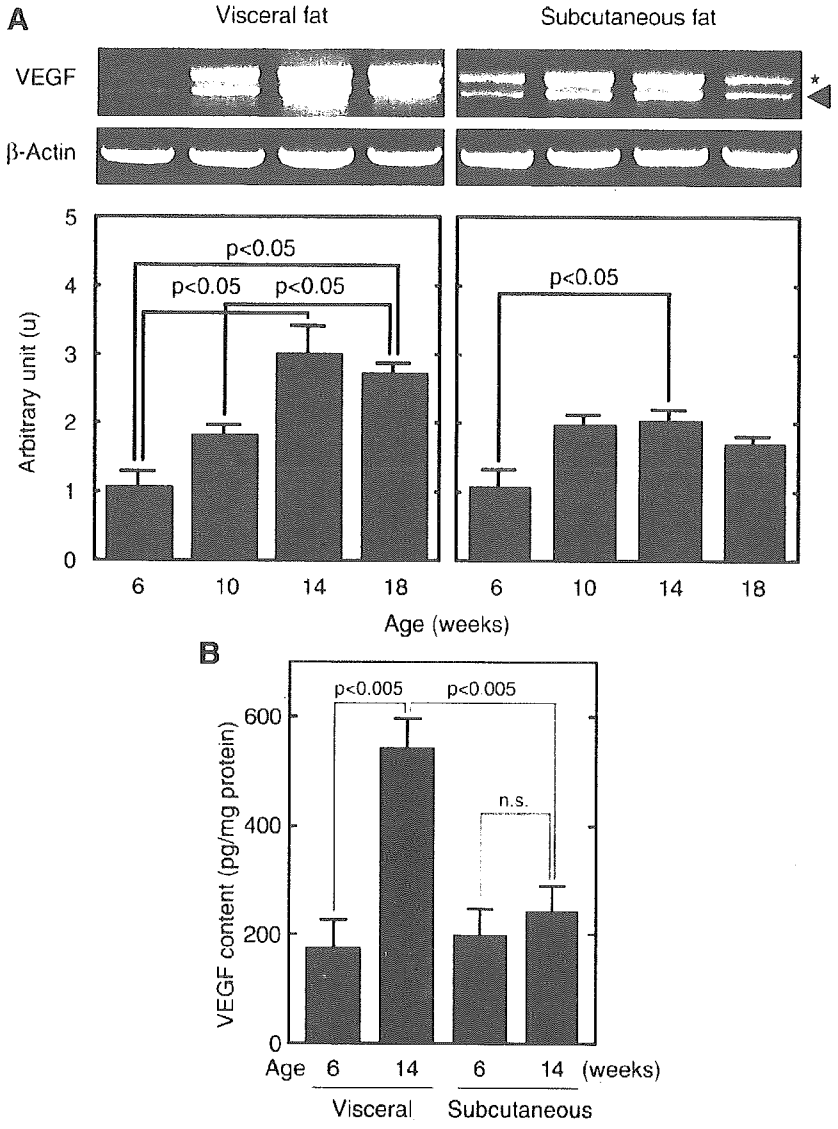

Fig. 2. Growth-dependent changes of mRNA expressions and protein contents of VEGF in mesenteric and subcutaneous adipose tissues of *db/db* mice. *A*: Time course changes of VEGF gene expressions in visceral and subcutaneous adipose tissues of *db/db* mice. Images show RT-PCR products of VEGF amplified from total RNA in mesenteric and subcutaneous adipose tissues at 6, 10, 14, and 18 wk after birth. PCR products of VEGF were densitometrically analyzed, and relative amounts at 6 wk old were set to 1.0. Results are represented as means and SD. Arrowhead, 644 bp ($VEGF_{164}$); *, 716 bp ($VEGF_{188}$). *B*: Tissue VEGF contents in mesenteric and subcutaneous fat. Tissues were extracted with a buffer containing 50 mmol/l Tris·HCl (pH 7.4), 1 mmol/l EDTA, 1 mmol/l DTT, 5 mmol/l $MgCl_2$, 130 mmol/l NaCl, 1% NP-40, 10 μmol/l APMSF, and 5 μmol/l leupeptin. Tissue extracts were processed for VEGF measurement using an ELISA system.

anesthetization by intraperitoneal injection with pentobarbital sodium. Mice were housed in microisolator cages under specific pathogen-free conditions during whole experiments. Four weeks after implantation, the mice were killed by cervical dislocation under anesthetization before mesenteric or subcutaneous fat area was collected. Total RNA of mesenteric and subcutaneous fat was isolated using ISOGEN reagent. Blood samples were also obtained from the retroorbital venous plexus of the mice fasted more than 16 h.

Measurement of immunoreactive VEGF. Plasma samples were prepared by centrifugation at 1,500 *g* for 15 min at 4 C. After centrifugation, the plasma fraction was collected and stored at −70 C until use. The extracts of adipose tissues and the conditioned media from pre- and postdifferentiated 3T3-L1 cells were also stored at −70 C until use. VEGF concentrations of plasma, extracts from adipose tissues, and conditioned media were measured with an enzyme-linked immunosorbent assay system (R&D Systems, Minneapolis, MN).

RT-PCR. To evaluate the contents of VEGF expression in adipose tissues and 3T3-L1 cells, 0.4 μg of total RNA was amplified by OneStep RT-PCR kit using the indicated specific primers. To compare directly the VEGF expressions in adipocytes of mesenteric fat during growth, total RNA prepared from 2×10^4 cells was also amplified using the specific primers. The contents of GLUT4, peroxisome proliferator-activated receptor-γ (PPARγ), and β-actin were also amplified by RT-PCR. The RT-PCR products were run on 1.5% agarose and stained with ethidium bromide. The relative signal intensities of the PCR products were determined with luminescent image analyzer LAS-1000 (Fuji Photo Film, Tokyo, Japan). mRNA amounts were normalized to levels of β-actin mRNA, which served as endogenous standard.

Primers. The following primers were designed for RT-PCR analysis using in this study: VEGF, 5′-GCGGGCTGCCTCGCAGTC-3′ (forward) and 5′-TCACCGCCTTGGCTTGTCAC-3′ (reverse); β-actin, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ (forward) and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′ (reverse); GLUT4, 5′-GGCATGTGTGGCTGTGCCATC-3′ (forward) and 5′-GGGTTTCACCTCCTGCTCTAA-3′ (reverse); PPARγ, 5′-GACATCCAA-

GACAACCTGCTG-3′ (forward) and 5′-GCAATCAATAGAAG-GAACACG-3′ (reverse). RT-PCR products for VEGF were 716 bp ($VEGF_{188}$), 644 bp ($VEGF_{164}$), and 512 bp ($VEGF_{120}$), respectively. The signal intensity of the 644-bp product was analyzed in this study. Products of 349, 413, and 258 bp were predicted for β-actin, GLUT4, and PPARγ, respectively.

Statistical analysis. Statistical analyses were performed using Statview J-4.5. Statistical analysis was performed with a *t*-test. All of the results reported herein were confirmed by repeating the experiments with different occasions. A value of $P < 0.05$ indicated statistical significance.

RESULTS

Growth-dependent changes of plasma VEGF concentration in db/db mice. We measured circulating VEGF concentrations in *db/db* mice, a strain of the mouse models for obesity, to demonstrate the role of fat accumulation and its effect on VEGF levels in vivo. Plasma VEGF concentrations were increased during growth in both *db/+* and *db/db* mice (Fig. 1*A*). At 10 wk old, plasma VEGF concentrations in *db/db* mice were significantly higher than in *db/+* mice. Moreover, plasma VEGF concentrations were significantly correlated with body weight (Fig. 1*B*).

Growth-dependent changes of VEGF mRNA expressions and protein contents in visceral and subcutaneous fat of db/db mice. VEGF mRNA was detected in both visceral and subcutaneous fat in *db/db* mice. Expression levels of VEGF mRNA in visceral fat were increased 3.0-fold in 14-wk-old mice compared with those in 6-wk-old mice (Fig. 2*A*). VEGF expressions in subcutaneous fat were also increased during growth, but its enhancement was smaller than in visceral fat. Moreover, tissue contents of VEGF in visceral fat were significantly increased in 14-wk-old mice compared with those in 6-wk-old mice (Fig. 2*B*). However, the VEGF contents in subcutaneous fat were almost the same in 6- and 14-wk-old mice. These data suggest that an enhanced expression of the VEGF gene in visceral fat mainly contributes to the elevated plasma concentrations.

Effect of fat accumulation on VEGF expression in white adipose tissues of db/db mice. Whole tissue weights of mesenteric and subcutaneous fat were increased gradually during growth (Fig. 3*A*). Total cell counts were significantly decreased during growth only in mesenteric adipose tissues (Fig. 3*B*). A significant correlation between fat weight and VEGF expression levels was observed in mesenteric adipose tissue but not in subcutaneous adipose tissue (Fig. 4*A*). Moreover, cellular levels of VEGF expression were calculated from the results of mRNA expression levels and total cell counts in adipose tissues and positively correlated for adiposity in mesenteric adipose tissue but not in subcutaneous adipose tissue (Fig. 4*B*).

Growth-dependent change of VEGF expression in adipocytes of visceral area of db/db mice. VEGF expressions in adipocyte fraction were increased during growth (Fig. 5). Cellular expression levels of VEGF mRNA in visceral adipocytes were increased sevenfold in 18-wk-old mice compared

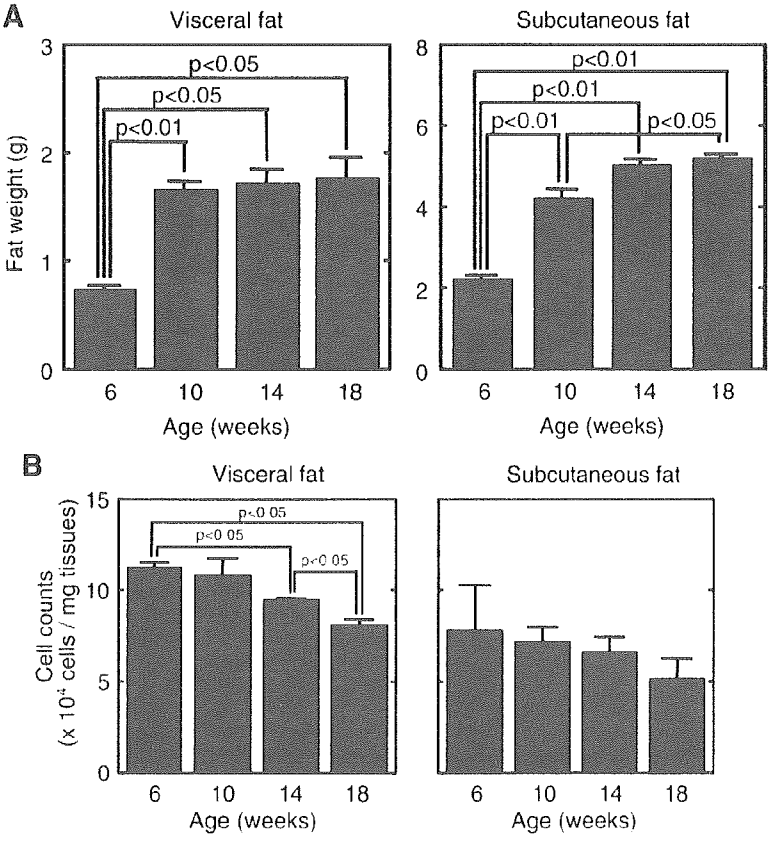

Fig. 3. Growth-dependent changes of fat weight and adiposity in mesenteric and subcutaneous adipose tissues. *A*: Time course changes of mesenteric and subcutaneous adipose tissue weights in *db/db* mice. The *db/db* mice were killed by cervical dislocation at 6, 10, 14, and 18 wk of age before adipose tissues were collected. Mesenteric and subcutaneous fat was weighed on an analytic balance. Results are represented as means ± SD. *B*: Time course change of adiposity of mesenteric and subcutaneous adipose tissues in *db/db* mice. Mesenteric and subcutaneous fat was digested with collagenase S-1 and processed for cell counts using a Neubauer chamber. Results are represented as means ± SD.

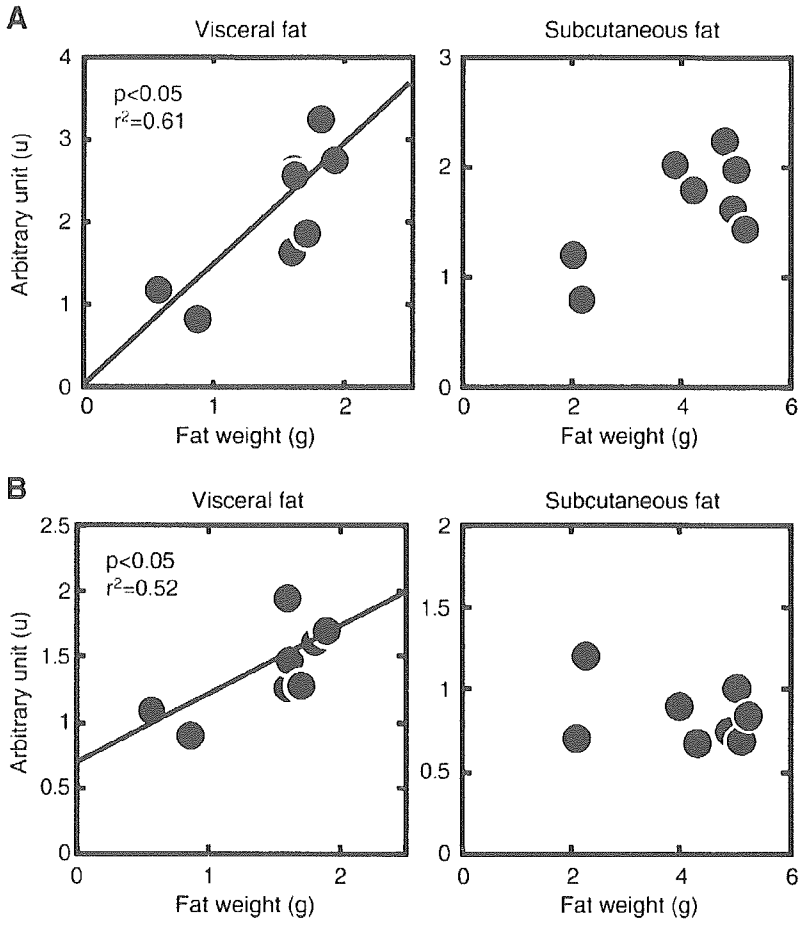

Fig. 4. Correlation between fat weight or adiposity and VEGF expressions in mesenteric adipose tissue. *A*: positive correlation between fat weight and tissue VEGF expression in mesenteric adipose tissues. *B*: positive correlation between fat weight and cellular VEGF expression in mesenteric adipose tissues.

with those in 6-wk-old mice. These results suggest that circulating VEGF concentrations in *db/db* mice were increased by the enhancement of VEGF mRNA expression in visceral adipocytes.

Plasma concentration and tissue expression of VEGF in KK-A^y mice. To demonstrate the correlation between VEGF expression and adiposity in another model of obesity, we analyzed KK-A^y mice. Plasma VEGF concentrations were significantly increased in both 8- and 16-wk-old KK-A^y mice compared with those in age-matched control mice (Fig. 6*A*). Moreover, expression levels of VEGF mRNA in visceral fat were significantly increased in KK-A^y mice compared with those in control mice (Fig. 6*B*). These results suggest that circulating VEGF concentrations in KK-A^y mice as well as in *db/db* mice were increased by the enhancement of VEGF mRNA expression in visceral fat.

Change of VEGF expressions during differentiation and maturation process in 3T3-L1 cells. We performed RT-PCR analysis for the gene expression of VEGF, PPARγ, and GLUT4 in cultured 3T3-L1 cells. VEGF mRNA was expressed even in the preadipocyte condition (Fig. 7*A*), and its expression was enhanced during adipocyte conversion. Especially, the expression levels of VEGF mRNA were significantly increased 14 days after differentiation (Fig. 7*B*). Both PPARγ and GLUT4 expressions were gradually enhanced during differentiation (Fig. 7*A*). These results suggest that expression levels of VEGF mRNA in 3T3-L1 cells were enhanced during lipid accumulation.

VEGF concentrations of conditioned media cultured with pre- and postdifferentiated 3T3-L1 cells. 3T3-L1 cells secreted VEGF proteins into culture medium even in the preadipocyte condition (Table 1), and the VEGF protein secretion was enhanced during adipocyte conversion. Especially, the VEGF concentrations in conditioned medium were increased fourfold 14 days after differentiation compared with those of predifferentiation. These results suggest that protein secretion as well as mRNA expression of VEGF in 3T3-L1 cells were enhanced during lipid accumulation. The biological activity of VEGF should be examined to know the role of VEGF in physiological and pathological conditions. Therefore, we demonstrated the angiogenic activity of conditioned medium from cultured adipocytes.

Enhancement of tube formation activity in HUVECs by addition of conditioned medium cultured with 3T3-L1 cells. VEGF secreted from both pre- and postdifferentiated 3T3-L1 cells had stimulatory activity toward HUVECs in tube formation (Fig. 8, *A* and *B*). The stimulatory activity in the condi-

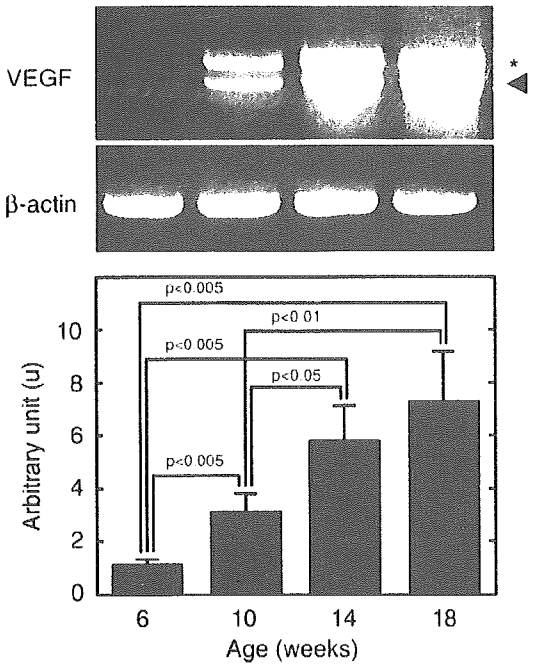

Fig. 5. Time course change of VEGF expressions in adipocyte fraction prepared from mesenteric fat of *db/db* mice. Visceral fat was cut into small pieces and digested using collagenase S-1. Then, the tissues were suspended with PBS and separated by centrifugation. The floating layer was collected as adipocyte fraction. Images show RT-PCR products of VEGF amplified from total RNA prepared from 2×10^4 cells of adipocytes at 6, 10, 14, and 18 wk after birth. The PCR products of VEGF were densitometrically analyzed, and the relative amounts at 6 wk old were set to 1.0. Results are represented as means and SD. Arrowhead, $VEGF_{164}$; *, $VEGF_{188}$.

tioned medium derived from postdifferentiated 3T3-L1 cells was three times higher than in predifferentiated cells. Moreover, anti-VEGF-neutralizing antibody apparently inhibited the stimulatory tube formation activity in both pre- and postdifferentiated 3T3-L1 cells. These findings suggest that 3T3-L1 cells secrete the bioactive form of VEGF protein.

Effect of implantation of 3T3-L1 preadipocytes into mesenteric or subcutaneous fat area of nude mice on VEGF expression. We performed RT-PCR analysis for gene expressions of VEGF, PPARγ, and GLUT4 in the mesenteric or subcutaneous fat area implanted with 3T3-L1 cells. As shown in Fig. 9*A*, the content of VEGF expression was increased fourfold in the mesenteric fat implanted with 3T3-L1 cells compared with those in sham-operated control mice. In contrast, VEGF expression of subcutaneous fat was almost the same in the mice implanted with 3T3-L1 cells into the subcutaneous area and controls. Moreover, PPARγ expression was enhanced only in mesenteric fat implanted with 3T3-L1 cells but not in subcutaneous fat. The expression levels of GLUT4 in both mesenteric and subcutaneous fat implanted with 3T3-L1 cells were higher than in controls.

The plasma VEGF concentration increased after implantation with 3T3-L1 cells into the mesenteric area, and these reached 381 ± 63 pg/ml at 4 wk. However, the mice injected in the subcutaneous area did not show any difference from control mice (Fig. 9*B*).

DISCUSSION

In the first set of experiments, we showed that the plasma VEGF concentrations gradually increased during growth in both *db/db* and *db/+* mice. After 10 wk of age, however, plasma VEGF concentrations were higher in *db/db* mice than in *db/+* mice. The *db/db* mice are considered to be an obesity model because fat deposition is the primary change. Then we analyzed the correlation between plasma VEGF concentration and body weight. The plasma VEGF concentration in *db/db*

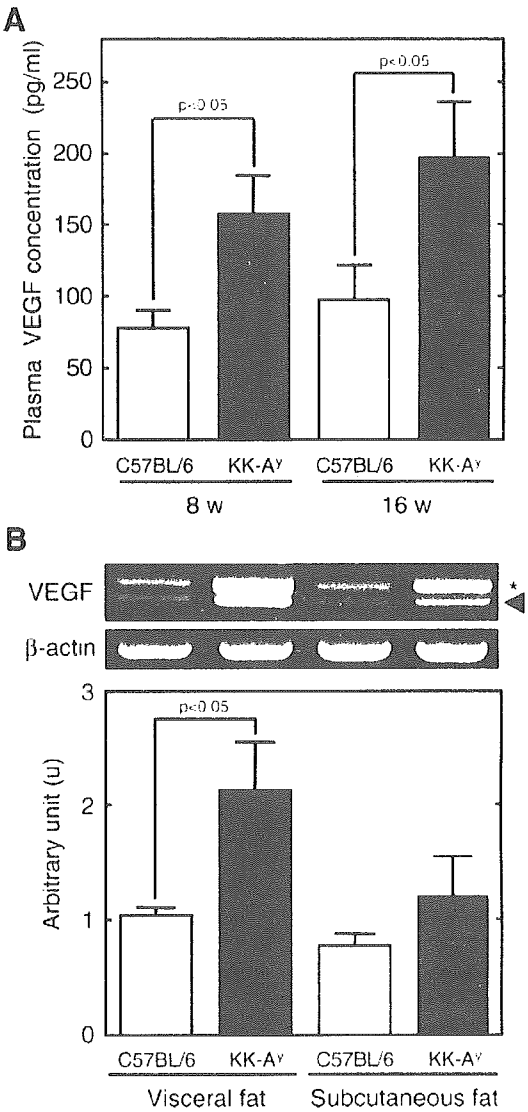

Fig. 6. Plasma concentration and tissue expression of VEGF in KK-A^y mice. *A*: comparison of plasma VEGF concentrations between KK-A^y and C57BL/6 mice. Plasma VEGF concentrations were measured in 8- and 16-wk-old KK-A^y and C57BL/6 mice. Results are represented as means ± SD. *B*: comparison of VEGF gene expressions in visceral and subcutaneous adipose tissues between KK-A^y and C57BL/6 mice. Images show RT-PCR products of VEGF amplified from total RNA in mesenteric and subcutaneous adipose tissues at 16 wk after birth. The PCR products of VEGF were densitometrically analyzed, and the relative amounts in visceral adipose tissues of control mice were set to 1.0. Results are represented as means ± SD. Arrowhead, $VEGF_{164}$; *, $VEGF_{188}$.