Methods

Each year about 7,000 examinees came to in Saku Health Doc Center for health checkups. Including all visits, the Saku Health Doc Center database contains approximately 197,000 records. We used the database to select initial examination records, and about 45,000 examinees were identified. For this study, the inclusion criteria were age 40–64 years and a body mass index (BMI:kg/m^2) within the upper quintile (28.3). Exclusion criteria were psychiatric conditions or physical conditions (i.e., significant hepatic or renal dysfunction and significant cardiovascular disease such as heart failure, stroke, and transient ischemic attacks) that would preclude full participation in the study; current treatment for obesity; current treatments known to affect eating or weight (e.g., medications). A total of 917 people whose BMI was more than 28.3 (upper quintile) were identified in the health checkup database, and 235 participants were enrolled in the Saku Control Obesity Program (SCOP). [9]

Five subjects who did not wear the accelerometer for 7 days or more were excluded from the study. Of the remaining 230 subjects, 111 were male and 119 were female. All research procedures of SCOP were performed according to the Helsinki Declaration. All subjects gave their written informed consent to participation in the study, and all procedures were reviewed and approved by the Ethical Review Board of the National Institute of Health and Nutrition.

To determine the baseline values of physical activity, each subject wore a uniaxial accelerometer on his or her belt from the time of waking to going to bed for 2 weeks. Measurements were as follows: daily step-count; PAEE; adjusted PAEE for body weight; and time spent in light, moderate, and vigorous physical activity. As the daily physical activities varied across the measurement period, daily mean values were calculated.

The activity monitor measures acceleration in the vertical direction. According to technical details provided by the manufacturer (Suzuken Co.,Ltd.), it samples the acceleration at 32 Hz and assesses values ranging from 0.06 to 1.94 g (where 1.00 g is equal to the acceleration of free fall). The acceleration signal is filtered by an analog band-pass filter and digitized. The frequency of acceleration signals is used to determine the step frequencies. Studies have shown that during walking the step frequencies measured by the accelerometer are within ±3% of the actual number of steps.[10] A maximum pulse over 4 s is taken as the acceleration value, and the activities are categorized into 11 activity levels based on the pattern of the accelerometer signal. The activity levels are subsequently converted by an algorithm to calculate EE (kcal) based on the following principle: when the sensor detects or more three acceleration pulses for 4 consecutive seconds, the activities are recognized as physical activity and are categorized into one of 9 activity levels (levels 1.0–9.0). The activity levels are calculated and counted every 4 s. The activity levels for ranges from 1.0 to 9.0 in steps of one unit corresponded to 1.465, 2.075, 2.808, 3.601, 4.537, 5.737, 7.324, 9.460, and 10.661 cal/kg/4 s, respectively.[7] There was a strong correlation between the activity levels and the measured EE while walking (r^2=0.93; P<0.001).[7] The daily PAEE (kcal) was calculated by summing the EE corresponding with activity levels every 4 s (cal/kg/4 s) and the product of the body weight (kg) of each subject.

If an acceleration pulse due to physical activity (*i.e.*, corresponding to activity levels 1.0–9.0) is not followed immediately by another acceleration pulse, it is not counted as 0.0 but level 0.5 is arbitrarily assigned for 3 min. It is assumed that the subject is standing up (or sitting down) and remaining in that state. These postures involve a higher EE than the resting supine position. Briefly, isolated spurts of acceleration are assumed to be due to acute changes in posture (lying down, sitting, and standing), because walking and moving around are typically rhythmic activities. EE due to very small trunk movements and posture effects (*e.g.*, changing from sitting to standing position, light deskwork) were not included in the PAEE. Thus, the PAEE measured by the accelerometer was systematically underestimated during a 24-h period, and the accelerometer assessed energy expenditure well during both the exercise period and the non-structured activities.[7]

As the PAEE is associated with body weight, PAEE adjusted for body weight (adjusted PAEE) was calculated as follows: adjusted PAEE (METs·h)=PAEE (kcal)/[W (kg) × 1.05].[11] The various activity levels are categorized as light (<3.0 METs), moderate (3.0–6.0 METs), and vigorous (>6.0 METs), and the time spent in each activity category per total time of physical activity (%) was calculated. In addition, the time spent in sedentary activity (sitting at a desk, visiting friends, reading, or watching television) was obtained from subjects' answers to the International Physical Activity Questionnaire (IPAQ).[12]

Anthropometric measurements (height, weight, and abdominal circumference) were determined in the standing position after the subjects removed their clothes, shoes, and socks. Abdominal circumference as a surrogate measurement of abdominal obesity was measured at the level of the umbilicus during expiration. Abdominal fat distribution was determined with subjects in the supine position using CT according to the procedure described previously.[13] Visceral fat areas were measured on one cross-sectional scan obtained at the umbilicus.

All statistical analyses were performed using SPSS® software (version 14.0; SPSS Inc., Chicago, IL, USA). All data are shown as means ± standard deviation. The differences between groups were analyzed by unpaired *t*-test. Linear regressions and Pearson's correlation coefficients were calculated. In addition, stepwise regression analysis was performed. Statistical significance was set at P<0.05.

Results

The subjects' characteristics are listed in ***Table 1***. Although there were no significant differences in age or BMI between men and women, height, body weight, and abdominal circumference in men were significantly greater than those in women. Using the Japanese diagnostic criteria, the prevalence of metabolic syndrome was 62.9% in men and 51.3% in women. These values

Table 1 Subject characteristics at baseline

Variables	Total (n = 235)	Men (n = 116)	Women (n = 119)
Age (years)	53.9 ± 6.6	53.4 ± 6.6	54.5 ± 6.4
Height (cm)	161.8 ± 8.6	168.4 ± 5.8	155.4 ± 5.5*
Weight (kg)	80.7 ± 12.1	86.4 ± 11.8	75.2 ± 9.5*
BMI (kg/m^2)	30.8 ± 3.4	30.4 ± 3.5	31.1 ± 3.1
Abdominal circumference (cm)	106 ± 9	105 ± 9	107 ± 8
SBP (mmHg)	138 ± 19	136 ± 17	140 ± 20
DBP (mmHg)	85 ± 14	84 ± 14	86 ± 13
FPG (mg/dL)	112 ± 26	112 ± 25	112 ± 27
TG (mg/dL)	158 ± 84	167 ± 89	148 ± 78
HDL cholesterol (mg/dL)	53 ± 11	50 ± 10	56 ± 12*
Visceral fat area (cm^2)	144 ± 53	159 ± 54	130 ± 47*

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; TG, triglyceride; HDL, high density lipoprotein
*: p < 0.05 vs. men

Table 2 Daily physical activity at baseline

Variables	Total (n = 230)	Men (n = 111)	Women (n = 119)
No. steps (steps/day)	7815 ± 3211	7601 ± 3300	8015 ± 3127
PAEE (kcal/day)	258 ± 115	271 ± 127	246 ± 102*
Adjusted PAEE (METs·h/wk)	3.09 ± 1.38	3.02 ± 1.43	3.15 ± 1.35
Time spent in light PA (%)	77.2 ± 12.2	76.1 ± 12.2	78.2 ± 12.2
Time spent in moderate PA (%)	21.5 ± 11.0	23.0 ± 11.9	20.0 ± 9.9*
Time spent in vigorous PA (%)	1.1 ± 1.4	0.9 ± 1.1	1.2 ± 1.5
Time spent in sedentary activity (min/day)	381 ± 230	436 ± 247	324 ± 188*

PAEE, physical-activity-related energy expenditure; METs, metabolic equivalents; PA, physical activity
*: $p < 0.05$ vs. men

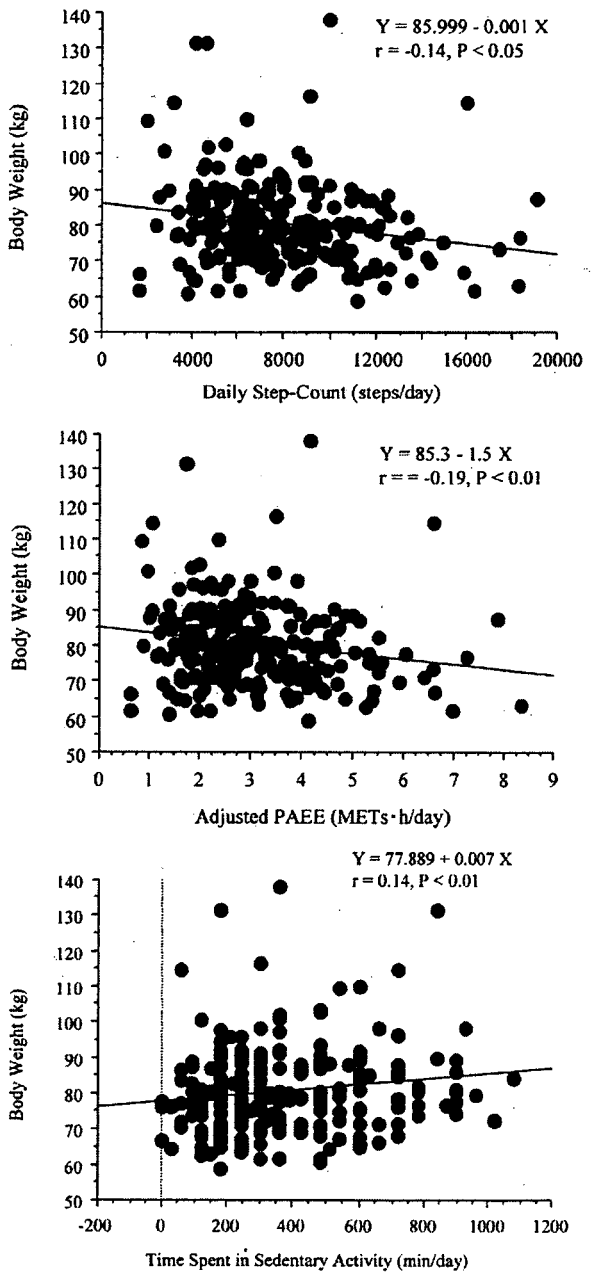

Fig. 1. Relationships between body weight and daily step-count (upper), adjusted physical activity-related energy expenditure (middle), and time spent in sedentary activity (bottom).

are notably lower in both men and women compared to the prevalence calculated using the International Diabetes Federation definition[14] based on waist circumference for Japanese (men: 77.6%, women: 72.3%), whereas only the values for women are lower using the American Heart Association/National Heart, Lung, and Blood Institute definition (men: 51.7%, women: 72.3%).[15]

The physical activity properties at baseline (*i.e.*, daily step-count, PAEE, adjusted PAEE, and time spent in light, moderate, and vigorous physical activity) are shown in ***Table 2***. The daily PAEE was significantly larger in men as compared with women. The time spent in moderate physical activity was longer in men than in women. In contrast, the time spent in sedentary activity in women was significantly shorter than that in men. There were no significant differences in other physical activity parameters between men and women. Although the association between occupation and PAEE was examined, there were no significant differences among the occupational categories (data not shown).

In all subjects, the daily step-count was closely related to the daily PAEE ($r=0.92$, $P<0.001$) and adjusted PAEE ($r=0.99$, $P<0.001$). The daily step-count was positively associated with the time spent in moderate physical activity ($r=0.35$, $P<0.001$), but negatively associated with time spent in light physical activity ($r=-0.30$, $P<0.001$). BMI was negatively correlated with the daily step-count ($r=-0.13$, $P<0.05$) and adjusted PAEE ($r=-0.14$, $P<0.05$). Moreover, body weight was negatively correlated to the daily step-count ($r=-0.19$, $P<0.01$, ***Figure 1, top***) and adjusted PAEE ($r=-0.18$, $P<0.01$, ***Figure 1, middle***). Visceral fat area was negatively and significantly correlated to the daily step-count ($r=-0.14$, $P<0.05$, ***Figure 2, top***) and adjusted PAEE ($r=-0.15$, $P<0.05$, ***Figure 2, bottom***). Abdominal

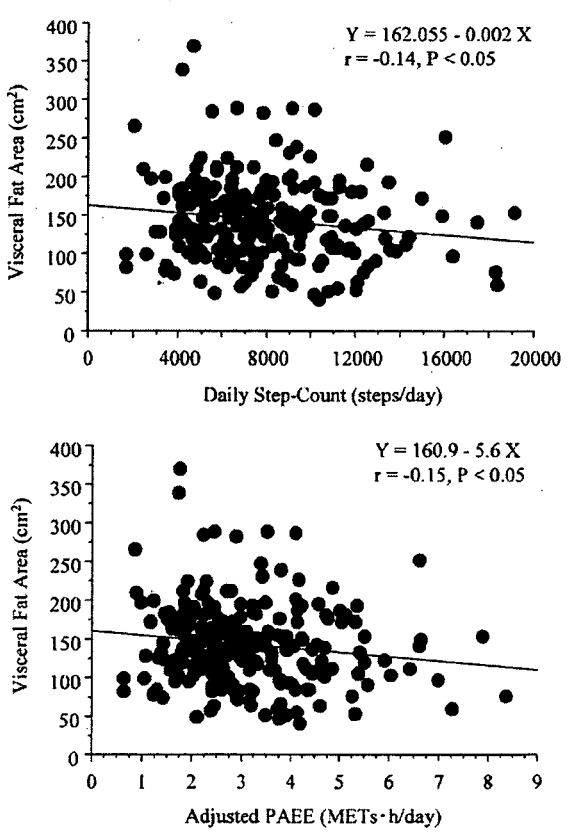

Fig. 2. Relationships between visceral fat area and daily step-count (upper), adjusted physical activity-related energy expenditure (middle), and time spent in sedentary activity (bottom).

circumference as a surrogate measurement of abdominal obesity was negatively and significantly related to the daily step-count ($r=-0.14$, $P<0.05$) and adjusted PAEE ($r=-0.16$, $P<0.05$). However, body weight had positive and significant correlations with daily PAEE ($r=0.15$, $P< 0.05$) and the time spent in sedentary activity ($r=0.14$, $P<0.05$, ***Figure 1, bottom***). If all activities were weight-bearing, the PAEE would only be expected to be directly related to body weight.

Stepwise regression analysis showed that the daily step-count could be adopted as an independent variable for BMI and body weight, and adjusted PAEE could be adopted as an independent variable for visceral fat area and abdominal circumference.

Discussion

The main findings of this descriptive study were as follows. First, the mean daily step-count was 7,815 steps in all SCOP subjects, with no difference between men (7,601 steps) and women (8,015 steps). Second, the adjusted PAEE for body weight was 3.09 METs·h/day in all subjects, and there was no sex-related difference. The adjusted PAEE was somewhat smaller than the reference values for the quantity of physical activity for primary prevention of lifestyle-related diseases (3.3 METs·h/day) established by the Ministry of Health, Labour, and Welfare of Japan.[16] Third, the amount of physical activity (daily step-count and adjusted PAEE) was significantly and negatively related to body size (body weight and BMI) and abdominal fat (visceral fat area and abdominal circumference) in the pooled subjects, although the correlation coefficients were weak ($r=-0.1$ to -0.2).

Average daily step-count in Japanese men is generally greater than that in Japanese women as assessed by a national health and nutrition survey.[17] In the present study, the daily step-count in female subjects was about 1,400 steps/day greater than that in male participants. The unexpectedly higher daily step-count in the female subjects may be related to their slower walking speed and shorter stride than the male subjects. In fact, the time spent in moderate physical activity (brisk walking) by women was significantly shorter than that by men, and the time spent in light physical activity (slow walking) tended to be longer in women as compared with men.

In 2006 the Ministry of Health, Labour, and Welfare reexamined the recommended quantity of exercise for primary prevention of lifestyle-related diseases (originally proposed in 1989) and set reference values for the quantity of physical activity and exercise for Japanese people between the ages of 20 and 69 years. Specifically, for individuals who intend to promote health mainly through physical activity, walking 8,000 to 10,000 steps/day (23 METs·h/week) was set as the target daily amount of physical activity.[16] In the present study, the daily step-count and adjusted PAEE for body weight were 7,815 steps/day and 3.09 METs·h/day, respectively, which were somewhat lower than the reference values described above.

Several previous studies from the USA and UK indicated that daily step-counts in overweight and obese adults are lower than those in normal-weight peers.[18,19] The present study showed that adjusted PAEE and daily step-count were significantly and negatively correlated with visceral fat and abdominal circumference in the pooled overweight and obesity subjects. This is the first evidence that the amount of physical activity is partly associated with not only systemic obesity but also abdominal obesity. Furthermore, in accordance with the results of stepwise regression analysis, although daily step-count was an independent predictor of weight and BMI, adjusted PAEE was an independent predictor of abdominal obesity, *i.e.*, visceral fat area and abdominal circumference. As adjusted PAEE is determined by the duration and intensity of physical activity, accumulation of abdominal fat may be associated with not only the duration but also the intensity of physical activity. We should emphasize that the relationships between amount of physical activity and obesity variables were weak ($r=-0.1$ to -0.2). This implies that factors other than physical inactivity (*e.g.*, overeating) may strongly contribute to obesity in the SCOP subjects. To clarify the cause of obesity in SCOP subjects, the results from the uniaxial accelerometer should be compared with the responses to dietary history questionnaires.

Increasing physical activity and reducing caloric intake are indispensable for the improvement of excess weight and obesity. SCOP is a randomized control crossover study aiming to reduce visceral fat of overweight and obese subjects by interventions of physical activity and diet. Our systematic review suggested that an increase in adjusted PAEE at 10 METs·h/week (1.38 METs·h/day) is necessary to reduce visceral fat of overweight and obese subjects. The increase in daily step-count corresponds to an increase of almost 3,000 steps/day as compared with the baseline. Therefore, all SCOP subjects receive physical activity modification education so that their daily step-count increases gradually by 3,000 steps/day, and it is necessary to set the mean value of action targets for 11,000 steps/day and 4.5 METs·h/day.

The validity and reliability of the uniaxial accelerometer have been established.[6,7,10] One methodological limitation, however, is that a uniaxial accelerometer cannot measure very light physical activity (<1.8 METs).[7] Daily life includes a great deal of very light physical activity, and very light PAEE occupies more than the half of total PAEE. Therefore, we should emphasize that the PAEE obtained in the present study was not total PAEE but PAEE at 2METs intensity or more. Moreover, the cross-sectional study design is another limitation of the present study. The results of the present cross-sectional study must be confirmed prospectively with exercise intervention studies in future.

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Original Article

Nutritional Education and Exercise Treatment Based on Cognitive Behavioral Treatment in the Saku Control Obesity Program (SCOP)

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Abstract

BACKGROUND: Long-term weight loss is difficult to maintain, but recently cognitive behavioral therapy has been shown to be effective for long-term weight loss and maintenance.

METHODS: The 119 participants, who had been assigned to program to lose weight, were interviewed by dieticians regarding their motivation for weight loss and psychological status and self-corrected problems with their eating activities and exercises, following recognition of problems, discussing solutions, and devising personal dietary plans and exercise plan to loose weight at 1.0-2.0 kg per month.

RESULTS: In women, the prevalence of motivation to resolve the situation (n = 44, 84.6%) was significantly higher than that of men (n = 33, 67.3%; $p < 0.05$). In men, awareness of the need to keep healthy by oneself was significantly associated with the motivation to resolve the situation ($p = 0.002$) and the availability of support from others ($p = 0.004$). Thirty problems and 29 dietary goals were set by participants. The percentages of intake of alcohol ($p < 0.05$) and intake of sweets ($p < 0.01$) as the problems and decrease of intake in specified foods ($p < 0.01$) and snacks ($p = 0.05$) as dietary goals were significantly different between men and women. Women with BMI over 31 kg/m^2 set fewer additional steps as exercise goals than those with BMI under 29 kg/m^2 ($p < 0.05$).

CONCLUSIONS: The characters of subjects such as psychological status and the problems and the target recognized by participants were different between gender and the degree of obesity.

KEY WORDS: cognitive behavioral treatment, health education, obesity, baseline data

Introduction

Obesity is increasing in prevalence in Japan, and it represents a major risk not only for metabolic syndromes, such as type 2 diabetes, ischemic heart disease, hypertension, gout, and dyslipidemias, but also some cancers.[1] Loss of 10% of starting weight is thought to be associated with amelioration of risk factors such as hypertension, hypercholesterolemia, and hyperglycemia.[2] Traditional dietary treatment of obesity consists of an energy-reduced diet prescribed by dietitians to achieve weight loss in a short period.[3] Long term evaluations of obesity interventions indicate that weight loss accomplished through changes in diet and physical activity is rarely maintained.[4]

Since Stuart first applied behavior therapy to weight loss in the obese,[5] more than 100 papers have been published in the field. The goal of behavioral treatment is for participants themselves to choose to reduce caloric intake and increase energy expenditures based on alternatives provided by professionals, such as the dietitians.

Recently, cognitive behavioral treatment has been applied to weight loss in the obese. Cognitive behavioral treatment (therapy) is a methodology for systematically modifying eating and activity habits, other behaviors, or negative thoughts that appear to contribute to obesity using a combination of self-monitoring, goal setting, stimulus control, cognitive restructuring, stress management, and social support. The cognitive behavioral treatment can be applied in an individual or group setting to achieve a long-term change in eating and physical activity behaviors. Studies suggest that frequent contact between professionals and patients is necessary to achieve the weight loss. Several studies in a group setting showed that the treatments, typically delivered in 15–26 weekly 30- to 60-min group sessions consisting of fewer than 10 patients, produced a mean post-treatment weight loss of approximately 8.5 kg a 1 year.[4-6,10] However, long-term evaluations of interventions indicated that maintenance of weight loss is difficult.[11-13] The U.S. Institute of Medicine defined success as a weight loss of 5% of body weight maintained for 1 or more years.[13] These results suggested that intensive intervention (weekly group sessions) is successful for weight loss up to 1 year, but long-term adherence to the weight-loss plan cannot be facilitated by these programs. Perri and Corsica evaluated several specific maintenance strategies to achieve better long-term outcomes, including a problem-solving model, relapse prevention training, motivation, and extended behavioral therapy.[14] However, a truly effective intervention treatment for obesity has not yet been established. Renjilian reported the benefits of group treatment as cost-effectiveness and greater weight loss in patients at the end of the program.[15] In the case of intensive intervention, such as weekly sessions for 6 months, individual treatment is more expensive than the group approach.

In this baseline survey, we clarified the characteristics of the psychological status of subjects and the problems and dietary goals that the subjects had realized and set at the first meeting in Saku Control Obesity Program (SCOP), because there were few reports about the problems and targets that the participants made, so far.

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Methods

The study is a randomized controlled trial comparing the effect of 1-year behavioral treatment and exercise versus a control group conducted at the Saku General Hospital Human Dock Center. Details of the aim and design of the study are described in other paper written by Watanabe et al.[16,17]

Participants

235 participants (116 males, 119 females) out of 976 people, who had received the regular medical checkup, were finally recruited through the Saku General Hospital Human Dock Center. Inclusion criteria were age 40–64 years, at body mass index (BMI) over 28.3kg/m^2 within top fifth percentile, stated with desire to lose weight, and no serious medical condition. A total of 119 participants (59 men, 60 women) attended the first group session, and 116 participants (57 men, 59 women) were placed on a waiting list control group for a second session. All participants provided written informed consent, and the ethical committees of the National Institute of Health and Nutrition and the Saku General Hospital approved the study.

Interventions

Dieticians interviewed participants about their motivation for losing weight and to assess psychological status by considering the following: benefits to losing weight, probability of losing weight, level of motivation to resolve the situation, availability of support from others, level of awareness of the need to keep healthy by oneself, obstacles to executing the plans, and the amount of stress felt.

Using a behavioral management treatment, teams of health professionals, including doctors, registered dietitians, and physical activity instructors, conducted a weight-loss intervention that focused on self-management of diet, exercise, and individually set behavior goals. Participants had a brief interview with doctors followed by an individual session for 30–45 min with dieticians and a group session with physical activity instructors; if necessary, participants also received individual instruction in physical activity.

Various strategies were used to modify participants' behaviors, including self-monitoring, problem solving, goal setting, cognitive restructuring, stress management, stimulus control, and social support. Following recognition of the specific dietary problems, participants were expected to self-correct problems with their eating activity and exercise, choose a solution, implement a plan, and evaluate the outcome. During the individual sessions with dieticians every 3 months, participants evaluate their ability to self-monitor weight, physical activity (number of steps per day), accomplishment of the dietary and exercise plan, and daily diet activity. The dietitians provide positive feedback to participants for their progress in weight loss and engaged participants in problem solving to deal with obstacles. Then the dieticians help participant to recognize any dietary problems and to make plans for the following month. Between these face-to-face sessions, participants report their progress for the previous month and their new plans for the following month by mailing records to the dieticians in the months when they have no meeting with dieticians. When the reports are sent from the participants, dieticians and physical activity instructors send back their comments to the participants within 1 week.

The goal of weight loss was set at 1.0–2.0 kg per month, with a final goal of 10 kg lost by the end of the program. Nutritional education focused on individual dietary behaviors as clarified by monitoring participants' dietary records, especially fat intake, amount of grains, cooking methods, snacking, eating out, skipping meals, and alcohol intake. Each month, participants are also required to make their exercise plans with 1,000 more steps per day into the actual steps last month, with aim at the final goal of 10,000 steps per day by the end of the program.

Data Analysis

Data were analyzed using the Statistical Package for Social Sciences® (SPSS/PC, version 12.0, SPSS Inc., Japan). Differences between men and women with regard to psychological status, problem-solving strategies, and goal setting were analyzed using chi-square tests. Analysis of variance was used to examine differences between initial goals for exercise with regard to BMI.

Results

In this baseline survey, the data collected from 119 participants in first intervention group were analyzed for psychological characteristics and the data from 117 participants except 2 dropout participants were analyzed for problems and dietary and exercise goals.

The prevalence of motivation to resolve the situation in women was significantly higher than that of men ($p < 0.05$; ***Table 1***). Compared to women, more men expected support from others ($p < 0.05$), particularly their wives. More women were aware of obstacles to executing their weight-loss plans, although the prevalence of seeing benefits to losing weight, probability of losing weight, awareness of the need to keep healthy by oneself, and stress were not different between men and women.

Table 1 Sample characteristics of psychological status

Variables	levels	Men		Women		
		n	%	n	%	p value *
Benefits to lose weight	High	46	85.2	51	91.1	0.34
	Low	8	14.8	5	8.9	
Probability to lose weight	High	32	61.5	28	53.8	0.43
	Low	20	38.5	24	46.2	
Motivation to resolve the situation	High	33	67.3	44	84.6	0.04
	Low	16	32.7	8	15.4	
Availability of support from others	High	32	66.7	22	45.8	0.04
	Low	16	33.3	26	54.2	
Awareness of necessity to keep healthy by oneself	High	37	84.1	39	76.5	0.35
	Low	7	15.9	12	23.5	
Obstacles to executing plans	Yes	20	45.5	30	66.7	0.06
	No	22	50.0	15	33.3	
Feel stress	Yes	31	77.5	35	71.4	0.52
	No	9	16.7	14	28.6	

* Results of chi-square tests to examine the distribution of psychological status with men and women.

Table 2 Association of awareness of necessity to keep healthy by oneself and other psychological variables

Variables		Awareness of necessity to keep healthy by oneself									
		Men					Women				
		High		Low			High		Low		
		n	%	n	%	p value *	n	%	n	%	p value *
Benefits to lose weight	High	32	72.7	6	13.6	0.96	39	76.5	7	13.7	<0.001
	Low	5	11.4	1	2.3		0	0.0	5	9.8	
Probability to lose weight	High	22	50.0	3	6.8	0.60	24	50.0	2	4.2	<0.01
	Low	15	34.1	4	9.1		12	25.0	10	20.8	
Motivation to resolve the situation	High	27	62.8	1	2.3	<0.01	35	72.9	6	12.5	<0.01
	Low	9	20.9	6	14.0		2	4.2	5	10.4	
Availability of support from others	High	26	60.5	1	2.3	<0.01	18	40.9	2	4.5	0.12
	Low	10	23.3	6	14.0		17	38.6	7	15.9	
Obstacles to executing plans	Yes	18	47.4	2	5.3	0.54	7	17.1	6	14.6	0.03
	No	15	39.5	3	7.9		24	58.5	4	9.8	
Feel stress	Yes	7	20.0	0	0.0	0.23	8	18.6	2	4.7	0.64
	No	23	65.7	5	14.3		24	55.8	9	20.9	

* Results of chi-square tests to examine the distribution of psychological status

Table 3 Recognition of problems related to diet

Classification	Contents	Men		Women	
		n	%	n	%
Behaviors for diet					
Behavior as a cause of eating too much	Eat fast	24	40.7	28	47.5
	Eat much at supper	7	11.9	4	6.8
	Eat much	9	15.3	9	15.3
	Can not leave food	2	3.4	7	11.9
	Eat continuously for long time	0	0.0	1	1.7
	Eat meal served in large plate	1	1.7	1	1.7
	Eat until feel full	1	1.7	1	1.7
Time of eating	Eat late supper	4	6.8	4	6.8
	Skip meal	3	5.1	4	6.8
Behavior related eating	Lay down immediately after eating meal	2	3.4	4	6.8
	Eat at any time when the others eat	0	0.0	1	1.7
	Eat something when I feel stress	1	1.7	0	0.0
	Can not refuse when the others offer	0	0.0	1	1.7
	Eat snacks surrounded myself	0	0.0	2	3.4
	Buy too much	0	0.0	1	1.7
	Eat often outside	1	1.7	1	1.7
Snacks	Eat sweet snacks too much	5	8.5	15	25.0
	Eat snacks after supper	6	10.2	5	8.5
Meal contents					
Eat specified foods too much	Eat too few vegetables	14	23.7	10	16.9
	Take much fat or fatty food	18	30.5	16	27.1
	Take much salt or salty food	7	11.9	7	12.1
	Eat grains too much	7	11.9	6	10.3
	Eat noodles too much	5	8.5	0	0.0
	Eat the main dish too much	4	6.8	2	3.4
	Eat meat too much	4	6.8	0	0.0
Alcohol	Drink alcohol too much	13	22.0	3	5.1
Sweets	Drink sweet beverage too much	3	5.1	5	8.5
	Eat fruits too much	1	1.7	3	5.2
	Eat sweets too much	6	10.2	18	30.5
Dietary balance	Eat unbalanced meal	2	3.4	0	0.0

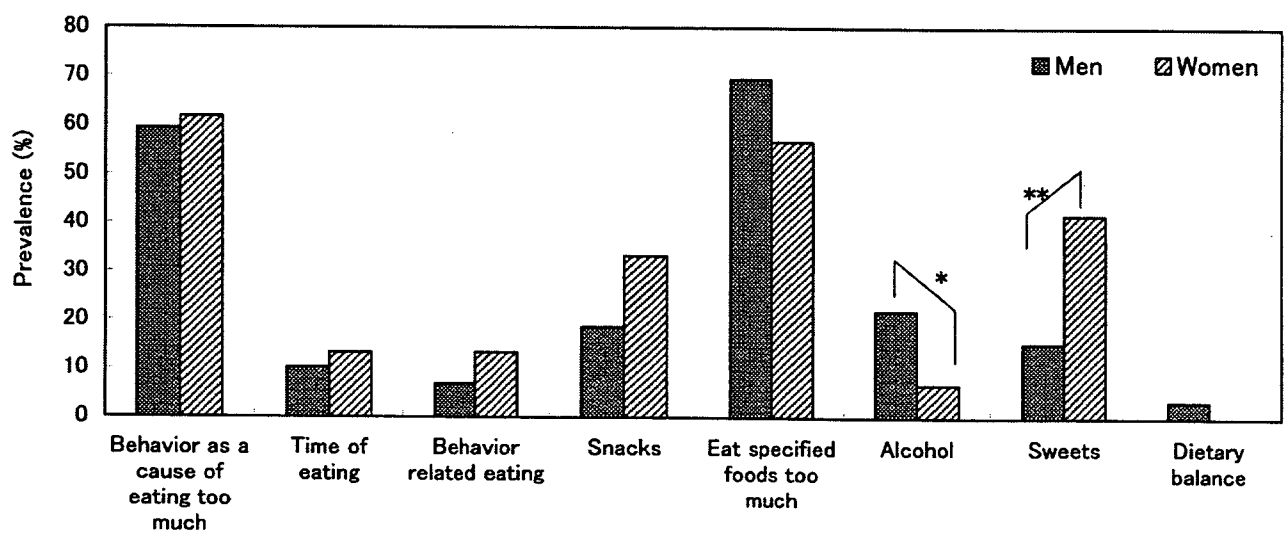

Fig. 1. Prevalence of dietary problems in men and women. The complete list of dietary problems is shown in *Table 3*. $*p < 0.05$, $**p < 0.01$

Table 2 shows the relationships between the awareness of the need to keep healthy by oneself and the other six psychological variables. In men, this awareness was significantly associated with level of motivation to resolve the situation ($p = 0.002$) and the availability of support from others ($p = 0.004$). In women, however, the awareness of the need to keep healthy by oneself was significantly associated with seeing the benefits of weight loss ($p < 0.001$), probability of losing weight ($p = 0.003$), motivation to resolve the situation ($p = 0.001$), and obstacle to executing the plans ($p = 0.027$).

Following the advice of dietitians, participants were asked to define the problems with their dietary habits and behaviors while setting personal goals for the following month. 30 recognized problems were classified into 8 categories and into two final categories of dietary behaviors and meal contents (***Table 3***). More than 40% of men and women recognized eating fast as a problem, and about 15% of men and women recognized eating too much as a problem. Insufficient intake of vegetables and excess intake of fat or fatty foods were recognized by both men and women. ***Figure 1*** shows the differences between men and women across the categories. With regard to meal contents, 41.7% of women reported eating too many sweets as a problem, whereas only 15.3% of men listed this as a problem ($p < 0.01$). In contrast, 22.0% of men reported drinking too much alcohol as a problem, whereas only 6.7% of women listed this as a problem ($p < 0.05$). The problems of behaviors as a cause of eating too much and eating specified food too mush were major common problems to men and women.

At their first meeting, dieticians and participants discussed which problems could be solved easily and which were the most important to solve. Following these evaluations, participants set and declared their personal dietary goals. Twenty-nine dietary goals were set and fell into the categories of dietary behavior and meal contents and were classified into seven categories (***Table 4***). The most prevalent goal of both men (31%) and women (39%) was chewing well during meal time. More women than men set goals of decreasing their snacking after supper and not eating snacks between meals. Similar percentages of men and women set the goals of eating more vegetables and decreasing their intake of fat or fatty foods. 23.7% of men set the goal of consuming less alcohol, whereas only 3.4% of women set this goal. Compared to men, more women set the goals of decreasing salt intake and replacing high-calorie snacks with low-calorie foods.

Compared to men, more women (30.5% vs. 50.0%) set a goal of decreasing the intake of snacks ($p < 0.05$). In contrast, more men (72.9%) set a goal of decreasing the intake of a specified food compared to women (48.3%; $p < 0.01$; ***Figure 2***). There were no differences between the genders with regard to the other five goal categories.

Table 4 The dietary goal-setting at first meeting

Classification	Contents	Men		Women	
		n	%	n	%
Behaviors for diet					
Decrease intake of snacks	Eat less snacks after supper	4	5.8	9	15.0
	Do not eat anything 2 hours before sleep	2	3.4	2	3.4
	Eat fruits on the time decided	2	3.4	3	5.1
	Quit a snack or eat less	9	15.3	19	32.2
	Do not eat nuts	2	3.4	0	0.0
Decrease the amount of meal	Eat with chewing food well	18	30.5	23	39.0
	Leave food when finish meal	0	0.0	5	8.5
	Do not eat once full	1	1.7	2	3.4
	Do not eat continuing for long time	0	0.0	1	1.7
	Serve a meal individually	3	5.1	2	3.4
	Record the energy displayed on foods	2	3.4	0	0.0
Eat regularly	Do not skip meals	3	5.1	2	3.4
Order of certain foods	Eat vegetables first	5	8.5	6	10.0
	Take some drinks or soup first	0	0.0	2	3.4
Meal contents					
Decrease intake of specified foods	Decrease intake of grains	10	16.9	7	11.9
	Decrease intake of main meal	1	1.7	2	3.4
	Decrease intake of meals	5	8.5	4	6.8
	Decrease side dish with alcohol	2	3.4	0	0.0
	Decrease intake of alcohol	14	23.7	2	3.4
	Decrease intake of salt or salty food	1	1.7	6	10.0
	Decrease intake of fat or fatty food	15	25.4	14	23.7
	Decrease amount of supper meals	2	3.4	0	0.0
Increase the amount of specified food	Increase intake of vegetables	16	27.1	14	23.7
Replace with low-calories from high-calories	Replace a main dish with other low-calories	5	8.5	1	1.7
	Replace with other kinds of grains	1	1.7	0	0.0
	Replace snacks with other low-calories	2	3.4	9	15.0
	Replace drinks with other low-calories	4	6.8	4	6.8
	Replace alcohol with other low-calories	0	0.0	1	1.7

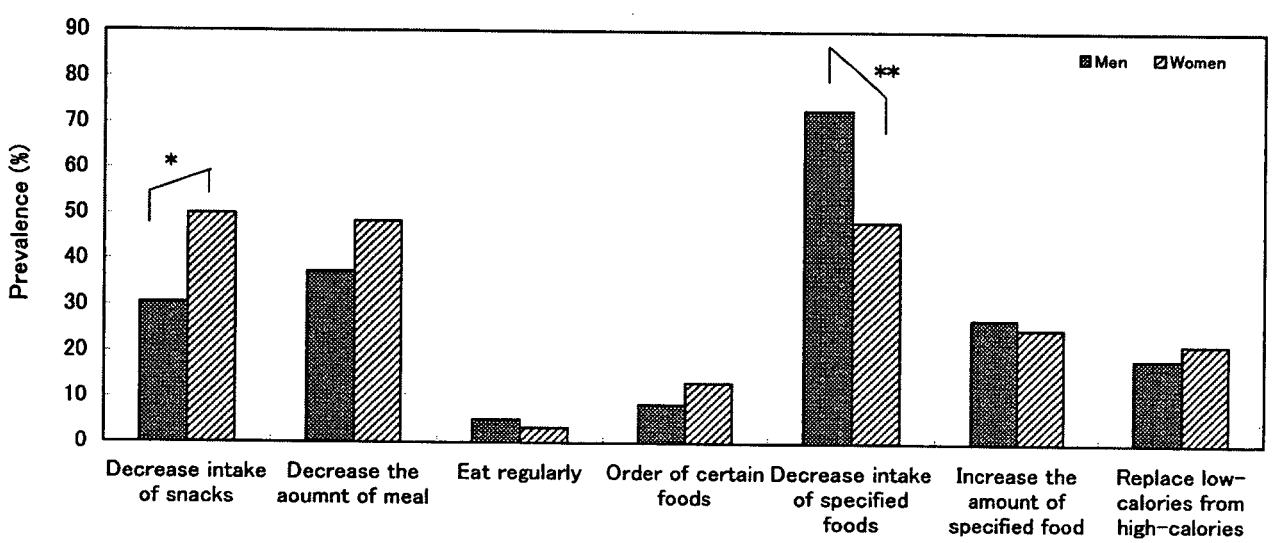

Fig. 2. Prevalence of dietary goals in men and women. The complete list of dietary goals is shown in ***Table 4***. $*p < 0.05$, $**p < 0.01$

The final exercise goal was basically set at 10,000 walking steps per day. At the beginning of the program, 21 participants already walked over 10,000 steps per day on average, and their exercise goal was to maintain this level of exercise. The remaining participants walked fewer than 10,000 steps, and their goal was to walk an additional 1,000 steps per day every month, except those participants who had knee or lower back pain. For those participants who were walking fewer than 10,000 steps per day, the average walking steps per day at the beginning of the program were 6314.6 ± 1915.7 (SD) steps in men and 6780.6 ± 1582.4 steps in women, the additional walking steps first goals were 1232.8 ± 789.4 steps in men and 928.1 ± 597.1 steps in women, and the target exercise goals per day were 7581.6 ± 1934.7 steps in men and 7662.8 ± 1470.8 steps in women; there were no significant differences between genders.

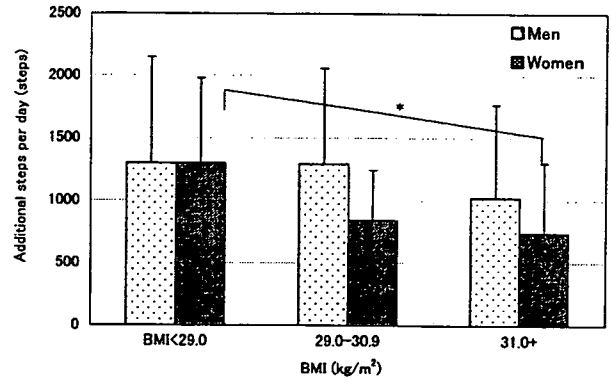

Fig. 3. The additional steps as an exercise goal among body mass index (BMI) tertiles in men and women.
Data represent subgroup mean ± SD. * $p < 0.05$

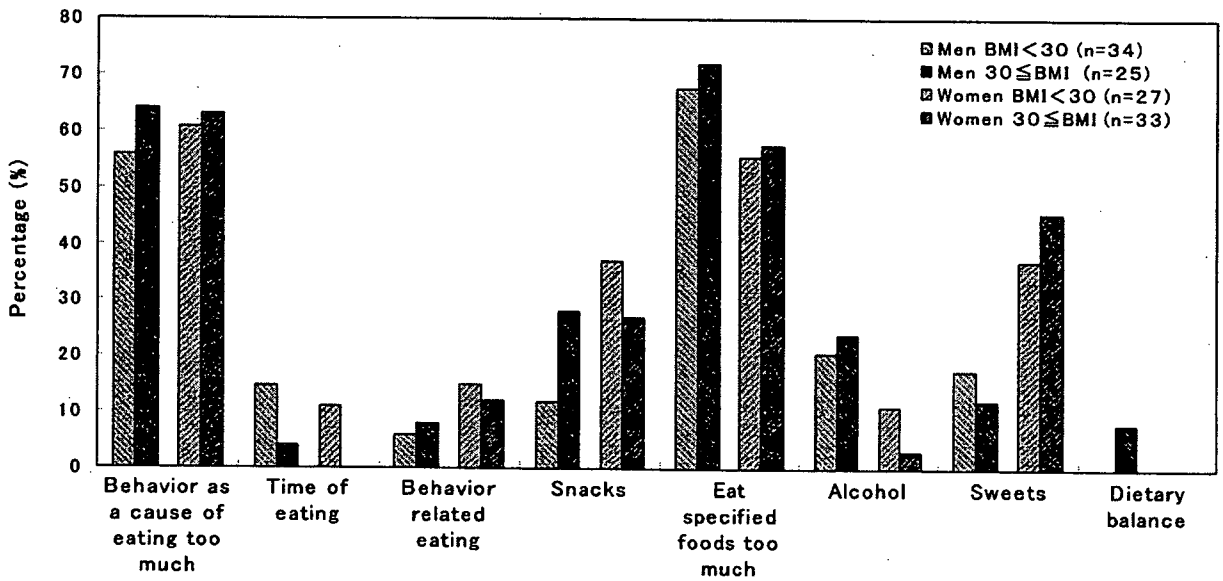

Fig. 4. Prevalence of the diet problems between body mass index (BMI) levels over or under 30 kg/m² in men and women.

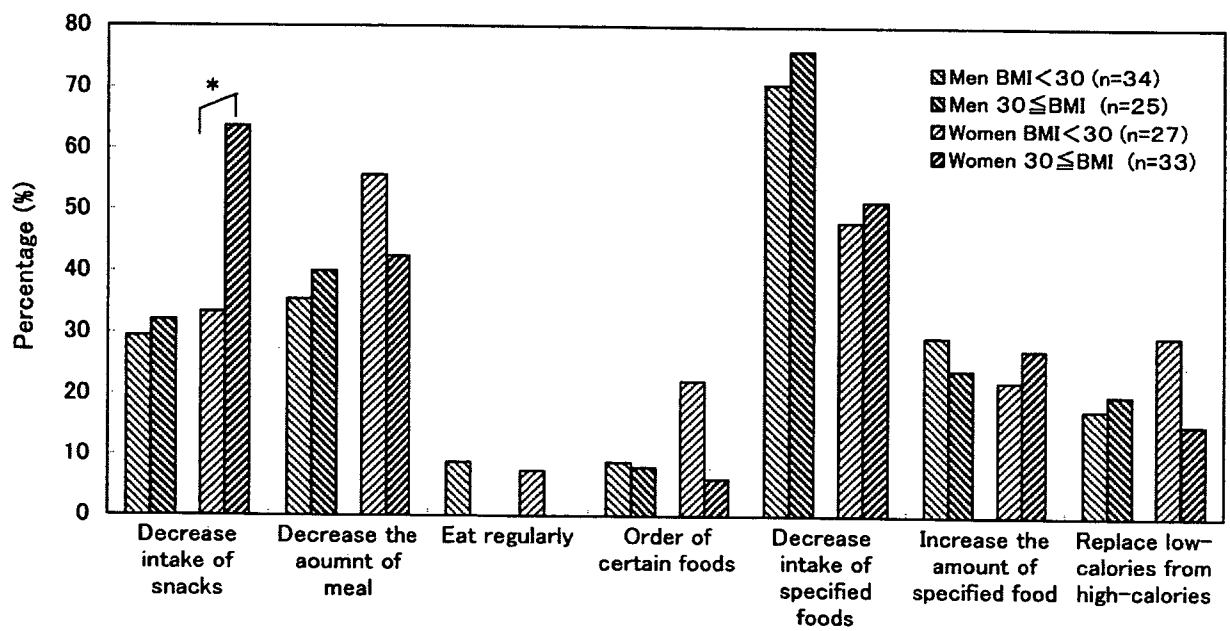

Fig. 5. Prevalence of the diet goals between body mass index (BMI) levels over or under 30 kg/m² in men and women. *$p < 0.05$; chi-square tests

Among those participants who set exercise goals, the relationship between BMI and the number of additional steps in the goal was analyzed by BMI tertiles. At baseline, there were no differences among BMI tertiles with regard to level of exercise. Those participants with BMI ≥ 31 set goals of significantly fewer additional steps compared to those with BMI < 29 ($p < 0.05$; ***Figure 3***).

The mean BMI was 30.5 kg/m^2 and the median was 29.8s kg/m^2. We divided two groups over and under 30 kg/m^2 BMI, in order to clarify relationships between BMI and the dietary problems and dietary goals. There were not significant differences of the percentage of dietary problems between the BMI levels over and under 30 kg/m^2 in women and men. (***Figure. 4***). Among the dietary goals, the percentage of the decreasing intake of snack was different between the BMI levels over and under 30 kg/m^2 in women ($p < 0.05$; ***Figure 5***).

Discussion

Most studies of traditional obesity treatment, including dietary restriction, nutritional education, and an increase in exercise, have demonstrated limited success. Other methods that have been used as an adjunct to dietary restriction in the treatment of obesity include lifestyle modification, drugs, therapeutic starvation, very low calorie diets, and surgical treatment. Long-term weight loss and maintenance requires management strategies including a combination of nutritional education and physical activity as well as behavioral interventions.[18]

In the SCOP study, we constructed a strategy for obesity treatment that mainly included cognitive behavioral treatment for nutritional education and physical activity. The dietitians interviewed participants about their levels of social support, stress, motivation, and self-efficacy. More men expected support from others, such as their wives and family than women (***Table 1***). In addition, in men, the awareness of the need to keep healthy by oneself was significantly associated with the motivation to resolve the situation and the availability of support from others (***Table 2***). Studies have shown that social support is an important aid in weight maintenance,[19,20] although the effect of family involvement on weight control is unclear. Wing et al. reported better weight maintenance when participants, especially women, were treated together with their spouses.[21] In contrast, Black and Lantz reported weight maintenance to be better when participants were treated alone, particularly men.[22] In the present study, however, men expected the support from others even though they were aware of the need to keep healthy by themselves in order to succeed in their weight loss.

The motivation to resolve the situation was significantly more prevalent in women than in men ($p < 0.05$; ***Table 1***). In women, the awareness of the need to keep healthy by oneself was also significantly associated with recognition of the benefits and probability of losing weight, the motivation to resolve the situation, and realizing the obstacles to success. Many studies have reported that a higher motivation for weight reduction was related to greater weight loss.[23] However, the Weight Loss Readiness Test (WLRT), which was developed to access weight loss readiness[24] and motivation, failed to predict weight loss.[23,25]

In the SCOP study, the dietary goals were well matched with problems, as participants were first asked to list potential problems and then set goals based on these. More than 40% of men and women recognized eating too fast as a problem and about 15% saw eating too much as an issue (***Table 3***). Likewise, both men and women recognized as problems eating too few vegetables, too much fat or fatty foods, and high salt intake. More women listed eating sweet snacks as a problem, whereas more men saw alcohol intake as an issue (***Figure 1***).

Compared to men, significantly more women set the goal of decreasing the intake of snacks, whereas more men aimed to decrease the intake of specified foods, including drinking alcohol, revealing that the favorite foods and the problem eating behaviors were different between men and women (***Table 4 and Figure 2***). The dietary goal of "Decrease intake of fat or fatty food" was set mainly in the category of "Decrease intake of specified foods" in both men and women. In women, more participants over 30 kg/m^2 BMI focused about decreasing intake of snacks including 5 goals related snacks than that under 30 kg/m^2 BMI, even though the percentage of snacks in the problems was not different between BMI levels. The goals set by participants were in line with sound dietary advice, as studies have shown that weight loss and maintenance is associated with reduced frequency of snacks,[26] less dietary fat,[26-30] and increased intake of vegetables and fruits.[26]

The dieticians usually proposed setting an exercise goal of an additional 1,000 steps per day to the actual steps last month. Although there was no difference among BMI tertiles with regard to the mean number of walking steps at the beginning of the intervention, women in the highest tertile (BMI = 31+) set significantly fewer additional steps as an exercise goal (***Figure 3***), suggesting that women in this tertile hesitate to walk. In women, there was a significant difference in the percentage of decreasing intake of snacks as a dietary goal between BMI levels over and under 30 kg/m^2 (***Figure 5***). This result may show that the participants in SCOP could set the successful dietary goal to lose weight by themselves with the guidance of the dieticians, as reported in previous study.[26]

Self-monitoring of body weight and food intake were reported as important factors in weight loss as well as weight maintenance.[27,31,32] In this study, the participants self-monitored and recorded items such as weight, daily food intake, and daily evaluations of their personal dietary and exercise goals and reported to dieticians the results of their efforts at the end of each month.

Obesity is recognized as a complex disorder involving appetite regulation and energy metabolism, and it is associated with a variety of comorbid conditions. Many studies have shown that traditional obesity treatment has been effective over the short term, but long-term outcomes do not mirror those satisfactory results. Lang and Froelicher concluded that the combination of a low-calorie diet, an increase of physical activity, and behavioral therapy should be incorporated in obesity treatments.[18] They also found that interventions involving frequent behavioral therapy, such as weekly sessions, seemed to improve the participants' adherence to changes in eating and exercise patterns and produced better outcomes. Elfhag and Rossner reviewed a variety of potential factors and concluded that weight maintenance was associated with an internal motivation to lose weight, social support, better coping strategies, a better ability to handle life stress, self-efficacy, autonomy, assuming responsibility in life, and greater overall psychological strength and stability.[33]

This survey baseline data revealed that the psychological characters were different in gender and the problems and dietary and exercise targets were also different in gender and BMI.

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ORIGINAL ARTICLE

Association of a single nucleotide polymorphism in *Wnt10b* gene with bone mineral density

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Background: Wnt signaling pathway regulates bone mineral density (BMD) through the lipoprotein receptor-related protein (LRP)5, a receptor of this signaling. Recently, we and several groups have shown that genetic variations at the *LRP5* gene locus are associated with osteoporosis. These data suggest that genetic variations in Wnt signaling genes may affect the pathogenesis of osteoporosis. To explore whether the Wnt signaling molecules are involved in the maturation of osteoblasts, we analyzed the expression levels of Wnt signaling genes, including LRP5, LRP6 and Wnt10b, in rat primary osteoblasts. Then, we studied an association of a single nucleotide polymorphism (SNP) in *Wnt10b* gene with BMD.

Methods: Expression levels of LRP5, LRP6 and Wnt10b mRNA were analyzed during the culture course of rat primary osteoblasts by real-time reverse transcription polymerase chain reaction (RT-PCR). Association of the *Wnt10b* gene polymorphism at 1059C/T (His353His), that is the only coding SNP found in J-SNP database with BMD, was examined in 221 postmenopausal Japanese women.

Results: LRP5, LRP6 and Wnt10b mRNA were detected during the differentiation of rat primary osteoblasts. As an association study of the SNP in the *Wnt10b* gene, the subjects without the T allele (CC; $n = 59$) had significantly higher total body and lumbar BMD than the subjects bearing at least one T allele (TT + TC; $n = 162$) (total body, $P = 0.0091$; lumber spine, $P = 0.0052$).

Conclusion: Wnt10b mRNA was expressed and regulated in rat primary osteoblasts. A genetic variation at the *Wnt10b* gene locus is associated with BMD, suggesting an involvement of the *Wnt10b* gene in the bone metabolism. SNP of Wnt signaling genes would serve to facilitate early diagnosis, treatment and prevention of osteoporosis.

Keywords: bone mineral density (BMD), LRP5, LRP6, osteoporosis, single nucleotide polymorphism (SNP), Wnt10b.

Introduction

Osteoporosis is a skeletal disorder characterized by low bone mineral density (BMD) and micro-architectural deterioration of bone tissue leading to an increased risk of fracture.[1] BMD is a complex trait that is influenced by both genetic and environmental factors. Heritability studies in twins and family studies have shown that genetic factors account for 50–90% of the variance in BMD.[2–6] In studies on osteoporosis-related genes, significant associations of the vitamin D receptor (*VDR*) gene,[7] estrogen receptor α (*ERα*) gene,[8] collagen type Iα1 (*COLIA1*) gene[9] and low density lipoprotein receptor-related protein 5 (*LRP5*) gene[10] polymorphisms with

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BMD in postmenopausal women have already been described. Identification of candidate genes, of which polymorphisms affect bone mass, will be useful for early detection of individuals who are at risk for osteoporosis and early institution of preventive measures.

The Wnt proteins represent a large group of secreted signaling proteins that are involved in cell proliferation, differentiation and morphogenesis.[11] Wnt proteins activate signal transduction through Frizzled which acts as receptors for Wnt proteins[12] and induce stabilization of cytoplasmic β-catenin protein. Meanwhile, low-density lipoprotein (LDL) receptor-related protein 5 and 6 (LRP5/6) were also found to be required for Wnt co-receptors.[13,14] Recent reports demonstrated that the Wnt signaling pathway regulates bone density through the LRP5.[15–18] We and several groups have shown that their is a significant association between BMD and polymorphisms in the *LRP5* gene.[10,19–21] We also have shown that a genetic variation of the *sFRP4* gene,[22] which is an inhibitor of Wnt signaling, affects the BMD among postmenopausal Japanese women. These data suggest that the single nucleotide polymorphism (SNP) in other Wnt signaling genes may affect the BMD.

Although these and other studies suggest that endogenous Wnt signaling regulates osteoblastogenesis and bone formation, Wnt molecules that are responsible for activation of this pathway in bone cells have to be determined. Recently, Wnt10b has demonstrated to regulate bone formation *in vivo*.[23] In this report, FABP4-Wnt10b mice, which overexpress Wnt10b in bone marrow, have shown increased bone.[23] It has also shown that Wnt10b$^{-/-}$ mice have decreased trabecular bone and serum osteocalcin.[23] These data suggest that Wnt10b may be a promising Wnt molecule as a determinant of BMD through the regulation of osteogenesis.

In the present study, we examined the expression of the Wnt10b in rat primary osteoblasts and the association of a polymorphism in the *Wnt10b* gene with BMD in Japanese women to investigate possible contribution of the Wnt10b in bone metabolism.

Materials and methods

Cell culture

Rat primary osteoblasts were isolated from calvaria of 5-day-old neonatal rats by enzymatic digestion as described previously[24] with some modification. Briefly, calvaria were minced and incubated at 37°C for 20 min in magnesium-free phosphate-buffered saline containing 0.1% collagenase and 0.2% dispase. The enzymatic digestion was repeated twice. The second digestion was performed for 70 min. Cells isolated at second digestion were cultured in α-minimum essential medium (MEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 IU/mL penicillin and 100 mg/mL streptomycin). Cells at the second passage were used for experiments.

Total RNA isolation and cDNA synthesis

Osteoblasts were cultured in 6-cm dishes with α-MEM containing 10% FBS, 50 μg/mL ascorbic acid and 5 mmol/L β-glycerophosphate for 3, 5, 8, 11, 13, 15 or 18 days. Total RNA were extracted from these cells using a ToTALLY RNA Kit (Ambion, Austin, TX, USA). cDNA was synthesized from 1 μg of total RNA of primary osteoblasts using a first strand cDNA synthesis kit (Amersham, Chicago, IL, USA).

SYBR green real time PCR

Primers were designed using PRIMER EXPRESS 1.0 software (Applied Biosystems, Foster City, CA, USA). Definitive primers were: rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5′-GGC ACAGTCAAGGCTGAGAAT-3′, reverse 5′-TCGCGC TCCTGGAAGATG-3′, rat alkaline phosphatase (ALP) forward 5′-TGACCACCACTCGGGTGAA-3′, reverse 5′-GCATCTCATTGTCCGAGTACCA-3′, rat LRP5 forward 5′-TGGATGGGCGTCAGAACA-3′, reverse5′-TGGGAGAGGTCAGCATGGA-3′, rat LRP6 forward 5′-AGCGTCCTCAAGCAGCTCTTC-3′, reverse5′-CGATGGTGGTGGGTTCAAA-3′ and rat Wnt10b forward 5′-TCTCTCGGGATTTCTTGGATTC-3′, reverse 5′-TGTTGTGGATCCGCATTCTC-3′. Quantitative polymerase chain reaction (PCR) was carried out using a 2× master mix composed from the SYBR Green PCR Core Reagents (Applied Biosystems) and 50 nmol/L primers. PCR reactions were performed using an ABI Prism 7000 system (Applied Biosystems) with the following sequence: 2 min at 50°C, 10 min at 95°C and 40 cycle of 15 s at 95°C and 1 min at 60°C. ALP, LRP5, LRP6 or Wnt10b signals were normalized to GAPDH signals.

Subjects

Genotypes were analyzed in DNA samples obtained from 221 healthy postmenopausal Japanese women. We chose postmenopausal women who were older than 50 years from volunteers (mean age ± SD; 61.8 ± 6.6). All women were non-related volunteers who lived in the Chubu district of Japan and provided informed consent before this study. Exclusion criteria included endocrine disorders and a metabolic bone disease other than primary osteoporosis such as hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease or unusual gynecological history. Women taking medicine related to bone metabolism such as active vitamin D, vitamin K, a vitamin K antagonist, estrogen, bisphosphonate, corticosteroids, anticonvulsants and

heparin sodium were also excluded. Ethical approval for the study was obtained from ethics committees of University of Tokyo and Research Institute and Practice for Involutional Diseases.

Measurement of BMD and biochemical markers

The lumbar-spine BMD and total body BMD (g/cm^2) of each participant were measured by dual-energy X-ray absorptiometry using fast-scan mode (DPX-L; Lunar, Madison, WI, USA). We measured serum concentration of calcium (Ca), ALP, intact-osteocalcin (I-OC, ELISA; Teijin, Tokyo, Japan), intact parathyroid hormone (PTH), calcitonin (CT) and $1,25(OH)_2D_3$. We also measured urinary ratios of urinary deoxypyridinoline (DPD, high-performance liquid chromatography method) to creatinine. The BMD data were recorded as "Z scores"; that is, deviation from the weight-adjusted average BMD for each age. Z scores were calculated using installed software (Lunar DPX-L) on the basis of data from 20 000 Japanese women.

Determination of a single nucleotide polymorphism in the Wnt10b gene

Because there was only one coding SNP in the *Wnt10b* gene in the Japanese-SNP database (J-SNP), we examined association of this SNP in the *Wnt10b* gene at 1059C/T (His353His) with BMD in 221 postmenopausal Japanese women. We also extracted this variation in the Wnt10b gene from the Assays-on-Demand SNP Genotyping Products database (Applied Biosystems) and, according to its localization on the gene, denoted it 1059C/T. We determined the 1059C/T polymorphism of the *Wnt10b* gene using the TaqMan (Applied Biosystems) PCR method.[22] To determine the *Wnt10b* SNP we used Assays-on-Demand SNP Genotyping Products C_7470505_1 (Applied BioSystems), which contains sequence-specific forward and reverse primers and two TaqMan MGB probes for detecting alleles. During the PCR cycle, two TaqMan probes competitively hybridize to a specific sequence of the target DNA and the reporter dye is separated from the quencher dye, resulting in an increase in fluorescence of the reporter dye. The fluorescence levels of the PCR products were measured with the ABI PRISM 7000, resulting in clear identification of three genotypes of the SNP.

Statistical analysis

Comparisons of Z scores and biochemical markers between the group of individuals possessing one or two chromosomes of the T-allele and the group with only C-allele encoded at that locus were subjected to statistical analysis (Student's *t*-test; StatView-J 4.5). A *P*-value less than 0.05 was considered statistically significant.

Results

Wnt10b mRNA expression is regulated during the course of primary osteoblast differentiation

At the inception of this study, we measured the Wnt10b mRNA levels during the course of differentiation in rat primary osteoblasts. In the presence of ascorbic acid and β-glycerophosphate, primary osteoblasts proceed to differentiation normally with the deposition of a collagenous extracellular matrix that mineralizes.[25,26] The continual maturation of the osteoblasts was reflected by the increase of ALP mRNA (Fig. 1A). The Wnt10b mRNA was detected at day 2 and then decreased in primary osteoblasts (Fig. 1B). Inversely, the LRP5 mRNA increased persistently during the time-course of osteoblastic differentiation until day 28 (Fig. 1C). The levels of LRP6 mRNA were almost parallel to those of LRP5 mRNA (Fig. 1D).

Association of the Wnt10b gene polymorphism with BMD

We examined a *Wnt10b* polymorphism at 1059C/T (His353His) in postmenopausal Japanese women, using the TaqMan methods. Among 221 postmenopausal women, 42 were TT homozygotes, 120 were CT heterozygotes, and 59 were CC homozygotes. The genotype distribution was found to be in the Hardy–Weinberg equilibrium.

We compared Z scores for BMD of total body and lumbar spine between the subjects bearing at least one T allele (TT + TC) and subjects without the T allele (CC). Those with the T allele had significantly lower Z scores for total body BMD (Z score; 0.24 ± 0.99 vs 0.65 ± 1.11; $P = 0.0091$) (Figs 2A and 1A) and lumbar spine BMD (Z score; -0.42 ± 1.35 vs 0.16 ± 1.41; $P = 0.0052$) (Fig. 2B). The background and biochemical data were not statistically different between these two groups (Table 1).

Discussion

During the course of primary osteoblast differentiation, Wnt10b mRNA levels showed gradual decrease and sustained at certain levels during the observation period. Recent reports demonstrated that during the course of adipogenetic differentiation in 3T3L1 cells, Wnt10b rapidly falls to an undetectable level by the first 0–1 day.[27,28] The differential expression of Wnt10b in osteoblasts and adipocytes may imply a different role of Wnt10b in the cell differentiation. The increase of LRP5 and LRP6 expression was accompanied by the increase of ALP expression, which is a marker of osteoblast differentiation.[29] A previous report also demonstrated that BMP2 induced the osteoblastic differentiation markers,

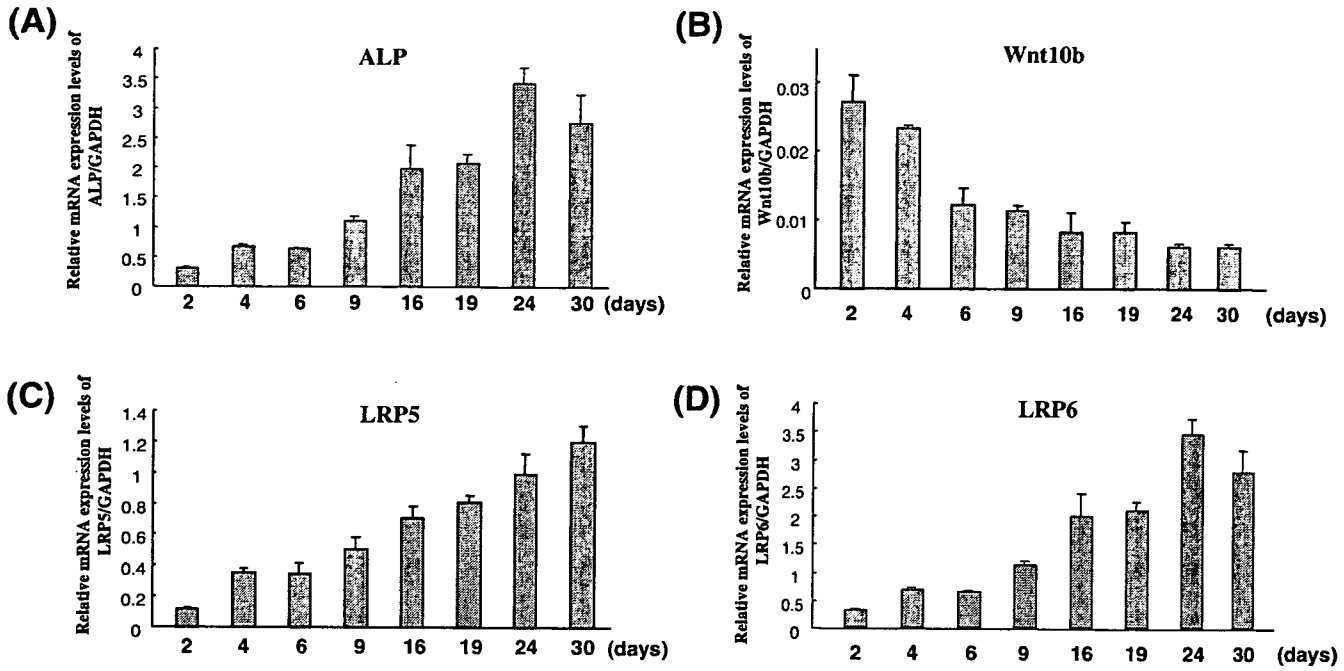

Figure 1 Expressions of the alkaline phosphatase (ALP), Wnt10b, lipoprotein receptor-related protein (LRP)5 and LRP6 mRNA during culture course of rat primary osteoblasts were analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR). Rat primary osteoblasts were cultured with α-minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 50 μg/mL ascorbic acid and 5 mmol/L β-glycerophosphate up to 18 days. At the indicated times, RNA were extracted and the expression levels of ALP (A), Wnt10b (B), LRP5 (C) and LRP6 (D) were analyzed by real-time RT-PCR, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression ($n = 4$ for each group). Values are shown as means ± SD.

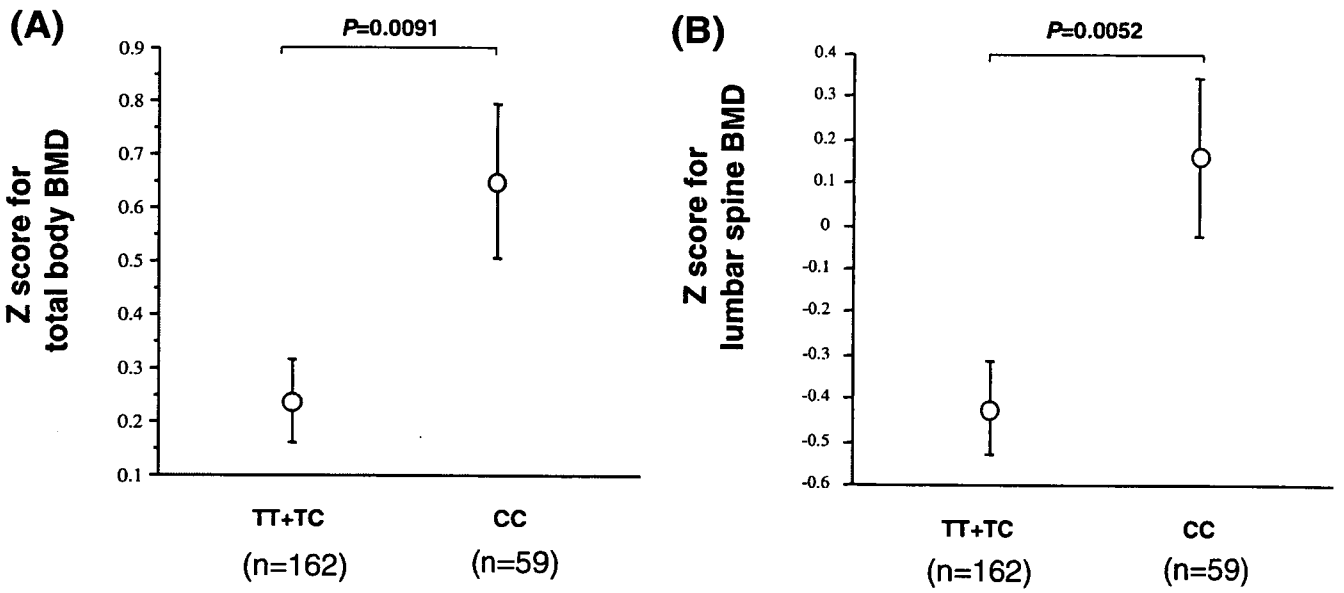

Figure 2 "Z score" values of total body and lumbar spine bone mineral density (BMD) in the groups of genotype TT + TC and genotype CC of the *Wnt10b* polymorphism in exon 6 (1059C/T). (A) Z score values for total BMD are shown for genotype TT + TC and for genotype CC. Values are expressed as mean ± SE. Numbers of subjects are shown in parentheses. (B) Z scores for lumbar BMD are shown in the same manner as (A).

followed by the increase of the LRP5 and LRP6 expression in ST2 cells.[15] Thus, the increase of LRP5 and LRP6 expression may have some roles in osteoblastic differentiation.

To our knowledge, the present study is the first to investigate the influence of a polymorphism of the Wnt10b gene on BMD. We demonstrated that the Japanese postmenopausal women who had one or two

Table 1 Comparison of background, bone mineral density and biochemical data between subjects bearing at least one T allele (TT + TC) and subjects with no T allele (CC) in the *Wnt10b* gene (1059C/T)

Items	Genotype (mean ± SD)		*P*-value
	TT + TC	CC	
No. of subjects	162	59	
Age (years)	61.4 ± 6.5	62.9 ± 6.7	NS
Height (cm)	152.3 ± 5.8	151.6 ± 5.8	NS
Bodyweight (kg)	52.2 ± 7.8	52.2 ± 7.0	NS
Lumber spine BMD (Z score)	−0.42 ± 1.35	0.16 ± 1.41	0.0052
Total body BMD (Z score)	0.24 ± 0.99	0.65 ± 1.11	0.0091
ALP (IU/L)	187.6 ± 58.8	182.3 ± 64.5	NS
I-OC (ng/mL)	8.2 ± 3.9	7.2 ± 3.2	NS
DPD (pmol/μmol/Cr)	6.3 ± 4.0	6.1 ± 3.3	NS
Intact PTH (pg/mL)	33.1 ± 10.7	36.4 ± 19.7	NS
Calcitonin (pg/mL)	23.0 ± 12.9	22.3 ± 7.9	NS
1,25 $(OH)_2D_3$ (pg/mL)	34.7 ± 10.2	35.2 ± 11.7	NS
% fat	32.6 ± 7.3	33.0 ± 7.0	NS
BMI	22.5 ± 3.1	22.7 ± 3.1	NS

ALP, alkaline phosphatase; BMD, bone mineral density; BMI, body mass index; DPD, deoxypyridinoline; I-OC, intact-osteocalcin; NS, not significant; PTH, parathyroid hormone. Statistical analysis was performed according to the method described in the text.

allele(s) of a synonymous change of C-T transition showed significant total body and lower lumbar BMD. Lower BMD in postmenopausal women could be considered as results of abnormally rapid bone loss and/or lower peak bone mass. The SNP analyzed in the present study would be useful as a genetic marker for low BMD and susceptibility to osteoporosis. Although the biological meanings of this polymorphism should be revealed by functional studies, several hypotheses could be proposed at present. First, this silent polymorphism may be linked with other mutations in exons, which contributes to the change of the Wnt10b protein function, such as in case of the *PADI4* polymorphisms in rheumatoid arthritis.[30] Second, the SNP may be linked with a mutation in regulatory elements affecting the levels of expression through variable transcriptional regulation, such as in case of the *LTA* exon 1 polymorphisms in myocardial infarction.[31] Third, this SNP in the *Wnt10b* gene may be linked with a mutation of another undefined gene adjacent to the *Wnt10b* gene that causes low BMD directly or indirectly, such as in case of the *ECM2* and *ASPN* polymorphisms in osteoarthritis.[32]

Because of the limited sample size and the number of SNP utilized in the present study, we need larger scale studies on this coding SNP and other polymorphisms in the Wnt10b gene in the future. The association study between multiple SNP and BMD using a statistical correction as well as functional analysis of SNP would be helpful.

The Wnt pathway has recently been implicated in the control of bone mass in adults in human and mice.[15–18] Activation of this pathway increases bone mass through a number of mechanisms including renewal of stem cells, stimulation of preosteoblast replication, induction of osteoblastgenesis, and inhibition of osteoblast and osteocyte apoptosis.[33] Taken together, these studies suggest that endogenous Wnt signaling plays an important role in osteogenesis and bone formation. However, the Wnt that are involved directly in the bone metabolism have to be identified among 19 members of the Wnt family. Recently, it was demonstrated that expression of Wnt10b in bone marrow increased bone mass and strength in mice.[22] Taking together with these data, our present finding of an association of a polymorphism in *Wnt10b* gene with BMD suggests that Wnt10b may be a specific ligand responsible for BMD among several Wnt.

In conclusion, our findings suggest that the *Wnt10b* gene may be a genetic determinant of BMD in postmenopausal women as is the case with its related co-receptor, *LRP5*. Examining the variation in the *Wnt10b* gene will hopefully enable us to elucidate one of mechanisms of involutional osteoporosis. Furthermore, the variation may be a potential genetic susceptibility factor that need to be further evaluated with regard to the condition of other metabolisms in which the Wnt signaling have been clearly implicated, including cholesterol, glucose and fat metabolisms.[34,35]

Acknowledgments

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ORIGINAL ARTICLES: EPIDEMIOLOGY, CLINICAL PRACTICE AND HEALTH

Association of a single nucleotide polymorphism in the steroid and xenobiotic receptor (*SXR*) gene (IVS1-579A/G) with bone mineral density

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Vitamin K2 plays an important role in the bone metabolism. The steroid and xenobiotic receptor (SXR) as a nuclear receptor activated by vitamin K2 as well as rifampicin could increase bone markers such as alkaline phosphatase in human osteoblastic cells. Thus, the SXR could mediate vitamin K2 signaling pathway in bone cells. Therefore, we analyzed expression of the SXR mRNA in human primary osteoblasts and chondrocytes. We also studied association of a single nucleotide polymorphism (SNP) in the *SXR* gene with bone mineral density (BMD). Expression levels of the SXR mRNA were analyzed during the culture course of human primary osteoblasts and chondrocytes. Association of a SNP in the *SXR* gene in intron 1 (IVS1-579A>G) with BMD was examined in 294 healthy postmenopausal Japanese women. The SXR mRNA increased at day 5 and then decreased at day 10 in human primary osteoblasts. Its mRNA gradually increased in human primary chondrocytes until day 10. As an association study of a SNP in the *SXR* gene (IVS1-579A/G), the subjects without the A allele (GG; $n = 47$) had significantly higher total BMD than the subjects bearing at least one A allele (AA + AG; $n = 247$) (Z score ± SD; 0.635 ± 1.031 *versus* 0.268 ± 1.061; $P = 0.0298$). The SXR mRNA was expressed and regulated in primary human osteoblasts and chondrocytes. A genetic variation at the *SXR* gene locus is associated with BMD, suggesting an involvement of the *SXR* gene in human bone metabolism.

Keywords: bone mineral density (BMD), osteoporosis, single nucleotide polymorphism (SNP), steroid and xenobiotic receptor (SXR), vitamin K2.

Introduction

Osteoporosis is a skeletal disorder characterized by compromised bone strength predisposing to an increased risk of fracture.[1] Twin and sibling studies have shown that bone mineral density (BMD) is under genetic control with estimates of heritability ranging 50–90%.[2–4] BMD is assumed to be determined by multiple genes with modest effects on bone mass and bone turnover as well as by environmental factors.[5,6] To date, various polymorphisms of candidate genes have been investigated in relation to BMD.[7,8] These include vitamin D receptor (*VDR*) gene,[9] estrogen receptor α (*ERα*) gene,[10] collagen type Iα1 (*COLIA1*) gene[11] and low-density lipoprotein receptor-related protein 5 (*LRP5*) gene.[12] Identification of candidate genes of which polymorphisms affect bone mass will be useful for early detection of

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individuals who are at risk for osteoporosis and early institution of preventive measures.

Vitamin K exerts an influence on the bone metabolism and is used as an anti-osteoporosis drug in Japan.[13] Moreover, vitamin K intake has been found to be associated with decrease of hip fracture risk.[14] In the bone homeostasis, a mechanism of vitamin K action is mediated through post-translation modification of proteins.[15] Vitamin K functions as an essential cofactor for carboxylation of glutamic acid residues to gammacarboxyglutamic acid residues. Recently, a novel mechanism was uncovered in the signaling that regulates the transcription of target genes by vitamin K through activation of a nuclear receptor, steroid and xenobiotic receptor (SXR, also known as PXR and NR1I2).[16] In the report, vitamin K2 was shown to bind to and activate the SXR that could induce bone markers such as alkaline phosphatase (ALP) and osteoprotegerin in the human osteoblastic cells.[16] Therefore, the SXR could be involved in the maintenance of bone homeostasis. In the present study, we examined the expression of the SXR in human primary osteoblasts and chondrocytes and the association between a polymorphism in the *SXR* gene and BMD in Japanese women to investigate possible contribution of the SXR in human bone metabolism.

Materials and methods

Cell culture

Primary human osteoblasts and chondrocytes were purchased from Cambrex (Charles City, IA, USA). Primary human osteoblasts were cultured 6-cm dishes in the osteoblast growth medium (OGM) medium (Cambrex) supplemented with SingleQuots for OGM, ascorbic acid and β-glycerophosphate for 2, 5, or 10 days according to the manufacturer's recommended protocol. Primary human chondrocytes were cultured in 6-cm dishes in the chondrocyte basal differentiation medium (CDBM) medium (Cambrex) supplemented with SingleQuots for CDBM (including insulin-like growth factor [IGF]-1, transforming growth factor [TGF]β1, insulin, transferrin and fetal bovine serum [FBS]) for 2, 5 or 10 days according to the manufacturer's recommended protocol.

Total RNA isolation and cDNA synthesis

Total RNA were extracted from the cells using a ToTALLY RNA Kit (Ambion, Austin, TX, USA). cDNA was synthesized from 1 μg of total RNA of primary osteoblasts using first strand cDNA synthesis kit (Amersham, Chicago, IL, USA).

SYBR Green real time PCR

Primers were designed using PRIMER EXPRESS 1.0 software (Applied Biosystems, Foster City, CA, USA). Definitive primers were: human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-TGG ACCTCATGGCCCACA-3′, reverse 5′-TCAAGGGG TCTACATGGCAA-3′; human ALP, forward 5′-TCCC ACGTCTTCACATTTGGT-3′, reverse 5′-AAGGGC TTCTTGTCTGTGTCACT-3′; human collagen type II alpha 1 (COL2A1), forward 5′-TTGCCTATCTGG ACGAAGCA-3′, reverse 5′-CGTCATTGGAGCCCT GGAT-3′; and human SXR forward 5′-ACTGCC TTTACTTCAGTGGGAATC-3′, reverse 5′-ATTCTC TTGCTTTTCTCACTGTGAAC-3′. Quantitative polymerase chain reaction (PCR) was carried out using a 2 × master mix composed from the SYBR Green PCR Core Reagents (Applied Biosystems) and 50 nmol/L primers. PCR reactions were performed using an ABI Prism 7000 system (Applied Biosystems) with the following sequence: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C. ALP, COL2A1 or SXR signal was normalized to GAPDH signal.

Subjects

Genotypes were analyzed in DNA samples obtained from 294 healthy postmenopausal Japanese women (mean age ± SD; 65.5 ± 8.9). Exclusion criteria included endocrine disorders (e.g. hyperthyroidism, hyperparathyroidism, diabetes mellitus, liver disease, renal disease), use of medications known to affect bone metabolism (e.g. corticosteroids, anticonvulsants, heparin sodium), or unusual gynecological history. All were non-related volunteers and provided informed consent before this study. Ethical approval for the study was obtained from the ethics committee of University of Tokyo Hospital and the ethics committee of Research Institute and Practice for Involutional Diseases.

Measurement of BMD and biochemical markers

The lumbar-spine BMD and total body BMD (in g/cm^2) of each participant were measured by dual-energy X-ray absorptiometry using fast-scan mode (DPX-L; Lunar, Madison, WI, USA). We measured serum concentration of Ca, ALP), intact-osteocalcin (I-OC, enzyme-linked immunosorbent assay [ELISA]; Teijin, Tokyo, Japan), intact parathyroid hormone (PTH), calcitonin (CT) and 1, 25(OH)2D3. We also measured urinary ratios of urinary deoxypyridinoline (DPD, high-performance liquid chromatography [HPLC] method) to creatinine. The BMD data were recorded as "Z scores"; that is, deviation from the weight-adjusted average BMD for each age. Z scores were calculated using installed software (Lunar DPX-L) on the basis of data from 20 000 Japanese women.

Determination of a single nucleotide polymorphism in the SXR gene

We extracted a polymorphic variation in the *SXR* gene intron 1 region from the Assays-on-Demand single nucleotide polymorphism (SNP) Genotyping Products database (Applied Biosystems), and, according to its localization on the gene, denoted it IVS1-579A/G. We determined the IVS1-579A/G polymorphism of the *SXR* gene using the TaqMan (Applied Biosystems) PCR method.[17] To determine the *SXR* SNP we used Assays-on-Demand SNP Genotyping Products C_1834250–10 (Applied BioSystems), which contains sequence-specific forward and reverse primers and two TaqMan Minor Groove Binder (MGB) probes for detecting alleles. During the PCR cycle, two TaqMan probes competitively hybridize to a specific sequence of the target DNA and the reporter dye is separated from the quencher dye, resulting in an increase in fluorescence of the reporter dye. The fluorescence levels of the PCR products were measured with the ABI PRISM 7000, resulting in clear identification of three genotypes of the SNP.

Statistical analysis

Comparisons of Z scores and biochemical markers between the group of individuals possessing one or two chromosomes of the A-allele and the group with only G-allele encoded at that locus were subjected to statistical analysis (Student's *t*-test; StatView-J 4.5). A *P*-value less than 0.05 was considered statistically significant.

Results

SXR mRNA expression is regulated during the course of primary osteoblasts and chondrocytes differentiation

At the inception of this study, we measured the SXR mRNA levels during the course of differentiation in human primary osteoblasts and chondrocytes. In the presence of ascorbic acid and β-glycerophosphate, primary osteoblasts proceed to differentiation normally with the deposition of a collagenous extracellular matrix that mineralizes.[18,19] The continual maturation of the osteoblasts was reflected by the increase of ALP mRNA (Fig. 1a). The SXR mRNA increased at day 5 and then decreased at day 10 in human primary osteoblasts (Fig. 1c). In the presence of insulin and transferrin, primary chondrocytes proceed to differentiation normally[20,21] and the continual maturation of the chondrocytes was reflected by the increase of COL2A mRNA (Fig. 1b). The SXR mRNA gradually increased in human primary chondrocytes until day 10 (Fig. 1c).

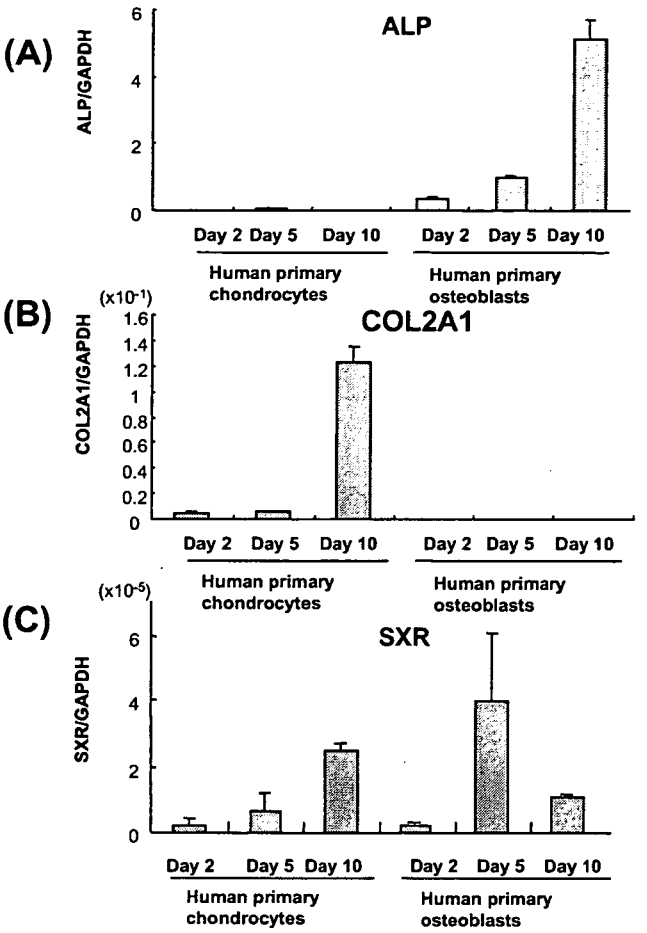

Figure 1 Expressions of the alkaline phosphatase (ALP), collagen type 2 α1 (COL2A1) and SXR mRNA during culture course of human primary osteoblasts and chondrocytes were analyzed by real-time reverse transcription polymerase chain reaction (RT-PCR). Human primary osteoblasts and chondrocytes were cultured with appropriate medium described in "Materials and methods" up to 10 days. At the indicated time, RNA was extracted and the expression levels of the ALP (a), COL2A1 (b) and steroid and xenobiotic receptor (SXR) (c) were analyzed by real-time PCR, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression ($n = 4$ for each group). Values are shown by means ± SD.

Association of the SXR gene polymorphism in intron 1 with BMD

During the search for SNP of human *SXR* gene by a SNP Genotyping database (http://www.appliedbiosystems.com), we noticed an SNP (IVS1-579A/G) in the *SXR* gene intron 1 region. We further studied this SNP for association analysis in Japanese women using the TaqMan methods, because it may affect transcriptional regulation of this gene. Among 294 postmenopausal volunteers, 112 were AA homozygotes, 135 were AG heterozygotes, and 47 were GG homozygotes.