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

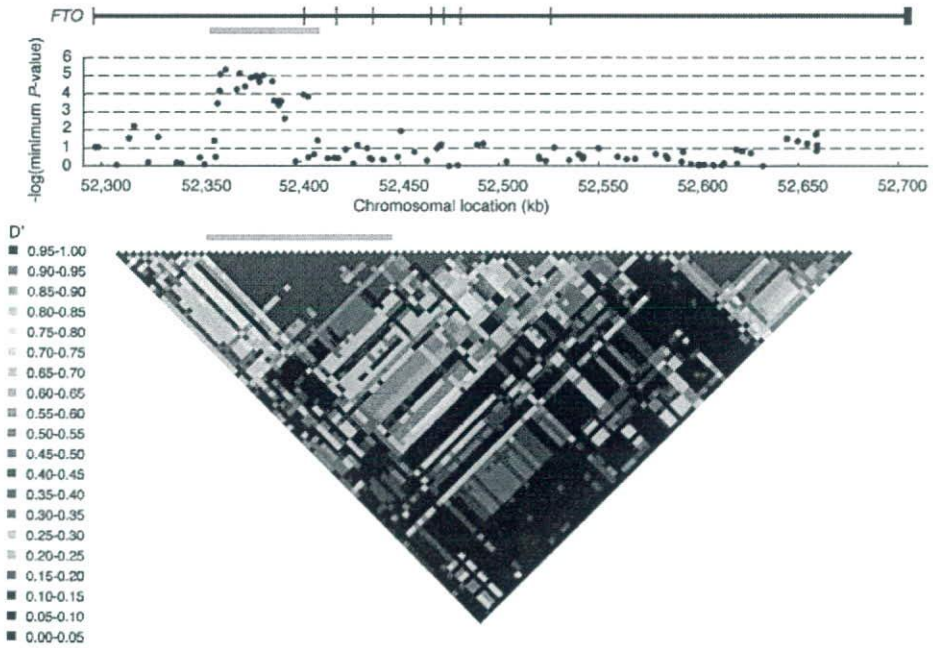

Fig. 1 Linkage disequilibrium (LD) mapping, polymorphisms, and *P* values obtained in the test of independence between the phenotype and genotypes of obesity at various single nucleotide polymorphisms (SNPs) in the fat-mass and obesity-associated gene (*FTO*) gene. *P* values are expressed as negative 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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Efficacy of Ghrelin as a Therapeutic Approach for Age-Related Physiological Changes

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Aging is associated with decreases in food intake and GH secretion, termed the anorexia of aging and somatopause, respectively. The mechanisms underlying these phenomena are not fully understood. Although many approaches have attempted to improve these age-related physiological changes, none have achieved satisfactory results. Ghrelin, a 28-amino-acid acylated peptide, was identified as an endogenous ligand for the GH secretagogue receptor. Ghrelin stimulates GH secretion and food intake in animals and humans. Previous studies have demonstrated that the mean plasma concentrations of ghrelin in normal-weight elderly people were lower than those in younger people. We hypothesized that ghrelin administration might improve the metabolic and physiological changes that accompany the anorexia of aging and somatopause. First, 75-wk-old mice fasted for 72 h, after which they resumed feeding with sc administration of ghrelin (360 μg/kg) twice daily for 4 d. Multiple administrations of ghrelin after a 72-h fast increased food intake and hastened body weight recovery with a high lean body mass ratio. Next, 50-wk-old mice were sc injected with rat ghrelin (40 μg/kg) twice weekly from 50–80 wk of age. Long-term administration of ghrelin kept aged mice with low body weight and low adiposity. These results suggest that ghrelin might be a novel approach for the therapy of age-related metabolic and physiological changes. (*Endocrinology* 149: 3722–3728, 2008)

AGING IS ASSOCIATED with progressive decreases in food intake (FI), termed the anorexia of aging (1–3). The physiological causes of the anorexia of aging are largely unknown and likely multifactorial (1, 2). One of the key factors of the anorexia of aging appears to be loss of appetite (4, 5). In comparison with healthy young people, elderly people feel less hunger when fasting and earlier satiety after initiating a meal (5–7). This insensitivity to the signs of appetite can lead to unintentional weight loss and undernutrition in response to acute and chronic illness, resulting in the increasing morbidity and mortality seen in elderly people (1, 8). Our understanding of the control of feeding has increased markedly in recent years. Animal and human studies have examined the roles of orexinergic and anorexinergic hormones, such as neuropeptide Y, orexin, ghrelin, CRH, and cholesystokinin (9–14). Of these, ghrelin has received great interest as a potential therapeutic agent for the anorexia of aging.

Ghrelin, an acylated peptide of 28 amino acids, was identified as an endogenous ligand for the GH secretagogue receptor (15). The major site of endogenous production of ghrelin is the stomach; this peptide is also expressed in the hypothalamus (16–19). Administration of ghrelin stimulates GH secretion and FI in both animals and humans (15, 16, 20–23). Plasma ghrelin levels are regulated by acute feeding states. They rise during fasting and are rapidly suppressed after feeding (12, 17, 24, 25). Ghrelin secretion is also regulated by chronic feeding states. Plasma ghrelin levels are elevated in food-restricted animals and patients with anorexia nervosa and are reduced in obese subjects (12, 17, 24–27). These data suggest a role for ghrelin in energy homeostasis. Previous studies have demonstrated that plasma concentrations of ghrelin in normal-weight elderly individuals were lower than those in younger people (28–30). GH responses to ghrelin administration in elderly people are also lower than those seen in young people (31). It has been speculated that aging is associated with reduced production of ghrelin or attenuation of endogenous ghrelin signaling (32).

Aging is associated with decreases in lean body mass (LBM) and increases in relative fat mass (33, 34). These changes can result in alternations of blood lipid profiles, which favor the development of vascular disease. Decreases in GH secretion are also seen in elderly people (35, 36), termed somatopause, which may contribute to these metabolic and physiological changes. Although the mechanisms inducing somatopause and leading to changes in body composition are not fully understood, clinical studies have attempted GH replacement in elderly persons. Such treatment has only occasionally been effective in increasing muscle mass and strength in elderly subjects (37, 38), and adverse effects, such as glucose intolerance and fluid retention, occurred frequently. Chronic administration of the ghrelin mimetic MK-677 to elderly people, however, restored pulse amplitude of episodic GH secretion and serum IGF-I levels to those seen in young adults (39) and increased bone mineral density (BMD) at the femoral neck with few adverse effects (40). These findings implicated a hypothesis that administration of ghrelin might safely restore intrinsic GH secretion

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Abbreviations: BMD, Bone mineral density; BW, body weight; CT, computed tomography; FI, food intake; LBM, lean body mass.

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and improve age-related metabolic and physiological changes.

Using an animal model of aging, we performed three examinations to evaluate the effectiveness of ghrelin on age-related metabolic and physiological changes. Using an experimental model of physical stress with a 72-h fast established by Wolden-Hanson and colleagues (41–43), we evaluated the influences of aging on FI. Second, we assessed the efficacy of ghrelin on the anorexia of aging; we investigated whether multiple ghrelin administrations can increase FI and hasten the recovery of body weight (BW) and body composition after the physical stressor of a 72-h fast. Third, we assessed the efficacy of long-term ghrelin administration to reverse the age-related metabolic and physiological changes; we investigated whether twice-weekly ghrelin from 50–80 wk of age improved the blood metabolic parameters and body composition.

Materials and Methods

Animals

All procedures using experimental animals were approved by the Kyoto University Graduate School of Medicine committee on animal research. Procedures were performed in accordance with the principles and guidelines established by that committee.

Male C57BL/6 mice were purchased from Japan CLEA (Tokyo, Japan). Animals were housed in air-conditioned animal quarters with lights on from 0800–2000 h. Except where noted, mice were allowed *ad libitum* access to water and standard rat chow (CE-2, 352 kcal/100 g; Japan CLEA).

Experiment 1: influence of aging on recovery of BW after a short-term fast (72-h fast)

Four age groups (n = 10 per age group) of mice (10, 25, 50, and 75 wk old) were analyzed. To evaluate the baseline characteristics of these mice, we measured BW and monitored daily FI for seven consecutive days. After a 7-d baseline period, food was withheld for 72 h. After *ad libitum* feeding was resumed, BW and FI were assessed daily for 5 d. Mice were allowed *ad libitum* access to water throughout the experiment.

Experiment 2: effect of ghrelin on recovery of BW after a short-term fast in aged mice (75 wk old)

Rat ghrelin was purchased from the Peptide Institute, Inc. (Osaka, Japan). After a 7-d baseline period, food was withheld from 75-wk-old mice (n = 30) for 72 h. *Ad libitum* FI was then resumed for 8 d (d 0–7). For the first half of the refeeding period (d 0–3), half of the animals (n = 15) were sc injected twice daily with rat ghrelin (360 μg/kg) at 0900 and 1800 h (ghrelin group), whereas the other half were injected with saline (saline group). BW and FI were measured daily in both groups. During the latter half of the refeeding period (d 4–7), BW and FI were measured in the absence of ghrelin or saline injections. Using computed tomography (CT) (laboratory CT; Lacita, Aloka, Japan), we examined the body compositions of 75-wk-old mice before the 72-h fast (d −3), after the 72-h fast (d 0), and after ghrelin or saline injection (d 4).

Experiment 3: effects of a long-term ghrelin injection on BW and body composition in aged mice (50–80 wk old)

The aim of this experiment was to investigate whether a long-term administration with low-dose ghrelin, which stimulates GH secretion without increase in cumulative FI, may lead to increase in LBM and decrease in fat mass via lipolytic and anabolic effects of elevated GH. We selected a dose and frequency on the basis of three points: 1) a dose that can stimulate GH secretion. 2) a dose that doesn't affect daily FI, and 3) a frequency that can avoid desensitization of GH in response to ghrelin

Because we have previously reported that sc administration of ghrelin at 40 μg/kg stimulated GH secretion and increased FI in 8-wk-old mice (21), we evaluated whether this dose of ghrelin could stimulate GH secretion and FI in 50-wk-old mice. Fifty-week-old mice were sc injected with rat ghrelin (40 μg/kg) or saline at 1000 h under *ad libitum* feeding conditions (n = 8 per group). Blood was collected from the tail veins of mice 15 min after injection. Fifty-week-old mice were sc injected with rat ghrelin (40, 120, and 360 μg/kg) or saline at 1000 h under *ad libitum* feeding conditions; daily FI was then measured. Previous reports have demonstrated that continuous administration of ghrelin with osmotic mini pump desensitizes GH secretion in response to ghrelin (16). To evaluate whether repeated administration of ghrelin desensitizes GH secretion in response to ghrelin, 20 mice were divided into two groups; half of them were sc injected with ghrelin at a dose of 40 μg/kg daily for 15 consecutive days, whereas the other half were injected with ghrelin at the same dose on d 1, 5, 8, 12, and 15 (twice weekly). On d 1 and 15, serum GH levels 15 min after ghrelin injection were measured. Fifty-week-old mice were also sc injected with rat ghrelin (40 μg/kg) at 0900 h twice weekly. Weekly FI was then measured.

Mice were scheduled sc administration of ghrelin twice weekly (Monday and Thursday). Fifty-week-old mice were also examined (n = 40). Half of the animals (n = 20) were sc injected with rat ghrelin (40 μg/kg) at 0900 h twice weekly (Monday and Thursday) from 50–80 wk of age, whereas the other half were injected with saline. BWs of both groups were measured weekly. CT was used to measure body composition before and after the experiment (50 and 80 wk old). After experimentation, blood samples were collected from the tail veins of mice at 1000 h under *ad libitum* feeding conditions for the measurement of blood glucose, serum insulin, triglycerides, total cholesterol, GH, and IGF-I levels. Blood glucose and serum insulin levels were also measured after an overnight fast (1700–0900 h).

Measurement of metabolic parameters and GH/IGF-I axis

Blood glucose was measured using a reflectance glucometer (One Touch II; Lifescan, Milpitas, CA). Serum total protein (Pierce, Rockford, IL), albumin (Albumin-E test; Wako, Osaka, Japan), triglycerides (Triglyceride-E test; Wako), total cholesterol (Amplex Red Cholesterol Assay Kit; Molecular Probes, Eugene, OR), serum GH levels (EIA kit; SPI-BIO, Bonde, France), and serum IGF-I levels (EIA kits; Diagnostic Systems Laboratories Inc., Webster, TX) were measured according to the manufacturer's instructions. Serum was isolated by centrifugation and stored at −20 C until assayed.

Statistical analysis

Results are expressed as the means ± SEM. The statistical significance of the differences in mean values was assessed by two-way ANOVA or Student's *t* test as appropriate. *P* values of <0.05 were considered to be statistically significant.

Results

Experiment 1: recovery of BW after a short-term fast was delayed in aged mice

We assessed the changes in BW and FI of aged mice after a 72-h fast and after refeeding (Fig. 1). The initial BWs of 10-, 25-, 50-, and 75-wk-old mice were 24.7 ± 0.3, 31.1 ± 1.0, 33.2 ± 1.3, and 34.9 ± 1.4 g, respectively. The average daily FI adjusted for BW declined markedly with age ($P < 0.01$). Those of 10-, 25-, 50-, and 75-wk-old mice were 136 ± 2.7, 106.1 ± 5.2, 104.5 ± 2.8, and 93.7 ± 6.3 mg/day·g BW, respectively. With fasting, younger mice lost more weight than older mice, both in absolute values and as percentages of BW before fasting. Ten-week-old mice lost 5.9 ± 0.2 g (23.9 ± 0.7% of prefasting BW), and 25-wk-old mice lost 5.9 ± 0.2 g (18.9 ± 0.6% of prefasting BW), whereas 50- and 75-wk-old mice lost 4.8 ± 0.3 g (14.5 ± 0.9% of prefasting BW) and 4.6 ± 0.2 g (13.1 ± 0.6% of prefasting BW), respectively. Despite a disproportionately increased weight loss, the re-

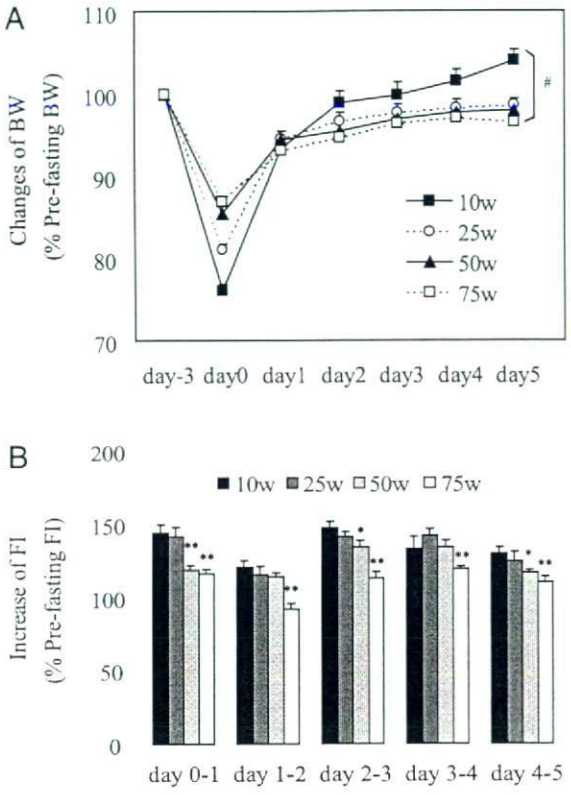

FIG. 1. A, Influence of aging on the recovery of BW after a 72-h fast. The 10-, 25-, 50-, and 75-wk-old mice fasted for 72 h, after which they resumed feeding. Data shown are the means ± SEM. Changes of BW are expressed as a percentage of prefasting weight. #, $P < 0.001$ (10- *vs.* 25-, 50-, and 75-wk-old mice). B, Daily FI for the first 5 d of the refeeding period. Data are expressed as a percentage of the prefasting FI. *, $P < 0.05$, **, $P < 0.01$ (*vs.* 10-wk-old mice on the same day).

covery of BW in 10-wk-old mice during refeeding was more rapid (on d 3) than that seen in all other age groups; the recovery of BW in 25-, 50-, and 75-wk-old mice was delayed. These animals did not recover to their average prefasting BW during the first 5 d of refeeding, reaching 98.6 ± 0.9, 98.0 ± 0.5, and 96.5 ± 0.9% of prefasting BW for 25-, 50-, and 75-wk-old mice, respectively. The difference in BW on d 5 between 10-wk-old mice and 25-, 50-, and 75-wk-old mice was significant ($P < 0.001$ for each group). The FI of 10-, 25-, 50-, and 75-wk-old mice in the first day of refeeding increased by 144 ± 5.7, 141.1 ± 6.6, 118.3 ± 3.2, and 116.2 ± 5.7% of prefasting FI, respectively. After adjustment for BW, the overall FI in 75-wk-old mice was significantly lower than that seen for 10-wk-old mice during the refeeding period ($P < 0.01$).

Experiment 2: multiple ghrelin injections restored BW loss after a short-term fast in aged mice

We monitored the changes in BW during experiment 2 (Fig. 2A). During the first half of the refeeding period (ghrelin or saline treatment period), BW recovery was more rapid in the ghrelin-treated subgroup in comparison with that seen in the saline-treated subgroup ($P < 0.01$). BW recovered fully by d 3 in the ghrelin-treated subgroup (100.6 ± 0.6% of

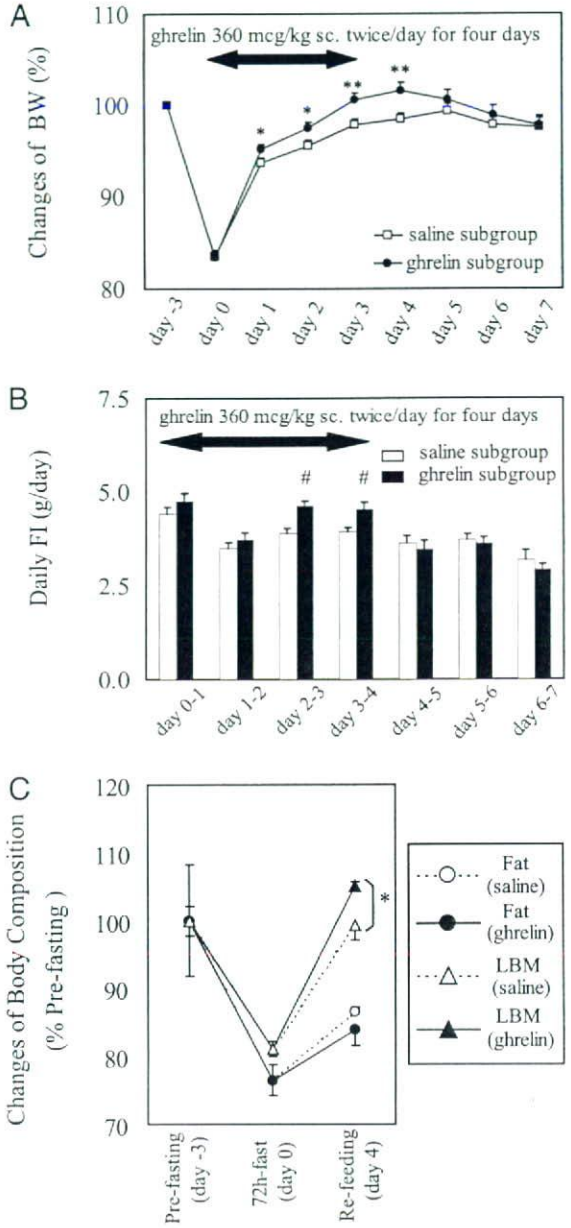

FIG. 2. Effect of repeated ghrelin administration on the recovery of BW and FI after a 72-h fast in aged mice. Seventy-five-week-old mice fasted for 72 h, after which they resumed feeding while being administered saline or ghrelin (360 μg/kg twice daily for 4 d). Data shown are the means ± SEM. Changes in BW (expressed as a percentage of the baseline BW) (A), daily FI (grams per day) (B), and CT-assessed body composition (expressed as a percentage of baseline fat mass or LBM) (C) were assessed in ghrelin-treated animals. *, $P < 0.05$; **, $P < 0.005$; #, $P < 0.001$ (*vs.* saline subgroup on the same day).

prefasting BW), whereas that of the saline-treated subgroup was 97.9 ± 0.6% of prefasting BW on d 3. After discontinuing the ghrelin injections, however, BW in the ghrelin subgroup decreased to levels equal to those of the saline subgroup. On d 7, the average BW of the ghrelin- and saline-treated subgroups were 97.8 ± 1.1 and 97.6 ± 1.0% of prefasting levels, respectively.

We evaluated daily FI during the refeeding period (Fig.

2B). During the first half of the refeeding period (ghrelin or saline injection period, d 0–4), daily FI increased in the ghrelin-treated subgroup in comparison with that seen in the saline-treated subgroup. The cumulative FI in the ghrelin-treated subgroup was 17.6 ± 0.6 g, whereas it was 15.7 ± 0.4 g in the saline-treated subgroup ($P < 0.005$). Daily FI during the latter half of the refeeding period (no-injection period, d 4–7), however, was similar between both groups. During this period, cumulative FI values in ghrelin and saline subgroups were 11.0 ± 0.5 g and 11.2 ± 0.6 g, respectively, which were not significantly different.

CT was used to measure body composition (Fig. 2C). As expected, adiposity and LBM decreased significantly after a 72-h fast (76.5 ± 2.2 and 81.1 ± 1.1% of prefasting levels, $P < 0.001$, respectively). After 4 d of refeeding and treatment, adiposity in the ghrelin-treated subgroup recovered to 83.9 ± 2.3% of prefasting levels and 86.6 ± 0.6% in the saline-treated subgroup; there were no significant differences between these values. LBM in the ghrelin-treated subgroup increased, surpassing the prefasting levels (105.1 ± 0.7%). This was significantly different ($P < 0.05$) from the saline-treated subgroup that returned only to prefasting levels (99.3 ± 2.2%).

Experiment 3: a long-term ghrelin injection decreased fat mass in aged mice

GH secretions of 50-wk-old mice were stimulated by sc ghrelin administration with a dose of 40 μg/kg. Fifteen minutes after ghrelin administration, serum GH levels were significantly higher than those seen after saline injection, 28.1 ± 7.6 and 8.4 ± 0.6 ng/ml, respectively ($P < 0.05$). Administrations of ghrelin with a dose of 40 μg/kg did not affect daily FI, 3.62 ± 0.18 g and 3.55 ± 0.25 g, respectively. Although those of 120 μg/kg or 360 μg/kg increased daily FI: 116.2 and 125.8% compared with control mice, respectively.

Repeated administration of ghrelin markedly attenuated the GH response to injected ghrelin; serum GH levels on d 1 and 15 were 20.4 ± 4.6 ng/ml and 5.8 ± 0.4 ng/ml, respectively (decrease to 28.6%) ($P < 0.05$). Although administration of ghrelin twice weekly did not attenuate the GH response to ghrelin; serum GH levels on d 1 and 15 were 21.6 ± 4.3 ng/ml and 22.3 ± 5.3 ng/ml, respectively. When mice were administrated ghrelin with a dose of 40 μg/kg twice weekly, weekly cumulative FI was equivalent for the saline-treated subgroups, 243.3 ± 2.5 g and 24.5 ± 2.3 g, respectively.

We followed the changes in BW from 50 to 80 wk old (Fig. 3A). The initial BWs of the saline- and ghrelin-treated subgroups were 33.2 ± 0.4 g and 32.8 ± 0.5 g, respectively. There were no significant differences between these values. Body weight increased gradually in both groups. Ghrelin-treated mice exhibited significantly lighter BW than the saline-injected mice throughout the course of the experiment ($P < 0.01$). By the end of the experiment, the average BW of the ghrelin-treated subgroup was 34.9 ± 0.9 g, whereas that of the saline-treated subgroup was 37.7 ± 1.0 g.

Body composition was assessed by CT before (50 wk old) and after (80 wk old) experimentation (Fig. 3B). As expected, we observed a significant effect of age on body composition. In the saline-treated subgroup, adiposity increased, whereas

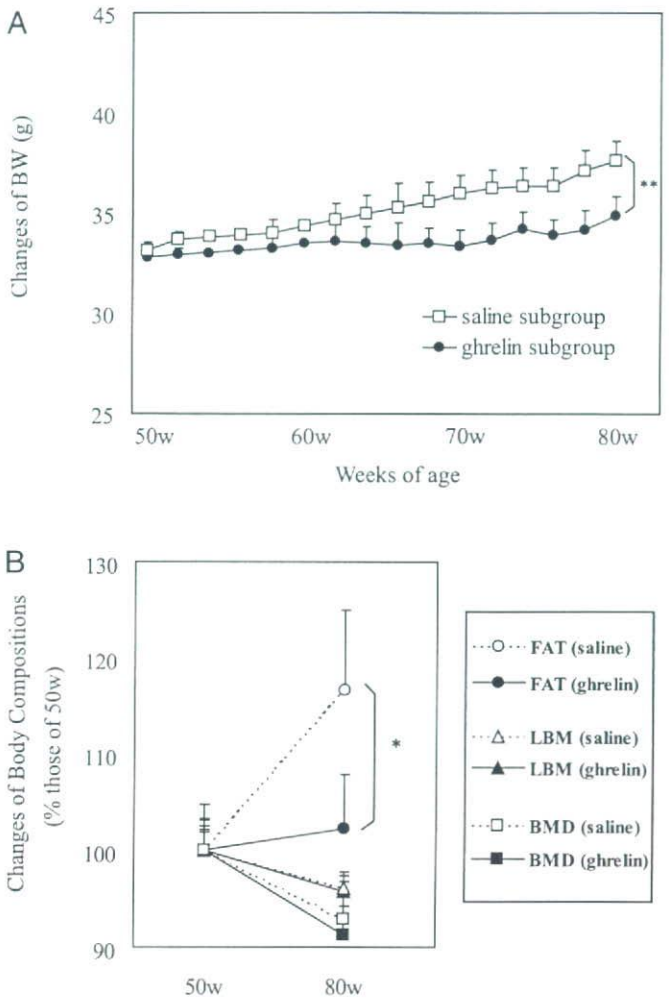

FIG. 3. Effect of long-term ghrelin administration on BW and body composition in aged mice. Fifty-week-old mice were injected with ghrelin (40 μg/kg twice weekly) for 30 wk (50–80 wk old). Data shown are the means ± SEM. Changes in BW (A) and body composition (B), as measured by CT (expressed as a percentage of the fat mass, LBM, or BMD of 50-wk-old animals), were measured in 80-wk-old mice. *, $P < 0.05$; **, $P < 0.005$ (*vs.* saline subgroup).

LBM and BMD decreased significantly with increasing age to 80 wk old (116.6 ± 8.4, 96.1 ± 1.6, and 92.9 ± 3.9% that seen for 50-wk-old animals, respectively) ($P < 0.01$). At the end of the experiment when animals were 80 wk old, LBM and BMD were similar for both the saline- and ghrelin-treated subgroups (saline *vs.* ghrelin subgroup, LBM was 96.1 ± 1.7 *vs.* 95.7 ± 2.1% of 50-wk-old mice, and BMD was 92.9 ± 3.9 *vs.* 91.2 ± 3.0% of 50-wk-old mice). Adiposity, however, decreased significantly in the ghrelin-treated subgroup (saline *vs.* ghrelin subgroup, 116.6 ± 8.4 *vs.* 102.2 ± 5.7% of 50-wk-old mice, $P < 0.05$).

Blood glucose (*ad libitum*), serum insulin (*ad libitum*), triglyceride, total cholesterol, GH, and IGF-I levels did not differ between the saline- and ghrelin-treated subgroups. Blood glucose measured after an overnight fast was significantly lower in ghrelin-treated animals than in the saline subgroup ($P < 0.05$). Serum insulin levels after an overnight fast were undetectable in both groups (Table 1).

TABLE 1. Blood glucose (*ad libitum* or overnight fast), serum insulin (*ad libitum* or overnight fast), triglyceride, total cholesterol, GH, and IGF-I levels at the end of experiment 3 (80-wk-old mice)

	Saline-injected mice	Ghrelin-injected mice
Ad libitum		
Blood glucose (mg/dl)	128.6 ± 4.7	128.1 ± 8.4
Immunoreactive insulin (ng/ml)	2.9 ± 0.5	2.0 ± 0.3
Overnight fast		
Blood glucose (mg/dl)	89.4 ± 5.1	73.2 ± 3.4[a]
Immunoreactive insulin (ng/ml)	ND	ND
Triglyceride (mg/dl)	164.3 ± 11.3	177.4 ± 12.1
Total cholesterol (mg/dl)	110.6 ± 2.8	110.2 ± 3.8
GH (ng/ml)	8.0 ± 0.8	8.9 ± 0.8
IGF-I (ng/ml)	377.1 ± 9.4	372.7 ± 14.0

Data are the means ± SEM. ND, Not detected.
[a] $P < 0.05$ (*vs.* saline subgroup).

Discussion

Many studies have demonstrated that as people age, they eat less food (1, 2). Elderly people have an impaired ability to recover BW fully after physical and mental stresses, such as acute and chronic illnesses, surgery, or bereavement (1, 8). Animal models of aging also reflect the decline in FI observed in humans both spontaneously and in response to disturbances in feeding (43, 45, 46). A spontaneous decline in FI with aging was also observed in C57BL6 mice in this study. Wolden-Hanson and colleagues (41–43) reported that, in comparison with young animals, aging male Brown Norway rats fail to regulate BW in response to the metabolic stressor of a 72-h fast and fail to increase FI appropriately after the fast. In accordance with those results, we observed that aging male C57BL6 mice recover BW more slowly than younger mice in response to the stressor of a 72-h fast. Thus, C57BL6 mice can serve as an animal model for the anorexia of aging.

As shown in Fig. 2, ghrelin treatment increased both BW gain and FI in 75-wk-old mice after the physical stress of a 72-h fast in comparison with untreated age-matched controls. The time course of BW recovery in ghrelin-treated 75-wk-old mice resembled that of 10-wk-old animals. CT measurement of body composition revealed that the improvement in BW resulted from increased LBM, which might include changes in body water, in ghrelin-treated mice. Yukawa and colleagues (47) demonstrated that ghrelin treatment prevented BW loss after surgery without increasing visceral fat mass in aging animals. Recent publications suggested that endogenous ghrelin may play a role in obesity and exogenous ghrelin-induced weight gain due to increased fat mass (23, 48–50). Most of these studies were performed under healthy conditions. On the other hand, under unhealthy conditions, it is reported that ghrelin administration to the patient with functional dyspepsia and chronic heart failure increased FI and LBM without fat accumulation (51, 52). As is often the case with an inpatient, when decreased BW recovers to the baseline level after physical and mental stress, such as acute and chronic illness and surgery, a nonfat component, which is necessary to maintain a normal physical function, recovers first, and then fat mass accumulates. We therefore think that the result of experiment 2, where ghrelin administration hastened the recovery of BW after 72-h fasting without fat accumulation, were not inconsistent with the reported effects of ghrelin. Involuntary weight loss in which loss of muscle predominates may predispose the elderly to muscle weakness and protein energy malnutrition, leading to increased risk for extended hospitalization, mortality, and morbidity (53). Several drugs have been suggested for the treatment of the anorexia of aging, such as cyproheptadine (an antihistaminergic, antiserotonergic drug), GH, ornithine oxoglutarate, and anabolic steroids. None, however, have been established in the management of weight loss in elderly people (1). This study and previous findings support a possible role for ghrelin as a means to prevent weight loss after acute and chronic illness, surgery, or bereavement in elderly patients.

As suggested by Toshinai *et al.* (46) and Sun *et al.* (54), sc administration of ghrelin at 40 μg/kg increased serum GH levels and did not affect daily FI in 50-wk-old mice. In addition, administration of ghrelin with a dose of 40 μg/kg twice weekly did not attenuate the GH response to ghrelin, whereas repeated administration of ghrelin markedly attenuated the GH response to injected ghrelin. These results strongly suggest that some intervals are needed to maintain the stimulating effect of ghrelin on GH secretion and support our regimen. The stimulating effect of ghrelin on GH secretion persisted at 80 wk of age (the end of experiment 3, data not shown).

In this study, long-term administration of ghrelin maintained low adiposity in aged mice without impairing glucose tolerance or lipid metabolism. Serum insulin levels and *ad libitum* and fasting blood glucose levels in ghrelin-treated mice tended to be lower than those seen in saline-treated mice. It is reported that peripheral ghrelin administration increased BW and cumulative FI and reduced insulin secretion (23, 55). This BW gain mainly results from increased fat mass and might be caused by decreased energy expenditure in addition to increased FI (50). Previous reports using mice showed that the effects of ghrelin on fat deposition were obtained by ghrelin administration at relatively high doses (50, 56). In this study, aged mice were administered low-dose ghrelin, which stimulates GH secretion without increase in cumulative FI. Discrepancy between previous reports and our result could be explained by differences in the doses and frequencies of ghrelin administration. Indeed, serum GH levels were increased by sc administration of ghrelin at a dose of 40 μg/kg, but daily and weekly FI were not increased in this study. Ghrelin-induced GH secretion may contribute to low adiposity. In contrast to our expectations, long-term administration of ghrelin did not increase either LBM or BMD. Clinical studies in elderly humans have indicated that MK-677 can reactivate the GH/IGF-I axis, increasing serum IGF-I levels, improving lean body composition (57), increasing BMD (40), and restoring lower-extremity function (44). In this study, serum IGF-I levels in ghrelin-treated mice were similar to those seen in saline-treated mice. The lack of change in IGF-I is likely why no increases in LBM or BMD were observed. Different results might have been obtained with ghrelin administration at higher doses and frequencies. In that case, however, fat mass would likely also have increased. Additional studies will be needed to investigate the

optimal dose and frequency of ghrelin administration to improve the age-related metabolic and physiological changes.

In conclusion, we demonstrated that repeated ghrelin administration increased FI and hastened the recovery of BW after a short-term physical stress. In addition, long-term administration of ghrelin maintained low adiposity in aged mice. Multiple approaches have attempted to improve such age-related metabolic and physiological changes. None of these, however, have yet achieved satisfactory results. Our results suggest that ghrelin may be a candidate therapeutic approach to combat such age-related metabolic and physiological changes.

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A Case of Cortisol Producing Adrenal Adenoma without Phenotype of Cushing's Syndrome due to Impaired 11β-Hydroxysteroid Dehydrogenase 1 Activity

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Abstract. This report concerns a case of cortisol-producing adrenocortical adenoma without the phenotype of Cushing's syndrome. A left adrenal tumor was incidentally detected in this patient. A diagnosis of adrenal Cushing's syndrome was based on the results of endocrinological and radiological examinations, although she showed none of the physical signs of Cushing's syndrome, glucose intolerance, hypertension or dyslipidermia. After a successful laparoscopic left adrenalectomy, the pathological diagnosis was adrenocortical adenoma. Slow tapering of glucocorticoids was needed to prevent adrenal insufficiency after surgery, and the plasma ACTH level remained high even though the serum cortisol level had reached the upper limit of the normal range. Further examination showed a urinary THF + allo-THF/THE ratio of 0.63, which was lower than that of control (0.90 ± 0.13, mean ± SD). Serum cortisol/cortisone ratios after the cortisone acetate administration were also decreased, and the serum half-life of cortisol was shorter than the normal range which has been reported. These findings indicated a partial defect in 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) activity, which converts cortisone to cortisol. Our case suggests that a change in 11β-HSD1 activity results in inter-individual differences in glucocorticoid efficacy.

Key words: 11β-Hydroxysteroid Dehydrogenase 1(11β-HSD 1), Cortisol, Adrenal adenoma, Cushing's syndrome

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THE phenotype of Cushing's syndrome consists of central obesity, abdominal striae, bruising, proximal myopathy, hirsutism, hypertension, dyslipidemia, glucose intolerance and edema. Patients with Cushing's syndrome show elevated serum cortisol levels, but the severity of the phenotype is not always comparable to that of hypercortisolemia. Patients with a genetic defect in the glucocorticoid receptor (GR) function, on the other hand, show "glucocorticoid resistance" with a compensatory increase in serum ACTH and cortisol and absence of the phenotype of Cushing's syndrome [1]. Instead, they exhibit hypokalemic alkalosis, hypertension and hyperandrogenism due to hyperproduction of cortisol, glucocorticoid precursors with mineralocorticoid activity, and adrenal androgens. It has recently been reported that metabolism in the periphery can modulate the biological activity of glucocorticoids in patients with Cushing's syndrome [2]. 11β-hydroxysteroid dehydrogenases (11β-HSDs) are the key enzymes of glucocorticoid metabolism in the peripheral tissue [3]. 11β-HSD has two isoforms, type1 and type2. Isozyme 1 (11β-HSD1) acts as a reductase converting inactive cortisone to active cortisol, while isozyme 2 (11β-HSD2) inactivates cortisol to cortisone. 11β-HSD1 is expressed in a wide variety of

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tissues and augments glucocorticoid action, while 11β-HSD2 is predominantly expressed in the distal nephron protecting the mineral corticoid receptors from cortisol. In addition, transgenic mice overexpressing 11β-HSD1 in the adipose tissue have been found to exhibit obesity, glucose intolerance, dyslipidemia and hypertension without elevation of serum glucocorticoids [4]. This report concerns a case of a middle-aged woman with cortisol-producing adrenocortical adenoma who completely lacked the phenotype of Cushing's syndrome because of impaired 11β-HSD1 activity.

Subject and Methods

Subject

A 55-year-old woman was admitted to our hospital because of an incidentally detected left adrenal tumor.

Until she underwent a hysterectomy because of myoma uteri at the age of 45, she had had regular menstruation. Since the operation, she had been in good health until six months before admission, when she had a periodic medical check-up. Abdominal ultrasonographic examination found a mass of 3 cm in diameter at the left adrenal gland. A low-density area on the computed tomogram confirmed the presence of a left adrenal tumor. The patient was 157.5 cm tall and weighed 45.3 kg, with a body mass index (BMI) of 18.3. Fat distribution was physiological and there was no evidence of hirsutism, abdominal striae, myopathy or abnormal pigmentation (Fig. 1). She was normotensive (120/70 mmHg) and there were no abnormalities in glucose tolerance at 75 g-OGTT, serum lipid composition, or plasma electrolytes. Endocrinological examination showed elevated serum cortisol, suppressed plasma ACTH and loss of circadian rhythm (at 0900 h, cortisol: 17.9 μg/dl, ACTH: <0.5 pg/ml; at 1500 h, cortisol: 19.7 μg/dl, ACTH: <0.5 pg/ml). The serum cortisol level was not reduced after a 1 mg-dexamethasone overnight suppression test (19.5 μg/dl) or following 2 mg- and 8 mg-dexamethasone suppression tests (Table 1). ^{131}I-adosterol scintigraphy showed a strong uptake by the left adrenal mass, while the uptake by the contralateral adrenal gland was completely suppressed. Serum levels of epinephrine (<5 pg/ml), norepinephrine (662 pg/ml), aldosterone (71 pg/ml) and plasma renin activity (1.6 ngAI/ml·h) were within normal range. These data strongly suggested the presence of a left adrenal cortisol-producing adenoma, so that a laparoscopic left adrenalectomy was performed, followed by glucocorticoid replacement therapy (hydrocortisone div. 300 mg/day). The histological examination of the removed tissue confirmed the diagnosis of adrenocortical adenoma (Fig. 2). After the surgery, the patient remained in good condition and the glucocorticoid dose was gradually reduced. On postoperative day 3, when she was receiving 10 mg of prednisolone i.m. daily, general malaise and low-grade fever emerged. On days 4 and 5, she suffered a hypoglycemic attack (plasma glucose: 49 mg/dl and 51 mg/dl) and needed glucose supplementation. When the glucocorticoid dose was temporally increased, these symptoms disappeared. We started tapering the glucocorticoid again, but could not reduce the dose of hydrocortisone to less than 40 mg without the appearance of adrenal insufficiency. The patient was discharged from hospital on postoperative day 37 with treatment for hydrocortisone replacement of 40 mg/day. At the CRH-loading test before discharge, neither ACTH nor cortisol responded to the CRH stimulation.

Table 1. Results of the dexamethasone suppression test

	Dexamethasone		
	Before	2 mg/48 h	8 mg/48 h
Urinary free-cortisol (μg/day)	429	435	203
Urinary 17-OHCS (mg/day)	26.4	25.4	15.7
Serum ACTH (pg/ml)	<5	<5	<5
Serum cortisol (μg/dl)	19.5	15.9	15.6

She continued to visit our hospital every month as an outpatient. Fig. 3 demonstrates the tapering of hydrocortisone and the changes in plasma ACTH and serum cortisol concentrations in the morning after discharge. Tapering of hydrocortisone was done very slowly and carefully. The plasma ACTH level started to elevate in the 58th week postoperatively when she was taking 20 mg/day of hydrocortisone, followed by the serum cortisol level in the 63rd week. Glucocorticoid replacement was discontinued successfully in the 98th week postoperatively. In the 103rd week postoperatively, the serum cortisol level reached 14.0 μg/dl, which is the upper limit of the normal range. Nevertheless, plasma ACTH concentration was still high (222 pg/ml). These findings suggest that the patient's physiological level of cortisol is above the normal range. The elevation of ACTH and cortisol levels was

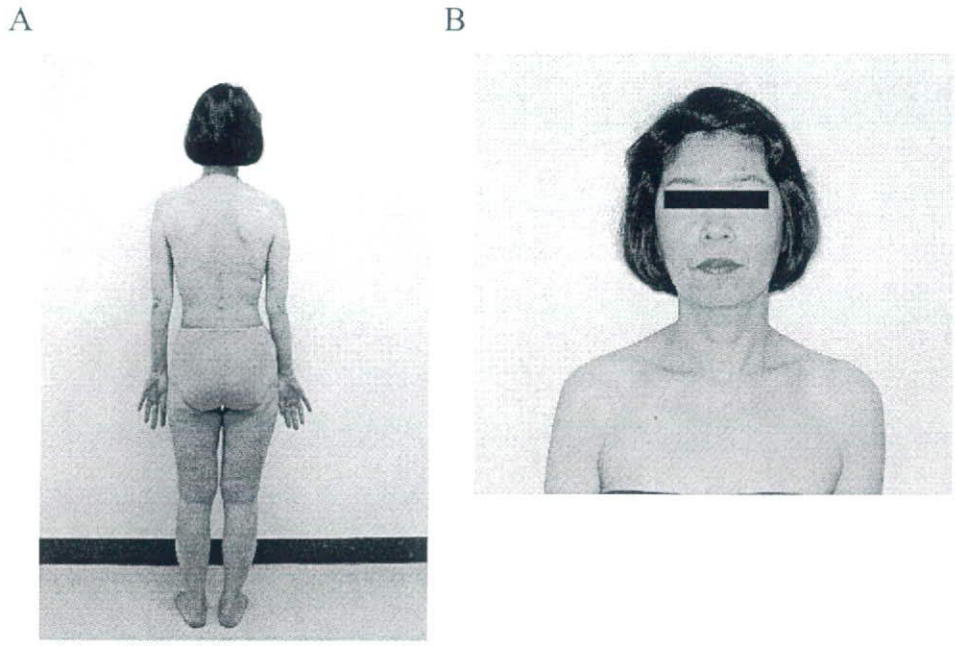

Fig. 1. Absence of the phenotype of Cushing's syndrome in a 55-year-old woman with cortisol producing adrenal adenoma is presented. A: whole body, posterior view. B: face.

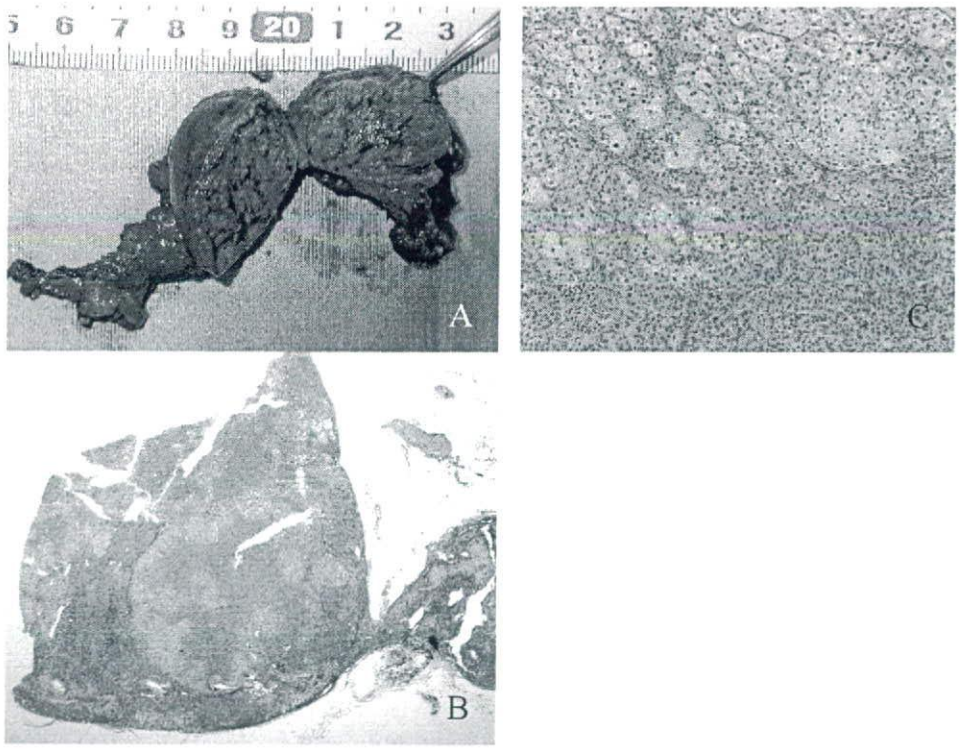

Fig. 2. Macroscopic (A) and microscopic (B, C) images of the removed adrenal tumor. Dissected left adrenal gland shows yellowish section of adrenocortical adenoma. Microscopic observation shows clear-type and compact-type cells growing in a nesting pattern. B: scanning view, C: high magnification (×100); hematoxylin-eosin staining

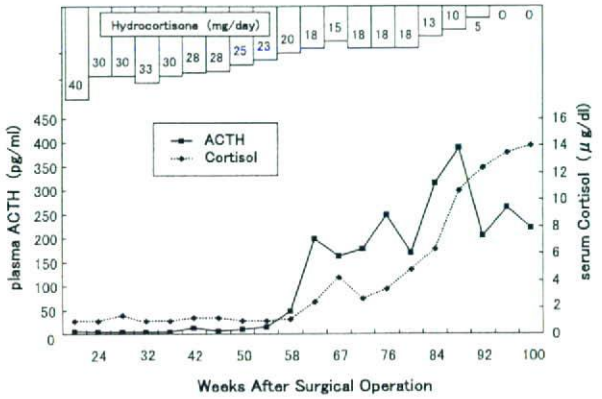

Fig. 3. Time course of hormone levels after discharge showing plasma ACTH concentration (solid line), serum cortisol concentration (dotted line) as well as prescribed doses of hydrocortisone (columns) are presented. Blood samples were always collected at 9:00 am before glucocorticoid administration.

completely suppressed after the 1 mg-dexamethasone overnight suppression test (ACTH <5 pg/ml, cortisol 0.3 μg/dl).

Analyses of cortisol metabolism

On day 22 postoperatively, when our patient was receiving 40 mg of hydrocortisone daily, 11β-HSD1 activity was assessed in several ways.

1) Urinary glucocorticoid metabolites analysis. Urinary excretion of tetrahydrocortisone (THE), 5β-tetrahydrocortisol (THF), and 5α-tetrahydrocortisol (allo-THF) was measured by means of gas chromatography-mass spectrometry [5]. Urine was also collected from 12 healthy non-obese females and was analyzed for normal control.

2) Cortisone acetate loading test. As previously reported, the evening replacement dose of hydrocortisone was omitted and at 0900 h the following morning cortisone acetate (25 mg) was administered po. Serum cortisol and cortisone levels were measured at 0, 30, 60, 90, 120, 180, and 240 min and were compared with published data [2].

3) Serum cortisol half-life. After oral administration of 2 mg of dexamethasone at 2400 h and at 0900 h the following morning, hydrocortisone (5 mg) was injected iv. Serum cortisol level was measured at 0, 5, 10, 15, 20, 30, 45, 60, 75, 90, 120, 150, 180, 210, and 240 min. Serum half-life was calculated by using nonlinear regression and was compared with published data [6].

Table 2. Analysis on urinary glucocorticoid metabolites

	Patient	Control
THF + allo-THF/THE ratio	0.61 0.66 0.61	0.91 ± 0.14

The data for the patient consist of the values obtained on three successive days.
The control value shows the mean value ± SD derived from healthy female volunteers (n = 11; BMI: 19.2 ± 1.3, mean ± SD)

Results and Discussion

The urinary THF + allo-THF/THE ratio for this patient was 0.63 (Table 2). This value was markedly lower than that for the normal control females (0.91 ± 0.14) or previously reported data (1.15 ± 0.11 for normal control and 1.70 for patients with adrenal Cushing syndrome) [5], indicating a reduction in 11β-HSD1 activity.

The serum cortisol/cortisone ratio after oral administration of cortisone acetate was also lower than that for the normal control (Fig. 4A).

Serum half-life of cortisol injected intravenously was estimated at 77 min according to the equation $y = 702.99e^{-0.0089848x}$, $R^2 = 0.9904$ (Fig. 4B). The normal range of the half-life has been reported as 78–102 min [6]. These findings also suggest that 11β-HSD1 activity in this patient is partially impaired.

We usually diagnose patients with adrenal incidentaloma, which autonomously secretes cortisol and lacks the phenotype of Cushing's syndrome, as subclinical Cushing's syndrome. However, two observations distinguish the case reported here from usual occurrence of subclinical Cushing's syndrome. First, a high dose of glucocorticoid replacement was needed to avoid adrenal insufficiency after resection of the adrenal adenoma. Second, her physiological level of serum cortisol seemed to be higher than the normal range.

The biological activity of glucocorticoids is determined by several factors. One is the function of the GR [1]. It has been reported that mutations in the GR gene lead to its functional abnormality and thus to glucocorticoid resistance [7]. In the case of our patient, glucocorticoid resistance could be ruled out because serum ACTH was suppressed throughout the clinical course and none of the signs including hypokalemic alkalosis, hypertension or hyperandrogenism was

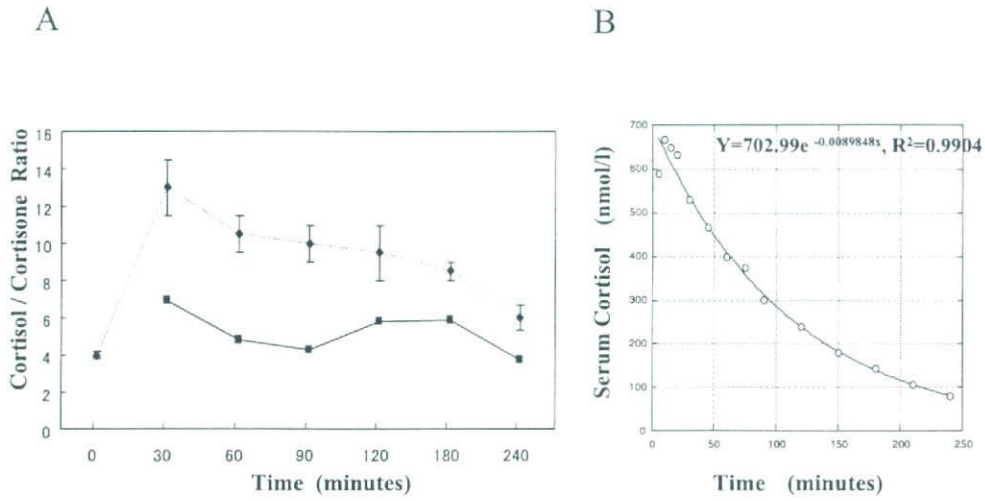

Fig. 4. A: Serum cortisol/cortisone ratios after oral administration of cortisone acetate (25 mg). Serum cortisol and cortisone levels were undetectable before administration. Normal control values (dotted line; mean ± SE) were previously reported (2). B: Serum cortisol concentrations after intravenous administration of hydrocortisone (5 mg). Mean serum half-life (77 min) was calculated with non-linear regression.

observed.

Another crucial factor is glucocorticoid metabolism by 11β-HSDs, especially re-activation of glucocorticoid by 11β-HSD1.

11β-HSD1 is expressed in a wide variety of tissues including liver, gonad, and adipose tissue, and converts cortisone to cortisol [8]. 11β-HSD2, on the other hand, is distributed mainly in mineralocorticoid target tissues, including kidney, and inactivates cortisol to cortisone. 11β-HSD2 prevents glucocorticoid from activating the mineralcorticoid receptors, so that a defect in 11β-HSD2 activity results in inappropriate activation of the mineralcorticoid receptors, "apparent mineralcorticoid excess (AME)". The activity of 11β-HSD can be assessed in several ways. Cortisol is metabolized to THF and allo-THF, and cortisone is converted to THE mainly in the liver. All these metabolites are excreted in the urine, so that the urinary THF + allo-THF/THE ratio indicates the 11β-HSD1 activity. For the normal female controls, this ratio was 0.91 ± 0.14, which is compatible with the value previously reported (1.15 ± 0.11) [5]. Patients with impaired 11β-HSD1 function exhibit the syndrome of apparent cortisone reductase deficiency (ACRD), presenting with oligomenorrhea, hirsutism and hyperandrogenism due to compensatory elevation of ACTH and activated adrenal androgen production [9–11]. The THF + allo-THF/THE ratios for these patients were less than 0.1. In contrast, this ratio was elevated for patients with Cushing's syndrome and has been reported to be 1.74 ± 0.24 and 1.70 (SD not available) for patients with pituitary Cushing's syndrome (Cushing's disease) and adrenal Cushing's syndrome, respectively [12].

The THF + allo-THF/THE ratio for our patient was 0.63, which is lower than the normal reference value and markedly below that for Cushing's syndrome patients. Tomlinson *et al.* reported a case of pituitary Cushing's syndrome without the Cushingoid phenotype [2], whose THF + allo-THF/THE ratio was 0.66, which is very close to that for our patient.

The serum cortisol/cortisone ratio after oral administration of cortisone acetate indicates the activity of cortisone to cortisol conversion. As shown in Fig. 2, the cortisol/cortisone ratio for our patient was lower than that reported previously for normal control by more than 2SD, and the serum half-life of cortisol was estimated at 77 min, which is slightly shorter than the lower limit of the normal range which has been reported. All of these data suggest that 11β-HSD1 activity is impaired in our patient, resulting in the accelerated metabolic clearance of cortisol.

Although it has been suggested that genetic defects in the HSD11B1 gene which encodes 11β-HSD1 to be the cause of impaired 11β-HSD1 activity, no exonic mutations in HSD11B1 have been detected in ACRD

patients [13]. Recently, however, it both intronic mutations in HSD11B1 and mutations in exon5 of H6PD, which encodes hexose-6-phosphate dehydrogenase (H6PDH), have been observed in individuals with ACRD [14]. Mutations in the intron of HSD11B1 were found to result in reduced gene transcription, and mutations in exon5 of H6PD to attenuate H6PDH activity and NADPH generation in endoplasmic reticulum, resulting in the loss of 11β-HSD1 oxo-reductase activity. It remains to be determined whether any mutations or polymorphism in HSD11B1 or H6PD is the cause of the partial defect of 11β-HSD1 activity in our patient.

The patient's clinical course after surgery (Fig. 3) suggests that her hypothalamo-pituitary-adrenal (HPA) axis had been constantly activated before she incurred the cortisol-producing adenoma. The causal relationship between the activation of the HPA axis and the adrenal adenoma is not clear. However, the possible occurrence of hyperplasia or adenoma in the contralateral adrenal gland warrants surveillance. Several 11β-HSD1 inhibitors have been developed for use in therapy for metabolic syndrome, although the compensatory activation of the HPA axis due to the diminished 11β-HSD1 activity can be an obstacle to their clinical application in the cases like ours.

The biological efficacy of glucocorticoids appears to differ among individuals. Our case demonstrates that variations in 11β-HSD1 activity can help identify such differences and suggests that prior evaluation of the 11β-HSD1 activity will help to determine the optimal glucocorticoid dose for steroid therapy.

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