With this background, the aim of this study was to clarify the effects of daily walking with a pedometer on APN levels as well as other metabolic parameters connected to life-style-related disease.

Subjects and Methods

We recruited 44 male volunteers (age, 37 ± 9 yrs) with a sedentary lifestyle who were instructed to wear a pedometer (TANITA FB-714) from the time they got up until they went to bed for 50 consecutive days and to record the number of steps they walked every day. All subjects were employees of a food company in Kanazawa city, Japan. We did not perform dietary therapy. Exclusion criteria included: abnormal liver or muscle enzymes, creatinemia, habitual alcohol intake >3 standard drinks/day or endocrinological disorder. Subjects who already walked daily using a pedometer were also excluded. Two subjects were on medication for hypertension (A-II receptor antagonist), three for hyperlipidemia (statins) and one for hyperuricemia (allopurinol). The dosage for these drugs did not change during the study period, and they had been on those medications for at least a few months before starting this study. Drinkers (consuming more than 30 grams of alcohol per day) and smokers (smoking more than 10 cigarettes) were also excluded from this study, because these factors are known to considerably affect plasma lipoprotein metabolism. Percent body fat (PBF) was determined from bioelectrical impedance analyses (BIA) using TANITA BC-118D (TANITA Corporation, Tokyo, Japan). Venous blood was obtained after a 12-h overnight fast. Serum total- and high-density lipoprotein (HDL) cholesterol and triglyceride levels were determined before and after 50 days. Serum cholesterol and triglyceride levels were measured by enzymatic methods. HDL-cholesterol was measured by a polyanion-polymer/detergent (PPD) method (Daiichi, Tokyo). Serum insulin concentrations were determined using a commercial enzyme immunoassay kit (Eiken, Tokyo, Japan). The homeostasis model assessment of insulin resistance (HOMA-R), a surrogate measure for insulin sensitivity[20], was calculated as fasting insulin X PG (mmol/L)/22.5. Serum levels of adiponectin [Otsuka Chemical] and TNF-α [R&D Systems] were measured by ELISA. A statement of institutional approval was granted for this study in accordance with the Declaration of Helsinki and informed consent was obtained from all participants.

Statistical Analysis

Statistical evaluation was performed using StatView-J 5.0 software (SAS Institute, Cary, NC, on a Macintosh Computer). All results are presented as the mean ± SD. Wilcoxon's rank test was used for evaluation of the significance of differences between before and after 50 days of walking with a pedometer.

Table 1. Baseline characteristics and changes in metabolic parameters in all subjects (n=44)

Variables	Before	After	*P* value
Body mass index, kg/m^2	24.2 ± 2.9	23.9 ± 2.9	0.0009
Percent body fat, %	22.7 ± 5.3	22.4 ± 5.4	0.21
Waist, cm	85.1 ± 7.7	84.7 ± 8.1	0.27
sBP, mmHg	122 ± 11	117 ± 10	0.0089
dBP, mmHg	77 ± 9	75 ± 9	0.176
TC, mg/dL	190 ± 26	189 ± 24	0.99
TG, mg/dL	119 ± 80	101 ± 52	0.019
HDL-C, mg/dL	56 ± 14	57 ± 13	0.25
FPG, mg/dL	96 ± 11	95 ± 11	0.36
Glycoalbumin, %	14.4 ± 1.0	14.3 ± 1.1	0.091
HOMA-R	1.87 ± 1.20	1.68 ± 1.15	0.19
TNF-α, pg/mL	1.58 ± 0.49	1.48 ± 0.51	0.023
Adiponectin, μg/mL	6.8 ± 2.3	6.6 ± 2.3	0.32

sBP, systolic blood pressure; dBP, diastolic blood pressure
TC, total cholesterol; TG, triglycerides; HDL-C, HDL-cholesterol
FPG, fasting plasma glucose; HOMA-R, homeostasis model assessment of insulin resistance

Results

Baseline Characteristics and Changes in Metabolic Parameters in All Subjects (Table 1)

The average number of steps of the 44 men over the 50-day period was 8211 ± 2084 steps per day. There was a subtle but significant reduction in body mass index (BMI), but no significant changes in PBF and waist circumference. There were significant reductions in systolic blood pressure (sBP), serum triglycerides (TG) and TNF-α levels; however, APN did not show a significant change during this period.

Changes in Metabolic Parameters in MT and LT Groups (Tables 2 and 3)

We divided the subjects into 2 groups according to the steps they walked per day, namely, more than 8000 steps (MT group, n=22) and less than 8000 steps (LT group, n=22). The average number of steps in the MT and LT groups was 9960 ± 1100 and 6462 ± 1128 steps per day, respectively. In the MT group, TG and sBP levels fell considerably after 50 days, wheseas in the LT group none of these metabolic parameters showed a considerable change after 50 days of walking.

Table 2. Changes in metabolic parameters in subjects walking more than 8000 steps per day (n=22)

	Before	After	*P* value
Body mass index, kg/m^2	23.9 ± 2.3	23.7 ± 2.2	0.0018
Percent body fat, %	22.0 ± 4.5	21.9 ± 4.1	0.52
Waist, cm	84.4 ± 7.3	84.0 ± 7.2	0.41
sBP, mmHg	124 ± 11	114 ± 11	0.0005
dBP, mmHg	78 ± 10	74 ± 9	0.085
TC, mg/dL	189 ± 26	187 ± 25	0.71
TG, mg/dL	125 ± 75	102 ± 61	0.034
HDL-C, mg/dL	53.0 ± 9.8	53.1 ± 10.6	0.45
FPG, mg/dL	96 ± 10	95 ± 8.5	0.42
Glycoalbumin, %	14.4 ± 1.0	14.3 ± 1.0	0.15
HOMA-R	1.78 ± 1.06	1.46 ± 0.64	0.11
TNF-α, pg/mL	1.5 ± 0.31	1.38 ± 0.26	0.09
Adiponectin, μg/mL	6.7 ± 2.3	6.4 ± 2.3	0.21

sBP, systolic blood pressure; dBP, diastolic blood pressure
TC, total cholesterol; TG, triglycerides; HDL-C, HDL-cholesterol
FPG, fasting plasma glucose; HOMA-R, homeostasis model assessment of insulin resistance

Table 3. Changes in metabolic parameters in subjects with less than 8000 steps per day (n=22)

	Before	After	*P* value
Body mass index, kg/m^2	24.4 ± 3.5	24.2 ± 3.5	0.17
Percent body fat, %	23.3 ± 6.1	24.2 ± 6.5	0.24
Waist, cm	85.8 ± 8.2	85.5 ± 9.0	0.47
sBP, mmHg	119 ± 11	119 ± 8.7	0.86
dBP, mmHg	77 ± 7.8	77 ± 9.2	0.95
TC, mg/dL	191 ± 27	191 ± 24	0.73
TG, mg/dL	114 ± 86	99 ± 44	0.22
HDL-C, mg/dL	59 ± 17	60 ± 15	0.47
FPG, mg/dL	96 ± 13	96 ± 13	0.64
Glycoalbumin, %	14.4 ± 1.1	14.3 ± 1.3	0.37
HOMA-R	1.97 ± 1.34	1.89 ± 1.48	0.76
TNF-α, pg/mL	1.7 ± 0.6	1.6 ± 0.6	0.12
Adiponectin, μg/mL	7.0 ± 2.4	6.9 ± 2.4	0.77

sBP, systolic blood pressure; dBP, diastolic blood pressure
TC, total cholesterol; TG, triglycerides; HDL-C, HDL-cholesterol
FPG, fasting plasma glucose; HOMA-R, homeostasis model assessment of insulin resistance

Table 4. Correlations of waist circumference, BMI and % fat (PBF) versus several metabolic parameters at baseline

	Waist		BMI		% fat	
Variables	r	p	r	p	r	p
Age	0.203	0.187	0.111	0.472	0.214	0.163
BMI	0.878	<0.0001	-	-	0.901	<0.0001
PBF	0.893	<0.0001	0.901	<0.0001	-	-
Waist	-	-	0.878	<0.0001	0.893	<0.0001
sBP	0.315	0.0373	0.306	0.0433	0.299	0.049
dBP	0.409	0.0058	0.287	0.0588	0.434	0.0033
TC	-0.047	0.760	-0.031	0.841	-0.017	0.914
TG*	0.165	0.285	0.085	0.583	0.201	0.192
HDL-C	-0.336	0.0256	-0.384	0.0101	-0.324	0.032
FPG	0.400	0.0071	0.374	0.0124	0.434	0.0032
HOMA-R*	0.662	<0.0001	0.596	<0.0001	0.667	<0.0001
Adiponectin*	-0.417	0.0049	-0.300	0.0477	-0.384	0.0102

*These values were logarithmically transformed before correlation analysis.
BMI, body mass index; PBF, percent body fat; sBP, systolic blood pressure; dBP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; HDL-C, HDL-cholesterol; FPG, fasting plasma glucose; HOMA-R, homeostasis model assessment of insulin resistance

Correlation of BMI, Percent Body Fat (PBF) and Waist Circumference with Metabolic Parameters at the Baseline (Table 4)

Significant associations were noted between BMI, PBF and waist circumference versus serum APN levels, among which the association between waist circumference and serum adiponectin levels was most pronounced. Similarly, there were considerable associations between BMI, PBF and waist circumference, and HOMA-R.

Correlation between the Number of Steps Per Day with % Changes in Metabolic Parameters During the Study Period

Among the metabolic parameters investigated, % changes in sBP showed a significant correlation ($r=-0.401$, $p=0.0069$) and BMI tended to show a correlation ($r=-0.276$, $p=0.068$) with the number of steps per day, whereas no other metabolic parameters showed a significant correlation.

Discussion

In this study we found that walking with a pedometer had a favorable effect on conventional metabolic parameters, such as blood pressure and serum triglycerides levels when the steps per day were greater than 8000. Meta-analysis of the effect of walking on serum lipids in 46074 subjects in 8 populations have shown that walking 6000 steps or more lowered TG by 10 mg/dL and increased HDL-C by 3 mg/dL compared to subjects walking less than 2000 steps per day[21]. In our study we did not find a significant effect of walking 6000 steps on lipid levels. Since our study subjects did not wear a pedometer before this study and thus we did not know the changes in the steps they walked between before and after this study, it was impossible to exactly explain the reason underlying this inconsistency between our study and their meta-analysis. This point might be a limitation of our study.

In our study, we measured several cytokines as well as conventional metabolic parameters. There was a significant reduction in plasma TNF-α levels after 50 days of walking, which could have a favorable effect on insulin sensitivities; however, there was no significant change in APN levels. There have been several studies conducted on the effects of exercise on plasma APN levels in obese or overweight subjects[17-19]. Yokoyama *et al.* reported that a 3-week intervention consisting of exercise, including walking and a bicycle ergometer, and diet therapy did not produce significant changes in APN levels in type 2 diabetic overweight individuals[17], which was consistent with previous findings[18, 19]. Our study differs from theirs in that we investigated the effect of walking with a pedometer. Also, we were able to compare the effects of walking on metabolic parameters between LT and MT groups. There is a report showing that considerable weight loss produces a significant increase in APN levels[22]. Yang *et al.* showed that 21% BMI reduction by gastric partition produced a 46% increase in plasma APN levels[22]. We presume that we did not observe significant changes in APN levels in our study because the weight loss produced by walking was too subtle.

In addition to the effect of walking on metabolic parameters, we also investigated the association between BMI, PBF and waist circumference versus various metabolic parameters. To our knowledge, there has been no previous study comparing the clinical significance of BMI, PBF and waist circumference versus metabolic parameters. Significant attention has been given to waist circumference, which is an important component for diagnosing metabolic syndrome[23]. In our study, there was a stronger association with APN levels than with BMI and PBF; thus, we presume that waist circumference could a better predictor of adiponectin levels than BMI or PBF.

It is intriguing that there was a positive correlation of % changes in sBP with the number of steps walked per day, indicating how important it is to walk with a pedometer to lower systolic blood pressure.

For the accuracy and reliability of PBF by BIA, which was applied in this study, Tsui *et al.*[24] showed a high correlation between PBF by BIA and that by DXA ($r=0.89$, $p<0.0001$) in 96 (48 men and 48 women) type 2 diabetic subjects. This is in line with the data previously shown in healthy individuals[25].

In conclusion, we have reported that walking with a pedometer considerably improved blood pressure and TG levels but had no effect on serum APN levels in Japanese middle-aged men.

Acknowledgments

We are indebted to Fujii Setsuro Memorial Foundation and Ishikawa Prefecture Local Government for their financial support to conduct this study.

References

1) Despres JP and Lamarche B: Low-intensity endurance exercise training, plasma lipoproteins and the risk of coronary heart disease. J Intern Med, 1994; 236:7-22.
2) Okada K, Hayashi T, Tsumura K, Suematsu C, Endo G, and Fujii S: Leisure-time physical activity at weekends and the risk of Type 2 diabetes mellitus in Japanese men: the Osaka Health Survey. Diabet Med, 2000; 17:53-58.
3) Hayashi T, Tsumura K, Suematsu C, Okada K, Fujii S, and Endo G: Walking to work and the risk for hypertension in men: the Osaka Health Survey. Ann Intern Med, 1999; 131:21-26.
4) Hu E, Liang P, and Spiegelman BM: AdipoQ is a novel adipocyte-specific gene dysregulated in obesity. J Biol Chem, 1996; 271:10697-10703.
5) Scherer PE, Williams S, Fogliano M, Baldini G, and Lodish HF: A novel serum protein similar to C1q, produced exclusively in adipocytes. J Biol Chem, 1995; 270:26746-26749.
6) Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y, and Matsubara K: cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (Adi Pose Most abundant Gene transcript 1). Biochem Biophys Res Commun, 1996; 221:286-289.
7) Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, Yamashita S, Noda M, Kita S, Ueki K, Eto K, Akanuma Y, Froguel P, Foufelle F, Ferre P, Carling D, Kimura S, Nagai R, Kahn BB, and Kadowaki T: Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med, 2002; 8:1288-1295.

8) Goldfine AB and Kahn CR: Adiponectin: linking the fat cell to insulin sensitivity. Lancet, 2003; 362:1431-1432.
9) Jansson PA, Pellme F, Hammarstedt A, Sandqvist M, Brekke H, Caidahl K, Forsberg M, Volkmann R, Carvalho E, Funahashi T, Matsuzawa Y, Wiklund O, Yang X, Taskinen MR, and Smith U: A novel cellular marker of insulin resistance and early atherosclerosis in humans is related to impaired fat cell differentiation and low adiponectin. FASEB J, 2003; 17:1434-1440.
10) Katsuki A, Sumida Y, Urakawa H, Gabazza EC, Murashima S, Matsumoto K, Nakatani K, Yano Y, and Adachi Y: Plasma levels of adiponectin are associated with insulin resistance and serum levels of triglyceride in Japanese metabolically obese, normal-weight men with normal glucose tolerance. Diabetes Care, 2003; 26:2964-2965.
11) Kern PA, Di Gregorio GB, Lu T, Rassouli N, and Ranganathan G: Adiponectin expression from human adipose tissue: relation to obesity, insulin resistance, and tumor necrosis factor-alpha expression. Diabetes, 2003; 52:1779-1785.
12) Cnop M, Havel PJ, Utzschneider KM, Carr DB, Sinha MK, Boyko EJ, Retzlaff BM, Knopp RH, Brunzell JD, and Kahn SE: Relationship of adiponectin to body fat distribution, insulin sensitivity and plasma lipoproteins: evidence for independent roles of age and sex. Diabetologia, 2003; 46:459-469.
13) Ezenwaka CE, Kalloo R, Uhlig M, and Eckel J: Relationship between adiponectin and metabolic variables in Caribbean offspring of patients with type 2 diabetes mellitus. Horm Metab Res, 2004; 36:238-242.
14) Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, and Matsuzawa Y: Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. Arterioscler Thromb Vasc Biol, 2000; 20:1595-1599.
15) Pellme F, Smith U, Funahashi T, Matsuzawa Y, Brekke H, Wiklund O, Taskinen MR, and Jansson PA: Circulating adiponectin levels are reduced in nonobese but insulin-resistant first-degree relatives of type 2 diabetic patients. Diabetes, 2003; 52:1182-1186.
16) Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, and Matsuzawa Y: Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. Circulation, 1999; 100:2473-2476.
17) Yokoyama H, Emoto M, Araki T, Fujiwara S, Motoyama K, Morioka T, Koyama H, Shoji T, Okuno Y, and Nishizawa Y: Effect of aerobic exercise on plasma adiponectin levels and insulin resistance in type 2 diabetes. Diabetes Care, 2004; 27:1756-1758.
18) Hulver MW, Zheng D, Tanner CJ, Houmard JA, Kraus WE, Slentz CA, Sinha MK, Pories WJ, MacDonald KG, and Dohm GL: Adiponectin is not altered with exercise training despite enhanced insulin action. Am J Physiol Endocrinol Metab, 2002; 283:E861-865.
19) Ryan AS, Nicklas BJ, Berman DM, and Elahi D: Adiponectin levels do not change with moderate dietary induced weight loss and exercise in obese postmenopausal women. Int J Obes Relat Metab Disord, 2003; 27:1066-1071.
20) Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, and Turner RC: Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia, 1985; 28:412-419.
21) Hata Y and Nakajima K: Life-style and serum lipids and lipoproteins. J Atheroscler Thromb, 2000; 7:177-197.
22) Yang WS, Lee WJ, Funahashi T, Tanaka S, Matsuzawa Y, Chao CL, Chen CL, Tai TY, and Chuang LM: Weight reduction increases plasma levels of an adipose-derived anti-inflammatory protein, adiponectin. J Clin Endocrinol Metab, 2001; 86:3815-3819.
23) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). JAMA, 2001; 285:2486-2497.
24) Tsui EY, Gao XJ, and Zinman B: Bioelectrical impedance analysis (BIA) using bipolar foot electrodes in the assessment of body composition in Type 2 diabetes mellitus. Diabet Med, 198; 15:125-128.
25) Sakamoto Y, Miura J, Yamaguchi Y, Ohno M, and Ikeda Y: Usefulness of bioelectrical impedence analysis for measurement of bodu fat. Int J Obesity, 1994; 18(suppl2):501.

Original Article

Prevalence of Metabolic Syndrome in the General Japanese Population in 2000

Hidenori Arai[1], Akira Yamamoto[2], Yuji Matsuzawa[3], Yasushi Saito[4], Nobuhiro Yamada[5], Shinichi Oikawa[6], Hiroshi Mabuchi[7], Tamio Teramoto[8], Jun Sasaki[9], Noriaki Nakaya[10], Hiroshige Itakura[11], Yuichi Ishikawa[12], Yasuyoshi Ouchi[13], Hiroshi Horibe[14], Nobuo Shirahashi[15], and Toru Kita[16]

[1]Department of Geriatric Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan.
[2]National Cardiovascular Center, Osaka, Japan.
[3]Sumitomo Hospital, Osaka, Japan.
[4]Clinical Cell Biology and Medicine, Graduate School of Medicine, Chiba University, Chiba, Japan.
[5]Institute of Clinical Medicine, Tsukuba University, Tsukuba, Japan.
[6]Third Department of Internal Medicine, Nippon Medical School, Tokyo, Japan.
[7]Department of Internal Medicine, Kanazawa University, Kanazawa, Japan.
[8]Department of Internal Medicine, Teikyo University, Tokyo, Japan.
[9]International University of Health and Welfare, Shizuoka, Japan.
[10]Nakaya Clinic, Japan.
[11]Ibaraki Christian University, Ibaraki, Japan.
[12]Faculty of Health Sciences, Kobe University, Kobe, Japan.
[13]Department of Geriatric Medicine, Graduate School of Medicine and Faculty of Medicine, the University of Tokyo, Tokyo, Japan.
[14]Keisen Clinic, Japan.
[15]Osaka City University Medical School, Osaka, Japan.
[16]Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan.

To determine the prevalence of metabolic syndrome in the Japanese general population, we analyzed data from a nationwide survey conducted in 2000. According to the Japanese new diagnostic criteria for metabolic syndrome in 2005, we analyzed 3,264 people aged from 20 to 79 (men, 1,917; women, 1,347) from the total participants. The incidence of metabolic syndrome was 7.8%. Men had a higher incidence (12.1%) than women (1.7%). Most of the women satisfying the criteria were 50 years old or over, while the incidence in men started to rise from their 30s. When we applied the criteria of Adult Treatment Panel III, the incidence was about 3-fold higher. In this population visceral obesity was associated with metabolic abnormalities, such as higher LDL-cholesterol, triglyceride, glucose, and blood pressure and lower HDL-cholesterol. Thus we determined the incidence of metabolic syndrome and each metabolic abnormality in the Japanese general population in 2000 and found an association of visceral obesity with metabolic abnormalities. Intervention to reduce the incidence of metabolic syndrome in Japan is necessary to reduce the risk of cardiovascular disease.

J Atheroscler Thromb, 2006; 13:202-208.

Key words; Metabolic syndrome, Dyslipidemia, Visceral obesity, Japanese

Introduction

Metabolic syndrome is a constellation of multiple risk factors, such as dyslipidemia, elevated glucose, and elevated blood pressure. This syndrome has received increased attention due to its association with increased risk for cardiovascular disease and type 2 diabetes[1]. Although the pathogenesis of metabolic syn-

Address for correspondence: Hidenori Arai, Department of Geriatric Medicine, Kyoto University School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.
E-mail: harai@kuhp.kyoto-u.ac.jp
Received: March 30, 2006
Accepted for publication: June 12, 2006

drome has not been fully understood, the predominant underlying risk factor is considered to be visceral obesity due to an atherogenic diet and physical inactivity in the presence of some unknown genetic background[2-4]. In women the incidence of metabolic syndrome increases after menopause; therefore, hormonal imbalance and aging are also associated with the development of metabolic syndrome[5].

Along with the westernization of lifestyle, the incidence of metabolic disorders, such as dyslipidemia, hypertension, and diabetes is increasing in Japan. In spite of the availability of many drugs, such as statins, angiotensin-converting enzyme inhibitors, and aspirin, the incidence of cardiovascular disease is not decreasing in Japan, probably due to these metabolic abnormalities, especially dyslipidemia and diabetes along with obesity according to the national survey by the Ministry of Health, Labour and Welfare (http://www.mhlw.go.jp/toukei/saikin/hw/kenkou/jyunkan/jyunkan00/gaiyo.html). In 2000, we conducted a lipid survey in various districts in Japan[6]. What we found in this survey was that the level of triglyceride increased in middle-aged men along with increased body mass index (BMI) compared with the data in 1990[7]. This increase in BMI also suggests an increase in the incidence of visceral obesity and metabolic syndrome; therefore, knowing the incidence of metabolic syndrome is very important from the standpoint of preventive medicine.

In the last few years, several expert groups have attempted to set forth simple diagnostic criteria to be used in clinical practice to identify patients with metabolic syndrome. The committee of International Diabetes Federation (IDF) adopted waist circumference as the surrogate marker for visceral obesity as an essential component of this syndrome (http://www.idf.org/webdata/docs/IDF_Metasyndrome_definition.pdf). In Japan the committee established diagnostic criteria under the same principle as that used in the IDF criteria, except that the cutoff point for high glucose is 110 mg/dL instead of 100 mg/dL[8]. The cutoff of waist circumference for visceral obesity was adopted as $\geqq 85$ cm in men and $\geqq 90$ cm in women. Meanwhile, the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (ATP III) criteria required no single factor for diagnosis, but instead required the presence of at least 3 out of 5 components for diagnosis[9]; thus, complete agreement on the definition and diagnostic criteria has not been achieved so far.

The purpose of this study is to examine the incidence of metabolic syndrome in the Japanese general population and the relationship with the risk factors included in the diagnostic criteria. We also compared the incidence of metabolic syndrome by using the NCEP-ATP III new diagnostic criteria.

Methods

Design and Data Collection

The Research Group on Serum Lipid Level Survey 2000 in Japan organized the members of 36 institutes from various areas around Japan. The project was designed to produce representative data about serum lipid levels in the civilian Japanese population. The subjects were people receiving annual health examinations in the general community, companies, and schools, and not patient-visiting hospitals. Among the 12,839 participants we measured the waist circumference of 3,264 people aged 20 to 79 (men 1,917; women, 1,357) and examined the incidence of metabolic syndrome.

Laboratory Methods

All serum and plasma samples were obtained in the fasting state. All lipid and other analyses were conducted on venous blood samples within one week of collection at BML (Saitama, Japan). Serum cholesterol and triglyceride levels were measured by enzymatic assay. HDL-cholesterol and LDL-cholesterol were measured enzymatically using a kit from Daiichi Kagaku Co. Ltd. (Tokyo, Japan). The results of lipid analyses in the four surveys were indirectly standardized according to the criteria of the CDC Lipid Standardization Program[10]. Thus, the cholesterol levels in these five surveys appear comparable. Plasma glucose was determined enzymatically and HbA1c was determined using a kit from Kyowa Medex Co.Ltd (Tokyo, Japan). Serum insulin was determined by immunoradiometric assay (Abbott Diagnostics Division, Abbot Park, IL). Waist circumference at the umbilical level was measured in the late exhalation phase in a standing position.

Definition of Metabolic Syndrome

According to the new definition released by the Japanese Committee for the Diagnostic Criteria of Metabolic Syndrome in April 2005, we defined metabolic syndrome as the presence of 2 or more abnormalities in addition to visceral obesity (waist circumference: 85 cm or more in men, 90 cm or more in women). These three abnormalities are as follows: 1, triglycerides $\geqq 150$ mg/dL and/or HDL-cholesterol < 40 mg/dL or under treatment for this type of dyslipidemia, 2, systolic blood pressure $\geqq 130$ and/or diastolic blood pressure $\geqq 85$, or under treatment for hypertension, 3, fasting glucose $\geqq 110$ mg/dL or under treatment for diabetes. People treated for dyslipid-

Table 1. Clinical characteristics of the study population

	men (n=1,917)	women (n=1,347)
age	46.3 ± 0.30	45.7 ± 0.46
BMI	23.4 ± 0.07	22.4 ± 0.07*
waist circumference (cm)	84.1 ± 0.20	73.2 ± 0.29*
systolic blood pressure (mmHg)	125 ± 0.40	120 ± 0.49*
diastolic blood pressure (mmHg)	76.3 ± 0.27	72.3 ± 0.31*
T-cho (mg/dL)	201 ± 0.78	200 ± 0.97
TG (mg/dL)	145 ± 2.97	92.1 ± 1.64*
HDLc (mg/dL)	54.8 ± 0.33	64.6 ± 0.39*
LDLc (mg/dL)	118.0 ± 0.99	113.5 ± 1.22**
HbA1c (%)	4.86 ± 0.02	4.82 ± 0.14
fasting glucose (mg/dL)	97.8 ± 0.43	91.1 ± 0.36*
insulin (IU/mL)	6.28 ± 0.11	7.16 ± 0.21*

Data are expressed as the means ± SEM. T-cho; total cholesterol, TG; triglyceride, HDLc; HDL-cholesterol, LDLc; LDL-cholesterol. *$p <$ 0.001, **$p < 0.01$

emia were excluded, because we could not obtain data as to whether they were treated for hypercholesterolemia or hypertriglyceridemia. We also analyzed the incidence of metabolic syndrome by ATP Ⅲ criteria published in 2005[9]. We modified the criteria by using the Japanese cutoff of waist circumference. Other differences are fasting glucose ≧100 mg/dL and HDL-cholesterol < 50 mg/dL in women. Metabolic syndrome in ATP Ⅲ criteria was defined as the presence of at least 3 abnormalities among visceral obesity, hypertriglyceridemia, low HDL-cholesterolemia, hypertension, and glucose intolerance.

Data Analysis

The results are expressed as the mean value ± standard deviation, and categorical data by the incidence and relation between visceral obesity and various factors were expressed by the odds ratio and 95% confidence interval. Differences in the means were evaluated by analysis of variance (ANOVA) or analysis of covariance (ANCOVA). The relation between visceral obesity and various factors was examined using multiple, logistic regression analysis for multivariate analysis. Analysis was performed using the statistical Package for Social Sciences (SPSS Japan Inc. ver. 11.5, Tokyo, Japan). A p value of 0.05 or less was considered to indicate significant difference.

Results

Table 1 shows the characteristics of the study population. The means of total cholesterol, triglyceride, HDL-cholesterol, and fasting glucose were 200 mg/dL, 123 mg/dL, 59 mg/dL, and 95 mg/dL. These data are almost the same as the means of the total participants (201, 115, 59, 95, respectively)[6]. The means of both genders were also equivalent to the means of the total participants, indicating that this population represents all participants in this Japanese lipid survey in 2000. Although we found no difference in the mean age, total cholesterol, and HbA1c between men and women, the means of BMI, waist circumference, blood pressure, triglyceride, LDL-cholesterol, and fasting glucose were higher in men than in women, while those of HDL-cholesterol and insulin were lower in men than in women.

Table 2. Incidence of metabolic syndrome and metabolic abnormalities by Japanese diagnostic criteria

	men (%)	women (%)	all (%)
metabolic syndrome	12.1	1.7	7.8
visceral obesity	48.2	9.7	32.3
hypertriglyceridemia	31.3	11.2	23.0
low HDL-cholesterolemia	12.4	2.2	8.2
dyslipidemia	35.2	12.1	25.6
hypertension	25.4	19.5	22.9
elevated fasting glucose	14.4	7.0	11.3

Dyslipidemia is defined as hypertriglyceridemia and/or low HDL-cholesterolemia

Using the Japanese diagnostic criteria for metabolic syndrome we determined the incidence of metabolic syndrome (**Table 2**). The incidence of metabolic syndrome in all participants was 7.8%. The incidence in men and women was 12.1, 1.7%, respectively. The incidence was about 7-fold higher in men than in women, reflecting the difference in visceral obesity defined by waist circumference, 48.2% in men and 9.7% in women. The incidence of dyslipidemia, hypertension, and glucose intolerance was also higher in men than in women in this population, indicating a higher prevalence of metabolic abnormalities in men.

It is important for us to intervene from the period of visceral obesity to prevent cardiovascular disease due to these metabolic abnormalities. Therefore, we compared the incidence of visceral obesity, visceral obesity plus one metabolic abnormality, and metabolic syndrome. **Fig. 1** shows the incidence of visceral obesity, visceral obesity plus one metabolic abnormality, and metabolic syndrome. The incidence of visceral obesity plus one metabolic abnormality was about twice the incidence of metabolic syndrome both in men and women.

To compare the incidence of metabolic syndrome

by Japanese and ATP Ⅲ criteria in this population, we determined the incidence of metabolic syndrome using these criteria in each generation from age 20s to 70s in men and women as shown in **Fig. 2**. The incidence of metabolic syndrome using ATP Ⅲ criteria was about 3 times higher than that by the Japanese criteria. Using both criteria the incidence of metabolic syndrome started to rise in men in their 30s and reached a plateau after their 40s. Meanwhile, the incidence of metabolic syndrome in women started to rise after their 50s using both criteria, indicating the increased prevalence of metabolic syndrome after menopause.

We next examined whether visceral obesity contributed to metabolic abnormalities in this study population. **Fig. 3** shows the difference of lipid profiles and fasting glucose levels with or without visceral obe-

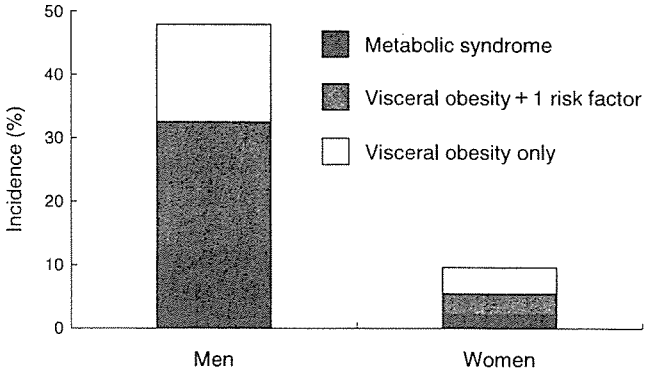


Fig. 1. Incidence of metabolic syndrome and visceral obesity in the lipid survey in 2000.

The percent incidence of metabolic syndrome, visceral obesity plus one risk factor, and visceral obesity in men and women is shown.

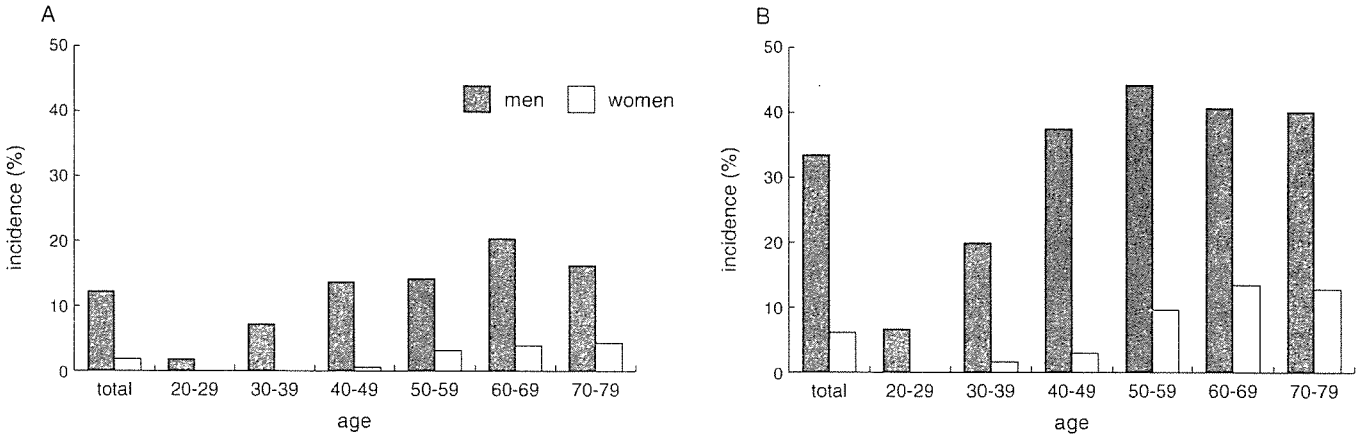


Fig. 2. Incidence of metabolic syndrome in each generation by Japanese and ATP Ⅲ criteria.

Each column shows the incidence of metabolic syndrome in each generation in men (closed column) and women (open column) by Japanese (A) and ATP Ⅲ (B) criteria. The incidence in the total population is shown on the left.

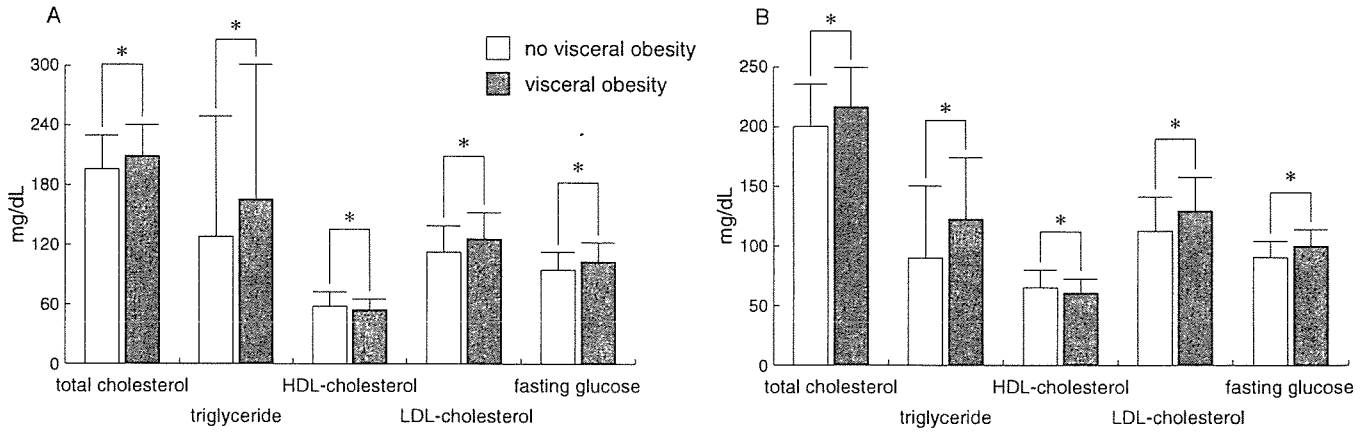


Fig. 3. Comparison of metabolic abnormalities with or without visceral obesity.

Each column shows the mean ± SD of total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, and fasting glucose with or without visceral obesity in men (A) and women (B). $^{*}p < 0.001$

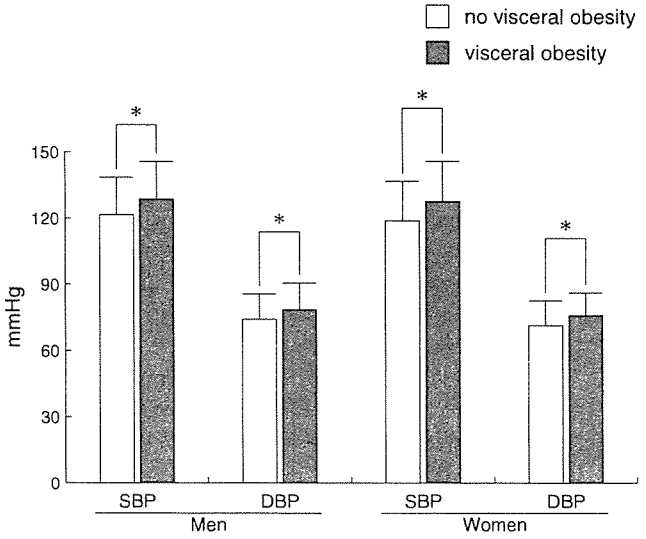


Fig. 4. Comparison of systolic and diastolic pressure with or without visceral obesity.

Each column shows the mean ± SD of systolic and diastolic blood pressure with or without visceral obesity in men and women. $^{*}p < 0.001$

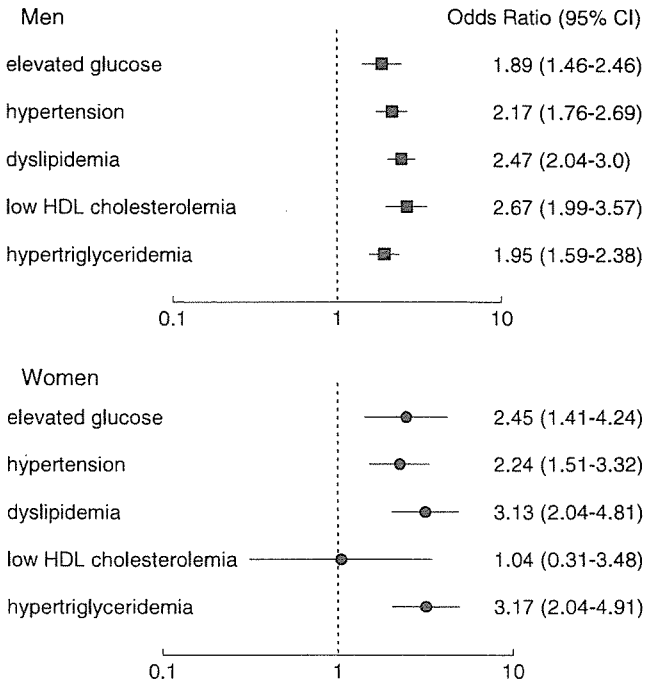


Fig. 5. Effect of visceral obesity on hypertriglyceridemia, low HDL cholesterolemia, dyslipidemia, hypertension, and glucose intolerance in men and women.

Odds ratios and 95% confidence interval are shown for each abnormality in the presence or absence of visceral obesity.

sity in this study population. The levels of total cholesterol, triglyceride, LDL-cholesterol, and fasting glucose were significantly higher, while the level of HDL-cholesterol was significantly lower in the group with visceral obesity than in the group without, indicating the contribution of visceral obesity to these metabolic abnormalities in both men and women. Systolic and diastolic blood pressure was also higher in the visceral obesity group in both genders (**Fig. 4**). We also determined the effect of visceral obesity on the development of each abnormality by calculating the odds ratios and 95% confidence interval (**Fig. 5**). Visceral obesity was significantly associated with the development of each metabolic abnormality in men and women except for low HDL-cholesterolemia in women. When we changed the cutoff of HDL-cholesterol to 50 mg/dL, visceral obesity was significantly associated with low HDL-cholesterolemia in women. The odds ratio was 2.10 and the 95% confidence interval was 1.35-3.27. Among dyslipidemia, hypertension, and glucose intolerance, visceral obesity was most associated with the development of dyslipidemia.

We also determined the age-adjusted difference of lipid profile in the presence or absence of visceral obesity in this population. Even after age adjustment we found a significant difference in total cholesterol, triglyceride, HDL-cholesterol, and LDL-cholesterol in men and in women, except for a difference in LDL-cholesterol in women (**Table 4**).

Discussion

In this study we determined the incidence of metabolic syndrome in the Japanese general population using a lipid survey performed in 2000 using new Japanese criteria to diagnose metabolic syndrome. We found that 3 times more people were diagnosed with metabolic syndrome using the new ATP Ⅲ criteria than the Japanese criteria and that visceral obesity contributed to metabolic abnormalities, such as dyslipidemia, glucose intolerance, and hypertension.

In our study the incidence of metabolic syndrome in Japanese men and women was 12.1 and 1.7%, respectively. The incidence of metabolic syndrome in our survey is lower than that from the latest National Health and Nutrition survey in 2004. In that survey the incidence of metabolic syndrome in Japanese men and women was 23.0 and 8.9%, respectively. In this national survey they used HbA1c (≧5.5) instead of FBS to diagnose glucose intolerance. This might explain the difference between the two surveys. This difference also indicates that the cutoff of FBS needs to be changed in the future. Although the mean age and the criteria used were different, Takeuchi *et al.*

Table 3. Incidence of each metabolic abnormality in the presence or absence of visceral obesity

	visceral obesity		no visceral obesity	
	men	women	men	women
hypertriglyceridemia	41.1%	25.4%	22.2%	9.7%
low HDL-cholesterolemia	17.6%	2.3%	7.4%	2.2%
dyslipidemia	45.7%	26.9%	25.4%	10.5%
hypertension	32.8%	33.1%	18.4%	18.1%
elevated fasting glucose	18.4%	14.6%	10.6%	6.2%

Dyslipidemia is defined as hypertriglyceridemia and/or low HDL-cholesterolemia

Table 4. Age-adjusted difference of lipid profile in the presence or absence of visceral obesity

		men		age-adjusted	women		age-adjusted	all		age-adjusted
		no visceral obesity	visceral obesity	*P*	no visceral obesity	visceral obesity	*P*	no visceral obesity	visceral obesity	*P*
T-cho	mean	195.6	205.9		198.8	214.2		197.3	206.9	
	number	994	923	<0.001	1217	130	0.082	2211	1053	<0.001
	SD	33.4	33.4		35.4	33.1		34.6	33.4	
TG	mean	128.7	162.0		88.9	121.7		106.8	157.0	
	number	994	923	<0.001	1217	130	<0.001	2211	1053	<0.001
	SD	119.3	138.8		60.2	51.5		93.7	131.8	
HDLc	mean	57.7	51.7		65.1	59.8		61.8	52.7	
	number	994	923	<0.001	1217	130	0.003	2211	1053	<0.001
	SD	14.2	13.9		14.5	12.5		14.8	14.0	
LDLc	mean	112.1	122.1		111.4	128.0		111.7	122.9	
	number	374	479	0.001	510	71	0.106	884	550	<0.001
	SD	26.0	30.1		29.0	28.8		27.8	30.0	

The mean, the number of samples, and SD are shown. *P* value was obtained by ANCOVA.

reported that the incidence of metabolic syndrome in men in the Tanno and Sobetsu study was 25.3%[11]. The mean age of their study population was 60.3 years, about 15 years older than that in our study population. Other studies reported a similar incidence of metabolic syndrome in Japanese. Considering that the incidence of metabolic syndrome in our population in their 60s was about 20%, the difference of the criteria used contributed to this difference. Similar to our study Urashima *et al.* reported an incidence of metabolic syndrome in Japanese men and women of 14.1% and 1.7%, respectively in central Tokyo[12]. Thus, the current incidence of metabolic syndrome in Japan would be around 15% in men and a few percent in women. In our study we found that about twice as many people with metabolic syndrome had visceral obesity and one risk factor in both men and women, indicating a potential for the incidence of metabolic syndrome to increase in the future. In our previous analysis we showed that the level of triglyceride in men dramatically increased from 1990 to 2000[6]. Therefore, we need to tackle this problem to prevent the increase in metabolic syndrome and cardiovascular disease in Japan.

In this population the incidence of metabolic syndrome in women was one seventh that in men. The incidence of visceral obesity, dyslipidemia, and glucose intolerance in women was one fifth, one third, and one half that in men, respectively. Furthermore, most of the women who satisfied this criteria were more than 50 years old, which means that few women are diagnosed with metabolic syndrome before the menopause. In Japan we adopted a cutoff of waist circumference of 90 cm for women, which is 5 cm more than that for men. This might explain why the incidence of metabolic syndrome in women was much less than in men. In contrast to the cutoff waist circumference in Japan, other criteria, such as in ATP Ⅲ,

generally have a larger cutoff in men than in women; however, our cutoff in women is based on the extensive study by Matsuzawa and his group using CT scan[13-15]. Therefore, in terms of detecting visceral obesity, 90 cm would be appropriate for Japanese women. However, we need to establish another method to select high-risk patients without visceral obesity. Our data also strongly indicate that visceral obesity using our cutoff is associated with metabolic abnormalities even after age adjustment, as shown in **Fig. 5** and **Table 4**. Therefore, we believe that visceral obesity is a useful surrogate marker for metabolic abnormalities and intervention to reduce abdominal circumference would lead to the prevention of cardiovascular disease. However, in terms of the cutoff of HDL-cholesterol, 50 mg/dL might be better than 40 mg/dL from the odds ratio in women (**Fig. 5** and Results) as in the cutoff of the ATP III criteria.

In summary we have shown that the incidence of metabolic syndrome in the Japanese general population is 7.8%, 12.1% in men and 1.7% in women. Intervention is required to prevent metabolic syndrome as well as metabolic abnormalities, such as dyslipidemia, hypertension, and glucose intolerance. The current criteria for metabolic syndrome should be assessed for the better diagnosis of women and elderly people.

Acknowledgements

This study was supported by research grants for health sciences from the Japanese Ministry of Health and a grant from the Japan Atherosclerosis Society. We thank Shizuya Yamashita (Osaka University) and Hideaki Bujo (Chiba University) for critical reading of the manuscript. We also thank Osaka Pharmaceutical Manufacturers Association for supporting our work.

References

1) Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report. Circulation, 2002; 106:3143-3421.
2) Matsuzawa Y: Therapy Insight: adipocytokines in metabolic syndrome and related cardiovascular disease. Nat Clin Pract Cardiovasc Med, 2006; 3:35-42
3) Lemieux I, Pascot A, Couillard C, Lamarche B, Tchernof A, Almeras N, Bergeron J, Gaudet D, Tremblay G, Prud'homme D, Nadeau A, and Despres JP: Hypertriglyceridemic waist: A marker of the atherogenic metabolic triad (hyperinsulinemia; hyperapolipoprotein B; small, dense LDL) in men? Circulation, 2000; 102:179-184.
4) Carr DB, Utzschneider KM, Hull RL, Kodama K, Retzlaff BM, Brunzell JD, Shofer JB, Fish BE, Knopp RH, and Kahn SE: Intra-abdominal fat is a major determinant of the National Cholesterol Education Program Adult Treatment Panel III criteria for the metabolic syndrome. Diabetes, 2004; 53:2087-2094.
5) Ford ES, Giles WH, and Dietz WH: Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. JAMA, 2002; 287:356-359.
6) Arai H, Yamamoto A, Matsuzawa Y, Saito Y, Yamada N, Oikawa S, Mabuchi H, Teramoto T, Sasaki J, Nakaya N, Itakura H, Ishikawa Y, Ouchi Y, Horibe H, and Kita T: Serum lipid survey and its recent trend in the general Japanese population in 2000. J Atheroscler Thromb, 2005; 12:98-106.
7) Yamamoto A, Horibe H, Mabuchi H, Kita T, Matsuzawa Y, Saito Y, Nakaya N, Fujioka T, Tenba H, Kawaguchi A, Nakamura H, and Goto Y: Analysis of serum lipid levels in Japanese men and women according to body mass index. Increase in risk of atherosclerosis in postmenopausal women. Research Group on Serum Lipid Survey 1990 in Japan. Atherosclerosis, 1999; 143:55-73.
8) Matsuzawa Y: Metabolic syndrome--definition and diagnostic criteria in Japan. J Atheroscler Thromb, 2005; 12:301.
9) Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC Jr., Spertus JA, and Costa F: Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation, 2005; 112:2735-2752.
10) Johnson CL, Rifkind BM, Sempos CT, Carroll MD, Bachorik PS, Briefel RR, Gordon DJ, Burt VL, Brown CD, Lippel K, et al.: Declining serum total cholesterol levels among US adults. The National Health and Nutrition Examination Surveys. JAMA, 1993; 269:3002-3008.
11) Takeuchi H, Saitoh S, Takagi S, Ohnishi H, Ohhata J, Isobe T, and Shimamoto K: Metabolic syndrome and cardiac disease in Japanese men: applicability of the concept of metabolic syndrome defined by the National Cholesterol Education Program-Adult Treatment Panel III to Japanese men--the Tanno and Sobetsu Study. Hypertens Res, 2005; 28:203-208.
12) Urashima M, Wada T, Fukumoto T, Joki M, Maeda T, Hashimoto H, and Oda S: Prevalence of metabolic syndrome in a 22,892 Japanese population and its associations with life style. JMAJ, 2005; 48:441-450.
13) Matsuzawa Y, Shimomura I, Nakamura T, Keno Y, and Tokunaga K: Pathophysiology and pathogenesis of visceral fat obesity. Diabetes Res Clin Pract, 1994; 24 Suppl: S111-116.
14) Tokunaga K, Matsuzawa Y, Ishikawa K, and Tarui S: A novel technique for the determination of body fat by computed tomography. Int J Obes, 1983; 7:437-445.
15) Yoshizumi T, Nakamura T, Yamane M, Islam AH, Menju M, Yamasaki K, Arai T, Kotani K, Funahashi T, Yamashita S, and Matsuzawa Y: Abdominal fat: standardized technique for measurement at CT. Radiology, 1999; 211:283-286.

High Frequency of a Retinoid X Receptor γ Gene Variant in Familial Combined Hyperlipidemia That Associates With Atherogenic Dyslipidemia

Atsushi Nohara, Masa-aki Kawashiri, Thierry Claudel, Mihoko Mizuno, Masayuki Tsuchida, Mutsuko Takata, Shoji Katsuda, Kenji Miwa, Akihiro Inazu, Folkert Kuipers, Junji Kobayashi, Junji Koizumi, Masakazu Yamagishi, Hiroshi Mabuchi

Objective—The genetic background of familial combined hyperlipidemia (FCHL) has not been fully clarified. Because several nuclear receptors play pivotal roles in lipid metabolism, we tested the hypothesis that genetic variants of nuclear receptors contribute to FCHL.

Methods and Results—We screened all the coding regions of the PPARα, PPARγ2, PPARδ, FXR, LXRα, and RXRγ genes in 180 hyperlipidemic patients including 60 FCHL probands. Clinical characteristics of the identified variants were evaluated in other 175 patients suspected of coronary disease. We identified PPARα Asp140Asn and Gly395Glu, PPARγ2 Pro12Ala, RXRγ Gly14Ser, and FXR −1g−>t variants. Only RXRγ Ser14 was more frequent in FCHL (15%, $P<0.05$) than in other primary hyperlipidemia (4%) and in controls (5%). Among patients suspected of coronary disease, we identified 9 RXRγ Ser14 carriers, who showed increased triglycerides (1.62±0.82 versus 1.91±0.42 [mean±SD] mmol/L, $P<0.05$), decreased HDL-cholesterol (1.32±0.41 versus 1.04±0.26, $P<0.05$), and decreased post-heparin plasma lipoprotein lipase protein levels (222±85 versus 149±38 ng/mL, $P<0.01$). In vitro, RXRγ Ser14 showed significantly stronger repression of the lipoprotein lipase promoter than RXRγ Gly14.

Conclusion—These findings suggest that RXRγ contributes to the genetic background of FCHL. (***Arterioscler Thromb Vasc Biol.* 2007;27:00-00.**)

Key Words: apolipoproteins ■ gene mutations ■ lipoprotein lipase ■ familial combined hyperlipidemia ■ nuclear receptors

Familial combined hyperlipidemia (FCHL) is the most common form of inherited hyperlipidemia. FCHL shows strong genetic susceptibility resembling an autosomal dominant disease,[1–3] but most of the underlying causal mechanisms remain to be elucidated. Lipoprotein lipase (LPL) has been implicated as one of the genes that modify the lipid phenotype in FCHL.[4,5] "Intra-individual variability" of the lipoprotein phenotype is often included as a criterion in diagnosis.[6] However, a recent prospective study of FCHL families suggests that this variability may even include normolipidemic periods in affected subjects.[7] This feature indicates that FCHL could be a "disease of regulation" rather than a genetic defect in certain peripheral components of lipid metabolism.

Nuclear receptors are transcription factors that can be activated by specific ligands. Recent studies have shown that nuclear receptors, especially retinoid X receptor (RXR) and its heterodimerization partners,[8] play important roles in maintenance of lipid homeostasis on their activation by a variety of ligands derived from dietary cholesterol and fatty acids.[9] The peroxisome proliferator-activated receptors (PPARs) family, the oxysterol sensor liver X receptor (LXR), and the bile acid sensor farnesoid X receptor (FXR) are all involved in control of plasma lipid concentrations.[10] Thus, we tested the hypothesis that variants of these nuclear receptors, ie, PPARα, PPARγ2, PPARδ, LXRα, FXR, and RXRγ, could constitute part of the genetic background of atherogenic dyslipidemia, particularly of FCHL.

Methods

Subjects

The study design consists of 2 parts. First, we screened for frequent variants in the nuclear receptor candidate genes among 180 patients with primary hyperlipidemia, including 60 unrelated patients with FCHL (clinical characteristics are presented in supplemental Table I, available online at http://atvb.ahajounals.org). Patients with familial

Original received August 28 2006; final version accepted January 6, 2007.

From the Departments of Lipidology (A.N., M.M., J. Kobayashi, H.M.) and Cardiovascular Medicine (M.K., M. Tsuchida, M. Takata, S.K., K.M., M.Y.), Graduate School of Medical Science, Kanazawa University, Japan; Center for Liver, Digestive, and Metabolic Diseases, Laboratory of Pediatrics (T.C., F.K.), University Medical Center Groningen, Groningen, the Netherlands; School of Health Sciences, Faculty of Medicine (A.I.), and Department of General Medicine (J. Koizumi), Kanazawa University Hospital, Japan.

Correspondence to Atsushi Nohara, Department of Lipidology, Graduate School of Medical Science, Kanazawa University, Takara-machi 13-1, Kanazawa 920-8641, Japan. E-mail a-nohara@med.kanazawa-u.ac.jp

Arterioscler Thromb Vasc Biol. **is available at http://www.atvbaha.org** **DOI: 10.1161/01.ATV.0000258945.76141.8a**

hypercholesterolemia and secondary hyperlipidemia were excluded. Diagnosis of FCHL was based on the fulfillment of all of the following three criteria: (1) Phenotype IIb, IIa, or IV hyperlipidemia according to the Fredrickson classification; (2) Presence of phenotype IIb, IIa, or IV hyperlipidemia in a first-degree relative and at least one family member with phenotype IIb; (3) Exclusion of familial hypercholesterolemia. Two hundred ninety-eight anonymous samples from healthy males were used as controls for frequency analysis of identified mutations. All blood samples in this study were obtained after an overnight fast.

Second, we evaluated the clinical impact of potentially relevant variants in another 175 patients who were suspected of having coronary artery disease based on any of the following reasons: ECG abnormalities, cumulative coronary risk factors, and/or chest symptoms. The group included 105 patients who had undergone coronary angiography. Patients with familial hypercholesterolemia were excluded because of their clear genetic background for hyperlipidemia. The extent and severity of atherosclerotic changes in coronary angiography were assessed by assigning scores to each of the 15 segments, according to the classification of the American Heart Association Grading Committee. The coronary stenosis index (CSI) was defined as the sum of the following scores[11]: A normal coronary angiogram was graded 0, stenosis of less than 25% was graded 1, 25% to 50% stenosis was graded 2, 50% to 75% stenosis was graded 3, and more than 75% stenosis was graded 4. CSI is a useful index for evaluating mild-moderate coronary atherosclerotic changes.

All the subjects and controls enrolled were inhabitants of the Hokuriku district of Japan. Written informed consent was obtained from each of the subjects. The study protocol was approved by the ethics committee of the Graduate School of Medical Science, Kanazawa University.

Laboratory Analyses

Total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL)-cholesterol, apolipoproteins, glucose, and thyroid hormones were measured according to standard clinical laboratory techniques. HDL-cholesterol fractions were obtained by dextran sulfate-magnesium chloride precipitation and assayed using a commercial kit (Daiichi, Tokyo, Japan).[12] Separation of lipoproteins by ultracentrifugation was performed as described by Havel et al.[13] Plasma remnant-like particle (RLP)-cholesterol was determined by immunoabsorption using the commercial RLP-C JIMRO kit.[14] Plasma cholesteryl ester transfer protein (CETP) concentrations were determined by enzyme-linked immunosorbent assay using the monoclonal antibody TP2 and a rabbit polyclonal antibody raised against recombinant human CETP.[15] For LPL assessment, blood samples were obtained 10 minutes after an intravenous injection of 30 IU heparin/kg body weight. LPL activity was measured using radiolabeled triolein emulsion after hepatic lipase (HL) inhibition by SDS as previously described.[16] LPL mass was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using specific monoclonal antibody against LPL (Daiichi Pure Chemicals Co Ltd, Tokyo, Japan).[17]

Genetic Analyses of Candidate Genes

Genomic DNA was isolated from peripheral white blood cells using standard phenol-chloroform extraction techniques. We screened all the coding regions of PPARα (NM_032644), PPARδ (NM_006238), PPARγ2 (NM_015869), LXRα (NM_005693), FXR (NM_005123), and RXRγ (NM_006917) genes with flanking exon-intron boundaries by polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) using the DCode system (Bio-Rad), which is highly accurate in detecting changes in nucleic acids.[18] The structural organization and nucleotide sequences of these genes were retrieved from the gene database of NCBI. Lists of all GC-clamped primers used in DGGE analysis are available online (supplemental Table II). Samples with a variant detected by DGGE analysis were directly sequenced on an ABI310 analyzer (Applied Biosystems). PCR-restriction-fragment-length polymorphisms analysis on the RXRγ Ser14 variant was performed with the primers 5′-AGCCGAGAGAGGCGGTAATA-3′ (forward) and 5′-TACAGGTCCACGCAGTGAAG-3′ (reverse) in patients suspected of coronary artery disease. Digestion with *AluI* resulted in a 76-bp fragment for Ser allele and a 120-bp fragment for Gly allele.

Cell Culture and Transfection Assays

Cos7 cells were grown in DMEM supplemented with 10% FCS, penicillin/streptomycin, sodium pyruvate, glutamine, and nonessential amino acids (Gibco BRL, Invitrogen). The medium was changed every 48 hours. Cos7 cells were transfected using FuGENE 6 reagent (Roche): 150 ng of the indicated LPL firefly luciferase reporter plasmid (a generous gift of Dr B. Staels), that contains the proximal 466-bp of the human LPL promoter in front of the ATG cloned into the *Hind*III site of the pGL3 plasmid, was cotransfected with or without 100 ng of the human RXRγ expressing vector (a generous gift of Dr W. Lamph). After an overnight incubation, cells were incubated with medium containing 10% FCS with or without the retinoid LGD1069, (1 μmol/L, Sigma) and luciferase activity was assayed 48 hours later using an Orion luminometer (Berthold). Transfection studies were performed at least 3 times in triplicate. Transfection efficiency was monitored by cotransfection of 150 ng of a SV40-driven β-galactosidase expression plasmid. A positive RXRE TKpGL3 construct was made by cloning 3 copies of the direct repeat AGGTCA spaced by 5 nucleotides in the TKpGL3 plasmid.

Plasmid Site-Directed Mutagenesis

Nucleotide substitution was introduced in the plasmid expressing human RXRγ using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, The Netherlands) and the primer 5′-CATGAAGTTTCCCGCAAGCTATGGAGGCTCCCCTGG C-3′ in which the nucleotide in bold indicates the mutation.

Statistical Analysis

The frequency distribution of genotypes was compared using standard χ^2 tests. Student *t* test was used for normally distributed parameters and the Kruskal–Wallis test was used for non-normally distributed parameters: triglycerides levels, LPL levels, and CSI. JMP 5.1.2 software (SAS Institute Inc) was used for statistical calculation.

Results

Identified Variants in Nuclear Receptor Genes

With PCR-DGGE analysis, we identified 4 variants with amino acid changes, ie, Asp140Asn and Gly395Glu in the PPARα gene, Pro12Ala in the PPARγ2 gene, Gly14Ser in the RXRγ gene, and 1 nucleotide substitution in a flanking

TABLE 1. Frequencies of Nuclear Receptor Genes Variants Identified in This Study

	FCHL n=60	Other Hyperlipidemia n=120	General Population n=298	*P* Value
PPARαGly395Glu				
Glu395	3 (5%)	1 (0.8%)	6 (2%)	ns
PPARαAsp140Asn				
Asn140	2 (3%)	1 (0.8%)	2 (0.6%)	ns
PPARγ2Pro12Ala				
Ala12	5 (8%)	10 (8%)	20 (7%)	ns
FXR −1g−>t				
−1g/t	19 (32%)	34 (28%)	108 (36%)	ns
−1t/t	2 (3%)	6 (5%)	27 (9%)	ns
RXRγGly14Ser				
Ser14	9 (15%)	5 (4%)	15 (5%)	0.03

TABLE 2. Clinical Characteristics of Patients With RXRγ Variant

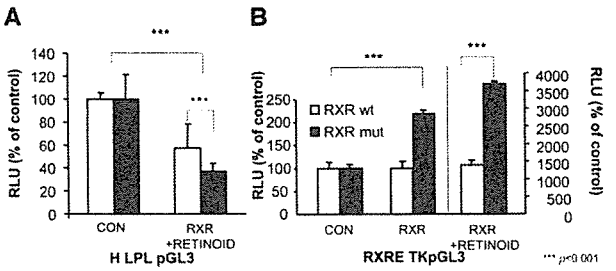
	RXRγ Gly14Ser		
	Gly/Gly	Gly/Ser	*P* Value
Number (M/F)	166 (78/88)	9 (5/4)	
Age, y	58±15	58±7	ns
BMI, kg/m^2	23.4±5	23.9±2	ns
Smoking, %	36	33	ns
Total cholesterol, mmol/L	5.98±1.4	5.96±1.55	ns
Triglycerides, mmol/L	1.62±0.82	1.91±0.42	*P*<0.05
HDL cholesterol, mmol/L	1.32±0.41	1.04±0.26	*P*<0.05
LDL cholesterol, mmol/L	3.94±1.27	4.07±1.45	ns
HDL2 cholesterol, mmol/L	0.78±0.28	0.54±0.10	*P*<0.05
HDL3 cholesterol, mmol/L	0.44±0.10	0.39±0.08	ns
ApoA-I, g/L	1.38±0.31	1.18±0.18	ns
ApoA-II, g/L	0.32±0.06	0.28±0.05	*P*<0.05
ApoB, g/L	1.31±0.38	1.35±0.31	ns
ApoC-II, g/L	0.06±0.02	0.05±0.02	ns
ApoC-III, g/L	0.11±0.05	0.10±0.03	ns
ApoE, g/L	0.06±0.02	0.05±0.01	ns
RLP cholesterol, mmol/L	0.15±0.10	0.21±0.10	*P*<0.01
CETP, mg/L	2.52±0.82	2.48±0.73	ns
Intraindividual lipoprotein phenotype variability, %	27	88	*P*<0.01
Fasting glucose, mmol/L	5.72±1.39	5.33±0.72	ns
HbA1c, %	5.6±1.0	5.8±1.0	ns
Fasting insulin, pmol/L	70.8±90.3	52.1±1.0	ns
HOMA-IR	2.28±2.1	2.19±1.7	ns
Diabetes, %	28	33	ns
HL activity, U/L	0.24±0.09	0.26±0.07	ns
LPL activiy, U/L	0.11±0.06	0.08±0.03	*P*<0.05
LPL mass, ng/mL	222±85	149±38	*P*<0.01
FT3, pmol/L	0.42±0.01	0.044±0.01	ns
FT4, pmol/L	15.2±5.15	13.3±2.57	ns
TSH, μU/mL	2.31±2.8	2.53±0.9	ns
Number (M/F)	100 (50/50)	5 (4/1)	
CSI	12.3±10	21.4±6	*P*<0.05
			mean±SD

BMI indicates body mass index; HOMA-IR, homeostasis model assessment; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid stimulating hormone.

coding region, ie, FXR −1g−>t variant. The PPARγ2 Pro12Ala polymorphism has already been well-described,[19] whereas the others represent novel variants identified in this study. In humans, variants in the RXRγ gene have been associated with elevated triglyceride levels in familial type 2 diabetes, but none of these variants showed an altered coding sequence.[20] Therefore, this is the first description of a RXRγ variant with an amino acid substitution. In the PPARα gene, the Leu162Val variant has been reported in Western countries,[21] but this variant was not identified in this study. We also identified some silent nucleotide substitutions, ie, 891C−>G (rs13306747) and 1431C−>T (rs1724155) in the PPARγ2 gene, 1233C−>T (rs9658166) in the PPARδ gene, and 1134A−>G (rs1131379) in the LXRα gene. We did not identify variants with amino acid changes in the PPARδ and LXRα genes. We further investigated the variants with amino acid substitutions and the −1g−>t FXR variant, because of the likelihood that these induced altered physiological function.

Higher Frequency of RXRγ Variant in FCHL

We evaluated the frequencies of the 5 identified polymorphisms in subjects with FCHL, subjects with other forms of primary hyperlipidemia and in the general population (Table 1). Only the RXRγ Ser14 variant was found to be significantly more frequent in FCHL patients (15%) compared with that in other forms of primary hyperlipidemia (4%) or the general population (5%).



A, Cos7 cells were cotransfected with RXRγ wild-type or the Ser14 variant and activated with retinoid in presence of the LPL promoter. B, Cos7 cells were cotransfected with RXRγ wild-type or the Ser14 variant and activated with retinoid in presence of a positive RXRE cloned in the TKpGL3 plasmid.

Atherogenic Plasma Lipids Profiles and Coronary Atherosclerosis Associated With the RXRγ Ser14 Variant

To establish the impact of the identified RXRγ variant on metabolic parameters and on coronary atherosclerosis, we evaluated anthropometric parameters and laboratory data from 175 patients suspected of coronary disease. The RXRγ Ser14 variant was identified in 9 patients, all of whom were heterozygotes. Eight of the RXRγ Ser14 carriers had hyperlipidemia, while the remaining 1 demonstrated an isolated low HDL cholesterol level. Clinical characteristics of patients with or without the RXRγ Ser14 allele are shown in Table 2. There was no difference in age or body mass index between the two groups. In their lipid profiles, RXRγ Ser14 carriers had higher TG, lower HDL cholesterol especially in the HDL2 subfraction, and lower apolipoprotein A-II levels. There was no difference in CETP protein levels between the groups. Furthermore, we found that the RLP cholesterol level was significantly higher in the RXRγ Ser14 carriers than in the wild-type. Subjects with this variant also showed significantly lower LPL activities and protein levels in post-heparin plasma. Separation of lipoproteins demonstrated that the Ser14 carriers had higher TG levels in very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) fractions, higher cholesterol levels in VLDL, and lower cholesterol levels in HDL (supplemental Table III).

Two RXRγ Ser14 carriers were diagnosed as FCHL (22%), and 2 additional carriers were suspected of FCHL with hyperlipidemic siblings without information on first-degree relatives. Among non-carriers, 22 of 166 patients were diagnosed as FCHL (13%). One hundred twenty-five patients suspected of coronary disease showed hyperlipidemia and the intraindividual variability of lipoprotein phenotype was significantly more frequent in RXRγ Ser14 carriers (7 of 8 hyperlipidemic patients; 88%) than in wild-type (32 of 117 hyperlipidemic patients; 27%, Table 2).

There was no significant difference in the thyroid hormone levels between the two groups.

Four males and 1 female were identified as RXRγ variant carriers among 105 patients who underwent coronary angiography. The carriers of RXRγ Ser14 demonstrated significantly higher CSI than those with the wild-type (Table 2).

RXRγ Variant Represses More Efficiently the LPL Promoter Activity

Because RXRγ Ser14 carriers showed significantly lower LPL activities and protein levels in post-heparin plasma, we hypothesized that activated-RXRγ downregulates LPL gene expression by a transcriptional mechanism and that RXRγ variant is more effective in repressing the LPL promoter activity. Therefore, transfection assays were performed using the LPL promoter cotransfected with either wild-type RXRγ or the variant (Figure). Interestingly, RXRγ Gly14 significantly repressed (−40%) the LPL promoter activity, whereas the RXRγ Ser14 repressed even more strongly (−60%, $P<0.001$, Figure A). Moreover, the RXRγSer14 was a more potent activator of a positive RXRE cloned in front of a TKpGL3 plasmid (note the different scales in Figure B). Taken together, our results indicate that RXRγ downregulates human LPL gene expression, at least partially by a transcriptional mechanism, and that the newly identified RXRγ variant is a more potent repressor than the wild-type in this respect, as well as a more potent transactivator of a positive RXR response element.

Gain of Function Variant of PPARα and Increased LDL-C Levels

The carriers of the PPARα variant Gly395Glu tended to have higher frequency in the FCHL population, although not statistically significant. Four subjects were identified as PPARα Glu395 carriers in the coronary artery disease-suspected group and showed significantly higher LDL-cholesterol levels (supplemental Table IV). On in vitro functional analysis, Glu395 showed a moderately but significantly increased transcriptional activity compared with wild-type PPARα (supplemental Figure I, available online at http://atvb.ahajournals.org). The previously described Leu162Val variant of the PPARα gene has been shown to give gain of function in in vitro,[24] has been associated with raised LDL-cholesterol levels.[21,22] Our results appear to be in accordance with these previous reports.

Discussion

The main findings of the present study are the following: (1) identification of novel polymorphisms in plasma lipid levels-associated nuclear receptor genes, (2) a higher frequency of the RXRγ gene variant Gly14Ser in subjects with FCHL, (3) RXRγ Ser14 variant carriers showed more atherogenic dyslipidemia associated with coronary atherosclerosis, (4) the RXRγ variant showed a stronger response to its ligand in repression of the LPL promoter than the wild-type RXRγ.

RXRs are major heterodimerization partners of nuclear receptors such as PPARs, LXRs, and FXR. Three RXR isotypes have been identified: RXRα, RXRβ, and RXRγ. Synthetic RXR ligands induce hypertriglyceridemia through decreased clearance of VLDL by LPL-dependent pathways,[23,24] except in 1 study.[25] In contrast to the embryonic lethality observed in RXRα- and RXRβ-deficient mice, RXRγ-deficient mice develop apparently normal.[26] Yet, RXRγ-deficient mice showed reduced fasting plasma TG levels and increased skeletal muscle LPL activity when fed a high fat diet.[27] The human RXRγ gene is located on chro-

mosome 1q21-q23, ie, the so-called "FCHL locus",[28] and both linkage analysis and a twin study have indicated that the RXRγ gene is linked with dyslipidemia in Chinese and German families.[29,30]

To our knowledge, there are only few data concerning the physiological roles and targets of RXRγ in humans. The RXRγ gene is mainly expressed in skeletal muscles, central nervous system, skin, intestine, and lung. In the present study, LPL protein mass and activity were significantly decreased in RXRγ variant carriers. Because LPL is mainly expressed in adipose tissues and in skeletal muscles, we assume that this is attributable to the fact that the presence of the RXRγ variant affects LPL expression in skeletal muscles. RXRγ mRNA is detectable in adipose tissue only at a low level,[31] but it has been reported that RXRγ could replace RXRα in heterodimerization with PPARγ in adipose tissue.[32] Therefore, there is a possibility that RXRγ variant expression in adipose tissue contributes to the changes in LPL.

It has been reported that RXRγ-deficient mice show a 17% increase in serum thyroid hormone (T4) and a 20% increase in thyroid-stimulating hormone (TSH) levels.[33] In the present study, thyroid hormone levels did not appear to differ sufficiently between variant carriers and non-carriers to explain the differences observed in lipid levels.

It has been shown that low LPL levels contribute to disorders associated with TG-rich lipoprotein catabolism with low HDL, especially in HDL2,[34,35] and are associated with increased risk for future coronary disease.[36] Thus, the low LPL could well contribute to the increase in TG and the decrease in HDL-cholesterol levels in subjects with the RXRγ variant.

We assessed the functional consequence of the RXRγ Ser14 variant in vitro. The activation function-1 (AF-1) domain of RXRγ is located between amino acids 1 and 103, and is required for optimal ligand-dependent transactivation of RXR response element.[37] Fourteen amino acids are located within the AF-1 domain and are conserved among humans, mice, and chickens. In a transfection assay, RXRγ Ser14 repressed LPL promoter activity more strongly than the wild-type RXRγ. In addition, the Ser14 variant was a more potent inducer of a positive RXR response element. Therefore, we speculate that the Ser14 variant induces a better recruitment and/or stabilization of RXR cofactors. Further studies will be required to understand the precise molecular mechanism(s) involved in the LPL regulation by RXRγ Ser14.

Within the so-called FCHL locus, on chromosome 1q21-q23, several genes have been reported to be associated with the FCHL phenotype[28,30,38] and with type 2 diabetes.[39] First, the thioredoxin interacting protein gene was shown to be associated with combined hyperlipidemia in mice, but no disease-causing mutation has been found in humans so far.[40,41] Currently upstream stimulatory factor 1 (USF1) is considered the most promising candidate gene of FCHL.[42] In the USF1 gene, no amino acid substitution has been identified in the coding regions, but single nucleotide polymorphisms in the 3′untranslated region and in intron 7 have been reported to be associated with FCHL, metabolic syndrome, or type 2 diabetes mellitus quite reproducibly.[43–45] However, populations did not show any such association have also been reported.[46–48] These reports emphasize the complexity of phenotypic expression in multi-factorial diseases such as FCHL. RXRγ had been reported to show an association with TG and cholesterol levels on linkage analysis,[29,30] and we identified novel RXRγ variant that associated with atherogenic dyslipidemia. However, the changes in lipid levels attributable to the RXRγ variant alone were not sufficient to cause FCHL. Thus, we suggest the RXRγ gene variant to be a strong modifier rather than a causative gene in development of the FCHL phenotype.

In conclusion, the present study suggests that a variant of RXRγ gene contributes to genetic dyslipidemia, including FCHL, based on the increased frequency of this variant in FCHL, its association with an atherogenic lipid profile, and initial functional studies.

Acknowledgments

The authors thank Sachio Yamamoto for technical assistance. Drs William W. Lamph and Bart Staels are kindly acknowledged for the generous gift of plasmids.

Sources of Funding

This work has been supported by a scientific research grant from the Ministry of Education, Science, and Culture of Japan (No.17790603) and ONO Medical Research Foundation. Thierry Claudel was supported by Grant 2002B017 from the Netherlands Heart Foundation.

Disclosures

None.

References

1. Goldstein JL, Schrott HG, Hazzard WR, Bierman EL, Motulsky AG. Hyperlipidemia in coronary heart disease. II. Genetic analysis of lipid levels in 176 families and delineation of a new inherited disorder, combined hyperlipidemia. *J Clin Invest.* 1973;52:1544–1568.
2. Rose HG, Kranz P, Weinstock M, Juliano J, Haft JI. Inheritance of combined hyperlipoproteinemia: evidence for a new lipoprotein phenotype. *Am J Med.* 1973;54:148–160.
3. Nikkila EA, Aro A. Family study of serum lipids and lipoproteins in coronary heart-disease. *Lancet.* 1973;1:954–959.
4. Hoffer MJ, Bredie SJ, Snieder H, Reymer PW, Demacker PN, Havekes LM, Boomsma DI, Stalenhoef AF, Frants RR, Kastelein JJ. Gender-related association between the -93T−>G/D9N haplotype of the lipoprotein lipase gene and elevated lipid levels in familial combined hyperlipidemia. *Atherosclerosis.* 1998;138:91–99.
5. Hoffer MJ, Bredie SJ, Boomsma DI, Reymer PW, Kastelein JJ, de Knijff P, Demacker PN, Stalenhoef AF, Havekes LM, Frants RR. The lipoprotein lipase (Asn291−>Ser) mutation is associated with elevated lipid levels in families with familial combined hyperlipidaemia. *Atherosclerosis.* 1996;119:159–167.
6. Gaddi A, Galetti C, Pauciullo P, Arca M. Familial combined hyperlipoproteinemia: experts panel position on diagnostic criteria for clinical practice. Committee of experts of the Atherosclerosis and Dysmetabolic Disorders Study Group. *Nutr Metab Cardiovasc Dis.* 1999;9:304–311.
7. Veerkamp MJ, de Graaf J, Bredie SJ, Hendriks JC, Demacker PN, Stalenhoef AF. Diagnosis of familial combined hyperlipidemia based on lipid phenotype expression in 32 families: results of a 5-year follow-up study. *Arterioscler Thromb Vasc Biol.* 2002;22:274–282.
8. Mangelsdorf DJ, Evans RM. The RXR heterodimers and orphan receptors. *Cell.* 1995;83:841–850.
9. Shulman AI, Mangelsdorf DJ. Retinoid x receptor heterodimers in the metabolic syndrome. *N Engl J Med.* 2005;353:604–615.
10. Claudel T, Staels B, Kuipers F. The Farnesoid X receptor: a molecular link between bile acid and lipid and glucose metabolism. *Arterioscler Thromb Vasc Biol.* 2005;25:2020–2030.

11. Mabuchi H, Koizumi J, Shimizu M, Takeda R. Development of coronary heart disease in familial hypercholesterolemia. *Circulation*. 1989;79: 225–232.
12. Talameh Y, Wei R, Naito H. Measurement of total HDL, HDL2, and HDL3 by dextran sulfate-MgCl2 precipitation technique in human serum. *Clin Chim Acta*. 1986;158:33–41.
13. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest*. 1955;34:1345–1353.
14. Nakajima K, Saito T, Tamura A, Suzuki M, Nakano T, Adachi M, Tanaka A, Tada N, Nakamura H, Campos E, et al. Cholesterol in remnant-like lipoproteins in human serum using monoclonal anti apo B-100 and anti apo A-I immunoaffinity mixed gels. *Clin Chim Acta*. 1993;223:53–71.
15. Kiyohara T, Kiriyama R, Zamma S, Inazu A, Koizumi J, Mabuchi H, Chichibu K. Enzyme immunoassay for cholesteryl ester transfer protein in human serum. *Clin Chim Acta*. 1998;271:109–118.
16. Baginsky ML, Brown WV. A new method for the measurement of lipoprotein lipase in postheparin plasma using sodium dodecyl sulfate for the inactivation of hepatic triglyceride lipase. *J Lipid Res*. 1979;20:548–556.
17. Kobayashi J, Hashimoto H, Fukamachi I, Tashiro J, Shirai K, Saito Y, Yoshida S. Lipoprotein lipase mass and activity in severe hypertriglyceridemia. *Clin Chim Acta*. 1993;216:113–123.
18. Grompe M. The rapid detection of unknown mutations in nucleic acids. *Nat Genet*. 1993;5:111–117.
19. Stumvoll M, Haring H. The peroxisome proliferator-activated receptor-gamma2 Pro12Ala polymorphism. *Diabetes*. 2002;51:2341–2347.
20. Wang H, Chu W, Hemphill C, Hasstedt SJ, Elbein SC. Mutation screening and association of human retinoid X receptor gamma variation with lipid levels in familial type 2 diabetes. *Mol Genet Metab*. 2002;76: 14–22.
21. Flavell DM, Pineda Torra I, Jamshidi Y, Evans D, Diamond JR, Elkeles RS, Bujac SR, Miller G, Talmud PJ, Staels B, Humphries SE. Variation in the PPARalpha gene is associated with altered function in vitro and plasma lipid concentrations in Type II diabetic subjects. *Diabetologia*. 2000;43:673–680.
22. Tai ES, Demissie S, Cupples LA, Corella D, Wilson PW, Schaefer EJ, Ordovas JM. Association between the PPARA L162V polymorphism and plasma lipid levels: the Framingham Offspring Study. *Arterioscler Thromb Vasc Biol*. 2002;22:805–810.
23. Vahlquist C, Lithell H, Michaelsson G, Selinus I, Vahlquist A, Vessby B. Plasma fat elimination tissue lipoprotein lipase activity and plasma fatty acid composition during sequential treatment with etretinate and isotretinoin. *Acta Derm Venereol*. 1987;67:139–144.
24. Davies PJ, Berry SA, Shipley GL, Eckel RH, Hennuyer N, Crombie DL, Ogilvie KM, Peinado-Onsurbe J, Fievet C, Leibowitz MD, Heyman RA, Auwerx J. Metabolic effects of rexinoids: tissue-specific regulation of lipoprotein lipase activity. *Mol Pharmacol*. 2001;59:170–176.
25. Mukherjee R, Davies PJ, Crombie DL, Bischoff ED, Cesario RM, Jow L, Hamann LG, Boehm MF, Mondon CE, Nadzan AM, Paterniti JR Jr, Heyman RA. Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. *Nature*. 1997;386:407–410.
26. Krezel W, Dupe V, Mark M, Dierich A, Kastner P, Chambon P. RXR gamma null mice are apparently normal and compound RXR alpha +/-/RXR beta -/-/RXR gamma -/- mutant mice are viable. *Proc Natl Acad Sci U S A*. 1996;93:9010–9014.
27. Haugen BR, Jensen DR, Sharma V, Pulawa LK, Hays WR, Krezel W, Chambon P, Eckel RH. Retinoid X receptor gamma-deficient mice have increased skeletal muscle lipoprotein lipase activity and less weight gain when fed a high-fat diet. *Endocrinology*. 2004;145:3679–3685.
28. Pajukanta P, Nuotio I, Terwilliger JD, Porkka KV, Ylitalo K, Pihlajamaki J, Suomalainen AJ, Syvanen AC, Lehtimaki T, Viikari JS, Laakso M, Taskinen MR, Ehnholm C, Peltonen L. Linkage of familial combined hyperlipidaemia to chromosome 1q21–q23. *Nat Genet*. 1998;18: 369–373.
29. Knoblauch H, Busjahn A, Muller-Myhsok B, Faulhaber HD, Schuster H, Uhlmann R, Luft FC. Peroxisome proliferator-activated receptor gamma gene locus is related to body mass index and lipid values in healthy nonobese subjects. *Arterioscler Thromb Vasc Biol*. 1999;19:2940–2944.
30. Pei W, Baron H, Muller-Myhsok B, Knoblauch H, Al-Yahyaee SA, Hui R, Wu X, Liu L, Busjahn A, Luft FC, Schuster H. Support for linkage of familial combined hyperlipidemia to chromosome 1q21–q23 in Chinese and German families. *Clin Genet*. 2000;57:29–34.
31. Kamei Y, Kawada T, Kazuki R, Sugimoto E. Retinoic acid receptor gamma 2 gene expression is up-regulated by retinoic acid in 3T3-L1 preadipocytes. *Biochem J*. 1993;293(Pt 3):807–812.
32. Metzger D, Imai T, Jiang M, Takukawa R, Desvergne B, Wahli W, Chambon P. Functional role of RXRs and PPARgamma in mature adipocytes. *Prostaglandins Leukot Essent Fatty Acids*. 2005;73:51–58.
33. Brown NS, Smart A, Sharma V, Brinkmeier ML, Greenlee L, Camper SA, Jensen DR, Eckel RH, Krezel W, Chambon P, Haugen BR. Thyroid hormone resistance and increased metabolic rate in the RXR-gamma-deficient mouse. *J Clin Invest*. 2000;106:73–79.
34. Patsch JR, Prasad S, Gotto AM Jr, Patsch W. High density lipoprotein2. Relationship of the plasma levels of this lipoprotein species to its composition, to the magnitude of postprandial lipemia, and to the activities of lipoprotein lipase and hepatic lipase. *J Clin Invest*. 1987;80:341–347.
35. Blades B, Vega GL, Grundy SM. Activities of lipoprotein lipase and hepatic triglyceride lipase in postheparin plasma of patients with low concentrations of HDL cholesterol. *Arterioscler Thromb*. 1993;13: 1227–1235.
36. Rip J, Nierman MC, Wareham NJ, Luben R, Bingham SA, Day NE, van Miert JN, Hutten BA, Kastelein JJ, Kuivenhoven JA, Khaw KT, Boekholdt SM. Serum lipoprotein lipase concentration and risk for future coronary artery disease: the EPIC-Norfolk prospective population study. *Arterioscler Thromb Vasc Biol*. 2006;26:637–642.
37. Dowhan DH, Muscat GE. Characterization of the AB (AF-1) region in the muscle-specific retinoid X receptor-gamma: evidence that the AF-1 region functions in a cell-specific manner. *Nucleic Acids Res*. 1996;24:264–271.
38. Coon H, Myers RH, Borecki IB, Arnett DK, Hunt SC, Province MA, Djousse L, Leppert MF. Replication of linkage of familial combined hyperlipidemia to chromosome 1q with additional heterogeneous effect of apolipoprotein A-I/C-III/A-IV locus. The NHLBI Family Heart Study. *Arterioscler Thromb Vasc Biol*. 2000;20:2275–2280.
39. Elbein SC, Hoffman MD, Teng K, Leppert MF, Hasstedt SJ. A genome-wide search for type 2 diabetes susceptibility genes in Utah Caucasians. *Diabetes*. 1999;48:1175–1182.
40. van der Vleuten GM, Hijmans A, Kluijtmans LA, Blom HJ, Stalenhoef AF, de Graaf J. Thioredoxin interacting protein in Dutch families with familial combined hyperlipidemia. *Am J Med Genet A*. 2004;130:73–75.
41. Bodnar JS, Chatterjee A, Castellani LW, Ross DA, Ohmen J, Cavalcoli J, Wu C, Dains KM, Catanese J, Chu M, Sheth SS, Charugundla K, Demant P, West DB, de Jong P, Lusis AJ. Positional cloning of the combined hyperlipidemia gene Hyplip1. *Nat Genet*. 2002;30:110–116.
42. Pajukanta P, Lilja HE, Sinsheimer JS, Cantor RM, Lusis AJ, Gentile M, Duan XJ, Soro-Paavonen A, Naukkarinen J, Saarela J, Laakso M, Ehnholm C, Taskinen MR, Peltonen L. Familial combined hyperlipidemia is associated with upstream transcription factor 1 (USF1). *Nat Genet*. 2004;36:371–376.
43. Coon H, Xin Y, Hopkins PN, Cawthon RM, Hasstedt SJ, Hunt SC. Upstream stimulatory factor 1 associated with familial combined hyperlipidemia, LDL cholesterol, and triglycerides. *Hum Genet*. 2005;117: 444–451.
44. Huertas-Vazquez A, Aguilar-Salinas C, Lusis AJ, Cantor RM, Canizales-Quinteros S, Lee JC, Mariana-Nunez L, Riba-Ramirez RM, Jokiaho A, Tusie-Luna T, Pajukanta P. Familial combined hyperlipidemia in Mexicans: association with upstream transcription factor 1 and linkage on chromosome 16q24.1. *Arterioscler Thromb Vasc Biol*. 2005;25: 1985–1991.
45. Putt W, Palmen J, Nicaud V, Tregouet DA, Tahri-Daizadeh N, Flavell DM, Humphries SE, Talmud PJ. Variation in USF1 shows haplotype effects, gene : gene and gene : environment associations with glucose and lipid parameters in the European Atherosclerosis Research Study II. *Hum Mol Genet*. 2004;13:1587–1597.
46. Ng MC, Miyake K, So WY, Poon EW, Lam VK, Li JK, Cox NJ, Bell GI, Chan JC. The linkage and association of the gene encoding upstream stimulatory factor 1 with type 2 diabetes and metabolic syndrome in the Chinese population. *Diabetologia*. 2005;48:2018–2024.
47. Gibson F, Hercberg S, Froguel P. Common polymorphisms in the USF1 gene are not associated with type 2 diabetes in French Caucasians. *Diabetes*. 2005;54:3040–3042.
48. Zeggini E, Damcott CM, Hanson RL, Karim MA, Rayner NW, Groves CJ, Baier LJ, Hale TC, Hattersley AT, Hitman GA, Hunt SE, Knowler WC, Mitchell BD, Ng MC, O'Connell JR, Pollin TI, Vaxillaire M, Walker M, Wang X, Whittaker P, Xiang K, Jia W, Chan JC, Froguel P, Deloukas P, Shuldiner AR, Elbein SC, McCarthy MI. Variation within the gene encoding the upstream stimulatory factor 1 does not influence susceptibility to type 2 diabetes in samples from populations with replicated evidence of linkage to chromosome 1q. *Diabetes*. 2006;55:2541–2548.

Cell surface-expressed moesin-like HDL/apoA-I binding protein promotes cholesterol efflux from human macrophages

Akifumi Matsuyama,* Naohiko Sakai,† Hisatoyo Hiraoka,† Ken-ichi Hirano,† and Shizuya Yamashita†,[1]

Medical Center for Translational Research,* Osaka University Hospital, 2-15 Yamada-oka, Suita; and Department of Cardiology,† Osaka University Graduate School of Medicine, B5, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan

Abstract **HDL and its major component, apolipoprotein A-I (apoA-I), play a central role in reverse cholesterol transport. We recently reported the involvement of a glycosylphosphatidylinositol anchor (GPI anchor) in the binding of HDL and apoA-I on human macrophages, and purified an 80 kDa HDL/apoA-I binding protein. In the present study, we characterized the GPI-anchored HDL/apoA-I binding protein from macrophages. The HDL/apoA-I binding protein was purified from macrophages and digested with endopeptidase, and the resultant fragments were sequenced. Cholesterol efflux, flow cytometry, immunoblotting, and immunohistochemical analyses were performed to characterize the HDL/apoA-I binding protein. Two parts of seven amino acid sequences completely matched those of moesin. Flow cytometry, immunoblotting, and immunohistochemistry using anti-moesin antibody showed that the HDL/apoA-I binding protein was N-glycosylated and expressed on the cell surface. It was termed *moesin-like protein.* Treatment of macrophages with anti-moesin antibody blocked the binding of HDL/apoA-I and suppressed cholesterol efflux. The moesin-like protein was exclusively expressed on macrophages and was upregulated by cholesterol loading and cell differentiation. Our results indicate that the moesin-like HDL/apoA-I binding protein is specifically expressed on the surface of human macrophages and promotes cholesterol efflux from macrophages.—** Matsuyama, A, N. Sakai, H. Hiraoka, K-i. Hirano, and S. Yamashita. **Cell surface-expressed moesin-like HDL/apoA-I binding protein promotes cholesterol efflux from human macrophages. *J. Lipid Res.* 2006. 47: 78–86.**

Supplementary key words apolipoprotein A-I • glycosylphosphatidylinositol-anchored protein • moesin-like protein • atherosclerosis

The risk of atherosclerosis is inversely correlated with plasma concentration of HDL-cholesterol (1). The most important mechanism through which HDL exerts its protective role against atherosclerosis is the removal of excess cholesterol from peripheral cells, especially from lipid-laden macrophages, and the transport of this excess cholesterol to the liver, a process called reverse cholesterol transport (2). There are at least two mechanisms in the initial step of this system (3). The first is a passive aqueous diffusion mechanism, by which cholesterol desorbes from the plasma membrane pool to extracellular phospholipid-containing acceptor particles (e.g., HDL) via a concentration gradient between the membrane and acceptors. The second mechanism involves the interaction between HDL/apolipoprotein A-I (apoA-I) and specific binding sites on the cell surface, which induces an intracellular signal leading to the translocation of cholesterol from intracellular sites to the plasma membrane and subsequent transport of cholesterol to extracellular lipid-poor acceptors (4).

A specific apo HDL/apoA-I binding to various cells has been shown by a number of investigators. Several candidate HDL/apoA-I binding proteins have been identified, such as scavenger receptor class B type I and ATP binding cassette transporter A1 (5–9). Cdc42Hs, a member of the Rho GTPase family, may also be implicated in the transport and efflux of cholesterol (10).

Recently, we succeeded in purifying a phosphatidylinositol-specific phospholipase C (PI-PLC)-sensitive 80 kDa protein that is involved in HDL/apoA-I binding and cholesterol efflux from human monocyte-derived macrophages (11). In this follow-up study, we characterized the HDL/apoA-I binding protein and demonstrated that it is a moesin-like protein expressed on the cell surface.

Manuscript received 21 September 2004 and in revised form 26 September 2005 and in re-revised form 25 October 2005.

Published, JLR Papers in Press, October 26, 2005.
DOI 10.1194/jlr.M500425-JLR200

Abbreviations: AcLDL, acetylated LDL; apoA-I, apolipoprotein A-I; GPI, glycosylphosphatidylinositol; IEF, isoelectric focusing; MIF, mean intensity of fluorescence; PI-PLC, phosphatidylinositol-specific phospholipase C.

[1]To whom correspondence should be addressed.
e-mail: shizu@imed2.med.osaka-u.ac.jp

Anti-moesin antibody decreased the binding and subsequent cholesterol efflux of HDL/apoA-I from macrophages, suggesting that the moesin-like protein is involved in the efflux of cholesterol from human macrophages.

MATERIALS AND METHODS

Amino acid sequencing

The purified 80 kDa apoA-I/HDL binding protein (11) was applied for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and extracted directly from the gel by an in-gel lysil-endopeptidase digestion method. The digested fragments were fractionated by high-performance liquid chromatography, and seven peaks were individually collected. They were subjected to amino acid sequencing in a Perkin-Elmer sequencer, and two amino acid sequences were identified.

Isolation of human monocyte-derived macrophages

Mononuclear cells were isolated from the buffy coats of blood collected from healthy volunteers using density gradient centrifugation with Lymphoprep (Nycomed; Oslo, Norway) (12) and cultured as described previously (11). After a 6 day culture, macrophages were loaded with cholesterol by incubation with 50 μg/ml of acetylated LDL (AcLDL), and then used for the experiments.

Cell culture

HepG2 and HEK293 cells, human fibroblasts, and THP-1 cells were obtained from American Type Cell Collection. HepG2 and HEK293 cells and human fibroblasts were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS). THP-1 cells were cultured in RPMI 1640 supplemented with 10% FCS and were transformed to macrophages by incubation for 24 h with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Nacalai Tesque; Kyoto, Japan).

Lipoprotein isolation, modification, and labeling

HDL3 was isolated from human plasma by ultracentrifugation at a density of 1.125–1.210 g/ml (13). Protein concentration was measured following the procedure of Lowry et al. (14). ApoA-I was purchased from Sigma (St. Louis, MO). HDL3 and apoA-I were labeled with fluorescein isothiocyanate (FITC) (Molecular Probes; Eugene, OR) as reported previously (11, 15). DiI-AcLDL was purchased from Molecular Probes. DiI-oxidized LDL was from Biomedical Technologies, Inc. (Stoughton, MA).

Immunoblotting analysis

Plasma membranes were prepared from human monocyte-derived macrophages, HepG2 cells, HEK293 cells, and human fibroblasts as described previously (11), and were applied for immunoblotting analysis with anti-moesin antibody (TK88, kind gift from S. Tsukita, Kyoto University Graduate School of Medicine). For two-dimensional immunoblotting analysis, the membrane proteins were separated using isoelectric focusing (IEF) gels (Amersham Pharmacia Biotech; Buckinghamshire, UK) according to the protocol supplied by the manufacturer and 4–20% polyacrylamide gradient gels (Daiichi Pure Chemicals; Tokyo, Japan), transferred onto nitrocellulose membranes, and blotted with anti-moesin antibody (TK88). After incubation with peroxidase-conjugated anti-rabbit IgG antibody, the blots were visualized with an ECL kit (Amersham Pharmacia Biotech).

PI-PLC treatment

To determine the effect of PI-PLC (Sigma), the macrophages were incubated at 37°C with 1 U/ml of PI-PLC for 1 h and applied for experiments.

Enzymatic deglycosylation

Concentrated PI-PLC-treated media (10 μl) were incubated for 24 h at 37°C with or without 50 units of N-glycosidase F (Sigma) in potassium buffer (200 mM K_3PO_4, 20 mM EDTA, pH 7.2) as indicated by the manufacturer, followed by the addition of 1 vol of 2× Laemmli sample buffer, and applied for immunoblotting analysis.

Flow cytometry

Cell surface expression of moesin-like protein was confirmed by flow cytometric analysis using anti-moesin antibody (TK88 and TK89, a kind gift from S. Tsukita). Competition analyses with anti-moesin antibody or control IgG were performed by incubating cells for 1 h at 4°C with FITC-labeled HDL3 or apoA-I. All fluorescence measurements were performed by flow cytometry using a FACScan (Becton-Dickinson; Mountain View, CA). Cell-bound fluorescence intensity was expressed as the mean intensity of fluorescence (MIF).

Immunohistochemistry

For immunohistochemical analysis to detect moesin, cultured human macrophages were incubated in a 2-well glass chamber slide (Falcon) and fixed for 30 min with 4% paraformaldehyde. The cells were blocked with 5% normal horse serum and incubated with anti-moesin antibodies (TK88 and TK89) in PBS containing 3% BSA for 1 h under detergent-free conditions, followed by sequential incubation with FITC-labeled anti-rabbit IgG antibody. The treated cells were observed using confocal fluorescence microscopy (Floview FV1000; Olympus, Tokyo).

Cell surface biotinylation

The cholesterol-laden macrophages and fibroblasts were washed with ice-cold phosphate buffer (10 mM, pH 7.4). They were subsequently treated with 1 ml of 0.8 mM NHS-biotin (water-soluble biotin; Pierce, Rockford, IL) in phosphate buffer for 15 min on ice, and washed three times with 0.192 M glycine-25 mM Tris (pH 8.3) to quench the reaction. The cells were lysed in MES buffer containing a cocktail of protease inhibitors (0.5 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A; Roche Applied Science), and membrane fractions were obtained from the materials as described previously (11). The membrane pellets were dissolved and applied for immunoprecipitation using anti-moesin antibody and control IgG. Immunoprecipitates were applied for SDS-PAGE and visualized with streptavidin-horseradish peroxidase (HRP) and the ECL kit.

Measurement of cellular cholesterol efflux from macrophages

Cellular cholesterol efflux via HDL3 or apoA-I was determined as described previously (11, 16). The cells were incubated with 20 μg/ml of HDL3 or 5 μg/ml of apoA-I diluted in RPMI with 5 mg/ml of BSA with 100 μg/ml of anti-moesin antibody or control IgG (Transduction Laboratories; San Jose, CA). After incubation for the indicated time, radioactivities in both the medium and the cells were measured separately. Fractional cholesterol efflux was calculated as the amount of radioactivity in the medium divided by the total radioactivity in each well and expressed as a percentage. The cellular cholesterol contents were

measured using a cholesterol-measuring kit (Kyowa Medics, Tokyo) according to the instructions provided by the manufacturer.

RESULTS

Sequencing of the 80 kDa HDL/apoA-I binding protein of macrophages

A single band of the 80 kDa apoA-I/HDL binding protein (5 pmol) was obtained from human macrophages derived from 25 healthy volunteers (10 l of whole blood) as reported previously (11), and applied for lysil-endopeptidase digestion followed by fractionation using HPLC. Seven peaks were individually collected and subjected to amino acid sequencing in a Perkin-Elmer sequencer. Two of them were successfully sequenced. The sequences of the peptides (DQWEERIQVWH and TANDEMHAEN) were the same as the internal sequences of human moesin (amino acid 164–174 and 540–549, respectively) (17).

Designation of the 80 kDa HDL/apoA-I binding protein as moesin-like protein

Previous studies reported that moesin is localized in the sub-membranous cytoskeleton, filopodia, and other membranous protrusions (17), but to our knowledge, cell surface expression of moesin has not been reported previously. To determine whether the HDL/apoA-I binding protein is identical to moesin, we first performed immunoblotting analyses using anti-moesin antibody (**Fig. 1**). The 80 kDa moesin-like protein was recovered in the conditioned media after PI-PLC treatment, whereas no immunoreactive mass was observed without PI-PLC treatment (Fig. 1Aa). It was noted that the molecular weight of the moesin-like protein was about 2 kDa larger than that located in the cell membrane. To confirm the loss of the moesin-like protein from the membrane after treatment with PI-PLC, membrane fractions of macrophages with or without PI-PLC treatment were applied for immunoblotting with anti-moesin antibody (Fig. 1Ab). The larger bands of two moesin-like proteins were reduced after PI-PLC treatment. These results suggest that the protein is a glycosylphosphatidylinositol (GPI)-anchored type and not identical to moesin.

To confirm that the 80 kDa HDL/apoA-I binding protein is not identical to moesin, two-dimensional immunoblotting was performed (Fig. 1B). The membrane fraction of human macrophages and the conditioned media after PI-PLC treatment were applied for two-dimensional immunoblotting with anti-moesin antibody. A massive amount of moesin at pH 6.0 (asterisk), as predicted by the amino acid content of moesin, and a small amount of protein with isoelectric points of pH 7.2 to 7.6 (arrowheads) were observed in membrane fraction proteins. The conditioned media with PI-PLC treatment showed moesin-like protein (arrowheads) but not moesin.

To further confirm that the 80 kDa HDL/apoA-I binding protein was not identical to moesin, enzymatic deglycosylation with N-glycosidase F was performed. After deglycosylation with the enzyme, the molecular weight of the 80 kDa HDL/apoA-I binding protein was shifted down compared with that of the nondigested protein (Fig. 1Ca), indicating that the protein was N-glycosylated. Next, the change in isoelectric point of the protein after deglycosylation was examined. If the protein was N-linked, charged glucans would be detached after deglycosylation and the isoelectric point would change. After N-glycosidase F treatment (Fig. 1Cb), the isoelectric point of the protein (pH 7.2 to 7.6) changed to pH 6.0. This result reinforced the notion that the protein was N-glycosylated. Because moesin is not expressed on the cell surface, as reported previously (17), the 80 kDa HDL/apoA-I binding protein was confirmed to be different from moesin and thus designated as moesin-like protein.

Expression of moesin-like protein on the cell surface of human macrophages

To determine whether the moesin-like protein was expressed on the cell surface of human macrophages, flow cytometry was performed using anti-moesin antibodies under detergent-free conditions. As shown in Fig. 2Aa, moesin-like protein was detected on the surface of macrophages by anti-moesin antibodies (TK88 and TK89). To confirm the loss of moesin-like protein from the cell surface, flow cytometric analysis was performed after PI-PLC treatment (Fig. 2Ab). PI-PLC treatment shifted the distribution to the left, indicating that the moesin-like protein was GPI-anchored and expressed on the cell surface.

To observe the distribution of the cell surface-expressed moesin-like protein, confocal microscopic analysis was performed using anti-moesin antibody (TK88) under detergent-free conditions (**Fig. 2B**). *En face* sections (*xy*) images were taken off the macrophage from the top to the bottom, and computer-reconstructed vertical section (*xz* and *yz*) images taken through the full thickness of the macrophage were obtained. Moesin-like immunoreactivities were observed only on the cell surface of the macrophages. No fluorescence signal was observed using control rabbit IgG (data not shown).

To further confirm that human monocyte-derived macrophages express moesin-like protein on their cell surface, cell surface biotinylation analysis was performed (Fig. 2C). After a monolayer of macrophages was treated with NHS-biotin (water-soluble biotin), the membrane fraction of the cells was collected and immunoprecipitated with anti-moesin antibody (TK88). The immunoprecipitates were applied for SDS-PAGE, transferred onto nitrocellulose membrane, and then visualized with avidin-HRP. As indicated by the arrow (Fig. 2C), the 80 kDa protein was labeled with NHS-biotin, suggesting that the moesin-like protein was expressed on the cell surface of macrophages. However, the biotinylated protein was not visualized on the fibroblasts, indicating that the expression of moesin-like protein is specific for macrophages.

Effect of anti-moesin antibody on HDL/apoA-I binding and HDL/apoA-I-mediated cholesterol efflux from macrophages

To determine whether moesin-like protein is an HDL/apoA-I binding protein, competition studies with anti-moesin