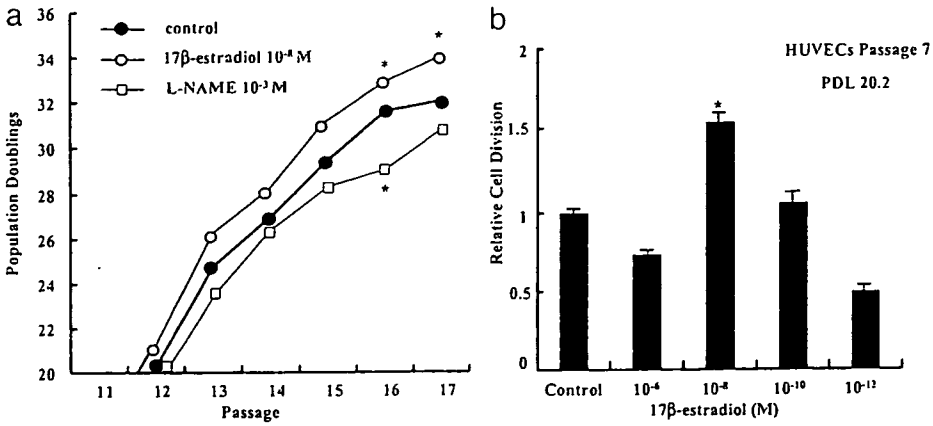

Fig. 4. Effects of E2 on endothelial cell proliferation. (*a*) The effects of E2 (10^{-8} M) and L-NAME (1 mM) on population doublings in each passage of HUVECs. The treatment time with E2 or L-NAME was 24 h. *, $P < 0.05$ vs. control. (*b*) The effects of different concentrations of E2 on relative cell division of HUVECs. Cells were used in PDL 20.2 at passage 7. *, $P < 0.05$: cell division vs. control.

Telomerase counteracts the shortening of telomeres and contains a catalytic subunit, the hTERT (4, 5). The introduction of hTERT into human cells extends both their lifespan and their telomeres to lengths typical of those of young cells (5, 6). The regulation of hTERT involves both transcriptional and posttranscriptional mechanisms. Transcriptional regulation is believed to be the main regulatory mechanism in cancer cells (24). Telomerase activity can be posttranscriptionally regulated by kinases such as protein kinase C (PKC), extracellular signal-regulated kinase 1/2 (ERK1/2), and Akt [Akt/PKB (protein kinase B)] in endothelial cells (8–10). ROS formation leads to an increase in Src-family kinase activation and a reduction of Akt expression in aging endothelial cells. It is speculated that phosphorylation by Akt keeps hTERT in an active status in the nucleus, whereas increasing the activation of Src-family kinases induces the nuclear export of hTERT, thereby reducing the ability to lengthen telomeres and protect from aging. Along with the enhanced ROS formation, we found that a decrease in telomerase activity preceded the onset of replicative senescence. Thus, ROS such as the superoxide radical and H_2O_2, which are formed during aerobic metabolism, are generally considered to be important regulators of the aging processes, and their production may be mainly due to the actions of NADPH oxidase and the mitochondria (9, 10, 24–26). In the present study, we showed that DETA-NO, an NO donor, and eNOS transfection activate hTERT and increase scavenging of ROS. L-NAME inhibited the effect of eNOS transfection. These results mean that telomerase activity was likely regulated by NO bioavailability. Our data indicated that eNOS transfection has comparable effects to hTERT transfection on both cellular aging and telomerase activity. In addition, these findings might also indicate that endothelial cell aging is linked to the balance between ROS formation and NO bioavailability, which in turn affects telomerase activity.

eNOS transfection has an antiatherosclerotic effect even in cases of advanced atherosclerosis, and the administration of L-arginine with the gene transfer of eNOS enhances the effect of eNOS transfection (27, 28). We showed that the coadministration of antioxidants with L-arginine and L-citrulline produces an enhanced antiatherosclerotic response in advanced atherosclerosis (29). L-arginine seems to increase the production of NO, whereas antioxidants most likely protect the newly formed NO against destruction by ROS. Recent evidence indicates that the bulk of intracellular endothelial L-arginine may not be available for NO production, because intracellular L-arginine for eNOS may be limited by uptake into plasmalemmal caveolae (30). The pathway by which L-citrulline is recycled to L-arginine is localized to the caveolae and it may be the main source of available L-arginine (21, 22, 29, 31). L-citrulline is converted to L-arginine by mammalian cells, including endothelial cells. This recycling pathway might, therefore, play an important role in sustaining the production of NO in endothelial cells by providing available L-arginine, especially in advanced atherosclerosis or diabetes mellitus, when plasma L-arginine levels are depleted.

Physiological concentrations of E2 activate telomerase activity and decrease the number of SA-β-gal-stained cells through the

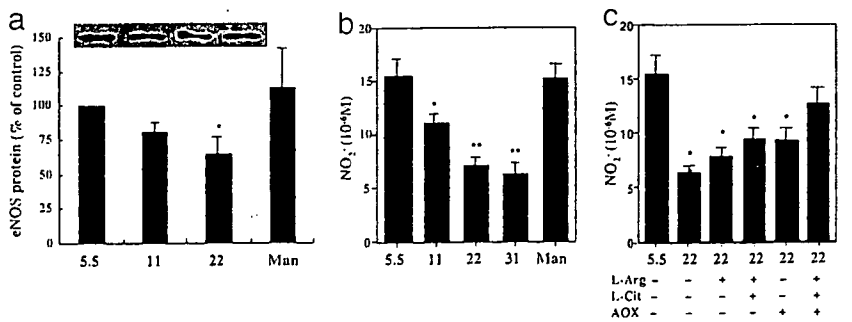

Fig. 5. Influence of high glucose on eNOS expression and nitrite production. (*a*) The effect of exposure to different concentrations of glucose on the level of eNOS protein expression in HUVECs. Mannitol (Man) was given as an osmolarity control. Cells were kept under different glucose conditions for 72 h. *, $P < 0.05$ vs. normal (5.5 mM) glucose. (*b*) The effect of exposure to different concentrations of glucose on nitrite levels in culture medium of HUVECs. *, $P < 0.05$; **, $P < 0.01$ vs. normal glucose. (*c*) The effects of L-arginine (L-arg, 1 mM), L-citrulline (L-cit, 300 μM), and antioxidants (AOX, 100 μM vitamin E plus 100 μM vitamin C) alone or in combination on nitrite levels in culture medium in HUVECs, which were reduced by 22 mM glucose. *, $P < 0.05$; **, $P < 0.01$ vs. normal glucose.

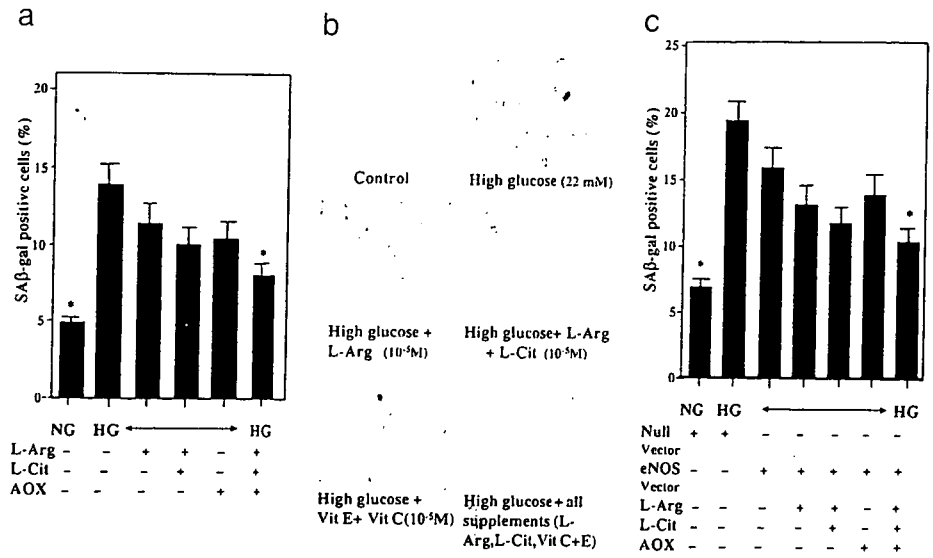

Fig. 6. Influence of high glucose for 72 h on cellular senescence of HUVECs. (*a*) The effects of L-arginine (L-arg, 1 mM), L-citrulline (L-cit, 300 μM), and antioxidants (AOX, 100 μM vitamin E plus 100 μM vitamin C) on the increase in β-gal-positive stained cells when exposed to high (22 mM) glucose. *, $P < 0.05$ vs. high glucose without any treatment. (*b*) Representative photographs showing cellular senescence by staining cells with SA-β- gal. (*c*) Modulation by transfection with eNOS of the effects of L-arginine, L-citrulline, and antioxidants on the increase in SA-β-gal-positive-stained cells when exposed to high glucose. Null vector is control vector of eNOS Vector. *, $P < 0.05$ vs. high glucose without any treatment. NG, normal glucose; HG, high glucose (22 mM).

estrogen receptor and NO-dependent mechanisms. E2 treatment also stimulated the proliferation of HUVECs through the estrogen receptor and NO-dependent mechanisms. We reported the possibility that such effects of estrogen were mediated by the direct effect on eNOS and the scavenging effect on ROS-producing enzymes such as NADPH oxidase, especially the p22phox subunit (32, 33). It is, therefore, proposed that estrogen exerts its effect on endothelial cell senescence by increasing NO bioavailability, which may then reduce ROS generation and subsequently prevent the nuclear export of TERT.

Atherosclerosis is an inflammatory disease characterized by endothelial dysfunction, impairment of NO production (1, 12 13), and oxidative stress (11), which can lead not only to cell membrane injury but also to the destruction of NO. Diabetic macroangiopathy occurs under almost the same conditions, with increased levels of superoxide from NADPH oxidase and impairment of NO production (34, 35). In the present study, high-glucose-induced endothelial dysfunction, oxidative stress, and cellular senescence were reversed with the administration of L-arginine, L-citrulline, and antioxidants. A lack of GTP cyclohydrolase I, which is the rate-limiting enzyme of tetrahydrobiopterin (BH4) synthesis, a cofactor of eNOS, also reduces NO production (36). We speculate that not only BH4 but also L-arginine, L-citrulline, and antioxidants are important in diabetic macroangiopathy. Although NO is known to be involved in reducing both oxidative stress and the progression of atherosclerosis, the present study also assessed the consequence of the NO-mediated delay of cellular senescence on the progression of atherosclerosis. The aforesaid notwithstanding, the local expression (bioavailability) of NO remains an important factor in the maintenance of normal tissue function. We also cannot exclude the possibility that other factors than NO is involved in the progressive cellular senescence in diabetes.

Taken together, the present data provide evidence demonstrating an NO-dependent mechanism in the delay of endothelial cell senescence. Consequently, the antiatherosclerotic action of NO is particularly profound under conditions of aging, estrogen depletion, and diabetes mellitus. NO could, therefore, scavenge the age-associated increase in ROS and thereby reduce the coronary risk factor-induced increase in ROS. Moreover, our data indicate that NO may also prevent endothelial cell senescence, possibly by interfering with the redox balance of endothelial cells.

Methods

Materials. We used 17β-estradiol (Sigma, St. Louis, MO), D-glucose, D-mannitol (Wako, Osaka, Japan), Takara One Step RNA PCR Kit (Takara, Kyoto, Japan), and eNOS monoclonal antibody (BD Biosciences, San Jose, CA). ICI 182780 was kindly provided by Zeneca Pharmaceuticals. L-NAME and DETA-NO were obtained from Sigma-Aldrich (St. Louis, MO). Monoclonal antibodies to β-galactosidase (Chemicon International, Lexington, NY) were used (7, 37).

Cell Culture. HUVECs were purchased from Clonetics (San Diego, CA) and cultured in low-glucose EBM-2 supplemented with 10% calf serum, EBM-2 including EGM-2 SingleQuots (Clonetics), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2, 95% air. These cells were positive for the endothelial cell-specific von Willebrand factor and angiotensin-1-converting enzyme activity. The cells were seeded into six-well plates, and subconfluent cell monolayers were studied within six to eight passages. Before starting the experimental procedures, the medium was removed and replaced with phenol red-free low-glucose D-MEM supplemented with 1% calf serum, 0.06% glutamine, and 1% penicillin–streptomycin. In some experiments accompanying eNOS transfection, HEK 293 cells were treated instead of HUVECs because of relative ease of transfection. The rate of PDL was calculated at each passage until growth arrest based on the following formula: $PDL = (\log_{10}Y - \log_{10}X)/\log_{10}2$ (Y indicates the number of cells counted at the end of the passage; X is the number of cells seeded). Cumulative population doubling was calculated as the sum of all of the changes in population doubling.

Measurement of Nitrite. The methods for measuring nitrite (NO_2^-) production by HUVECs have been previously described by our laboratory. In brief, samples of the incubation culture medium were recovered after centrifugation to remove any precipitated materials. The nitrite concentrations of the supernatants were determined by high-performance liquid chromatography (ENO10; EICOM,

Kyoto, Japan) as described (29, 38). The incubated medium was not completely free of nitrite; therefore, an aliquot of medium was assayed by the same process as the medium obtained from the cultured cells. We used the nitrite value obtained in the medium alone as a blank, and it was subtracted from all of the samples.

Flow Cytometric Analysis of ROS Generation. The determination of intracellular oxidant production in HUVECs was based on the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (H_2DCFDA) resulting in the formation of the fluorescent compound 2′,7′-dichlorofluorescein (DCF) (Molecular Probes, Eugene, OR) (29, 38). Carboxy-H2DCFDA freely diffuses across the cell membrane, is diacylated, and incorporates into hydrophobic lipid regions of the cell. HUVECs were incubated at 37°C for 30 min in PBS in which 2 μl of 5 mM H2DCFDA was added. After incubation, the dye was aspirated and the cells were trypsinized and washed once by centrifugation at 1,670 × *g* for 5 min to remove trypsin and extracellular H_2DCFDA. HUVECs were resuspended in PBS and transferred into 5 ml of polystyrene round-bottom tubes with cell-strainer caps (Becton Dickinson, Franklin Lakes, NJ). They were protected from light and kept cold until ready for analysis on a FACS caliber flow cytometer (Becton Dickinson) set at ≈515- to 545-nm excitation. The emission filters used a 530/30-nm bandpass.

SA-β-Gal. HUVECs and tissues were fixed and stained for SA-β-gal activity as described (37). In brief, the cells were fixed for 10 min in 2% formaldehyde, 0.2% glutaraldehyde in PBS, and incubated for 12 h at 37°C without CO_2 with fresh β-gal staining solution: 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$, pH 6.0. The cells were counterstained with 4′6-diamidinophenylindole (DAPI; 0.2 mg/ml in 10 mM NaCl) for 10 min to count the total cell number. The percentage of SA-β-gal-positive cells was determined by counting the number of blue cells within a sample of 1,000 cells. We also used the Flow Cytometric Analysis.

Human Telomerase Activity. The quantitative determination of telomerase activity was performed according to the manufacturer's protocol for the TeloTAGGG telomerase PCR ELISAPLUS Kit (Roche Diagnostics, Mannheim, Germany) based on the telomeric repeat application protocol (TRAP) assay. To measure telomerase activity, 2 μg of protein was used in the PCR.

Western Blot Analysis of eNOS. Total protein was extracted from the endothelial cells and then analyzed by Western blotting (38, 39). Briefly, the protein concentration was determined with a Dc protein assay kit (Bio-Rad Laboratories, Hercules, CA). Samples of cell homogenate (5 μg) were subjected to electrophoresis on polyacrylamide gels, and proteins were transferred to poly(vinylidine difluoride) filter membranes. To reduce any nonspecific binding, the membrane was preincubated for 30 min at room temperature in TTBS (150 mM NaCl/10 mM Tris, pH 8.0/0.05% Tween 20) containing 5% nonfat milk. The membrane was then incubated overnight with the primary antibody at 3:10,000 dilutions in PBS (0.075 μg/ml). The membrane was incubated with the horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution) for 60 min at room temperature. The blots were washed in TTBS and subsequently visualized with the aid of a SuperSignal West Dura Trial Kit (Pierce Biotechnology, Rockford, IL), exposed to x-ray film, and analyzed by the NIH Image Software program produced by Wayne Rasband (National Institutes of Health, Bethesda, MD). Loading of equal amounts of protein was confirmed by Coomassie brilliant blue and Amido black staining of protein in each lane of the same blot.

Construction of an Adenovirus Vector Carrying eNOS and Transfer into Cultured ECs. Recombinant adenoviruses containing eNOS cDNA were constructed by using the ADENO-QUEST Kit (Quantum, Quebec City, Canada) (27). Briefly, bovine eNOS cDNA (provided by T. Michel, Harvard University, Cambridge, MA) was cloned into the AdBM5pAG vector. The resulting plasmid was then cotransfected with viral DNA into HEK 293 cells. We incubated 5×10^5 HUVECs in a six-well plate for 24 h, then incubated cells with adenoviruses at a multiplicity of infection of 20 for 24 h. For all of the studies, the viral titers were adjusted to 2×10^9 pfu/ml. Adenoviruses carrying an *Escherichia coli lacZ* gene encoding a nucleus-localized variant of β-gal (Ad. β-gal) or no cDNA (Ad.-null) were also used. We also used eNOS/pcDNA3.1(+) and Qiagen Effectane Transferase Reagent.

Statistics. All data are given as means ± SEM from at least three independent experiments. Comparisons between the two groups were made based on the nonparametric Mann–Whitney *U* test. Statistical significance was evaluated with repeated-measures ANOVA by using a least-significant difference (LSD) post hoc test or ANOVA for multiple comparisons (SPSS Software 11.0). Differences were considered to be significant at a value of $P < 0.05$.

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Original Article

Polymorphisms of Apolipoprotein E and Methylenetetrahydrofolate Reductase in the Japanese Population

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Aim: The aim of this study is to analyze the effect of apolipoprotein E (apo E) and methylenetetrahydrofolate reductase (*MTHFR*) gene polymorphisms on serum lipid and homocysteine levels in the general Japanese population.
Methods: We analyzed the polymorphisms in individuals randomly selected from among participants of Serum Lipid Survey 2000.
Results: The frequency of the $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$ alleles of *APOE* was 4.2, 85.3, and 10.5%, respectively. Individuals with the genotype $\varepsilon 4/\varepsilon 4$ had the highest total and low-density lipoprotein (LDL) cholesterol levels, while those with $\varepsilon 2/\varepsilon 2$ had the lowest. Individuals with the $\varepsilon 2/\varepsilon 2$ and $\varepsilon 2/\varepsilon 4$ genotypes had higher remnant-like particles (RLP)-cholesterol levels than those with $\varepsilon 2\varepsilon 3$, $\varepsilon 3\varepsilon 3$, and $\varepsilon 3\varepsilon 4$. There was a trend for individuals with the $\varepsilon 2/\varepsilon 4$ and $\varepsilon 2/\varepsilon 2$ genotypes to have higher triglyceride levels, although the difference was not significant. The presence of the T allele in a *MTHFR* polymorphism (C667T) was associated with higher homocysteine levels, which is more prominent in men than in women.
Conclusion: Thus in our large-scale analysis we have shown that RLP-cholesterol is better associated with *APOE* genotype than triglyceride and the effect of the T allele on *MTHFR* polymorphism (C667T) homocysteine levels is more prominent in men than in women among Japanese.

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Key words; Hyperlipidemia, Polymorphism, Apolipoprotein E, *MTHFR*, Homocysteine

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Introduction

Apolipoprotein E (apo E) is an important structural constituent of serum chylomicrons, very low-density lipoproteins, and high-density lipoproteins (HDL) and plays a critical role in lipoprotein metablism, where it can facilitate the clearance of remnant lipoprotein and cellular efflux of cholesterol[1]. Apo E has three polymorphisms, $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$, which affect lipoprotein metabolism and atherosclerosis[2]. The $\varepsilon 4$ allele is associated with higher low-density lipoprotein (LDL) cholesterol levels than the other alleles and with a higher incidence of coronary heart disease[3]. Apo E4 is also shown to be involved in the development of Alzheimer's disease[4], while homozygosity for apo E2 is associated with the development of type III hyperlipidemia[5].

We also studied the *MTHFR* gene because its polymorphisms affect serum homocysteine levels and homocysteine is also associated with cardiovascular disease and Alzheimer's disease[6-9]. An elevated homocysteine level is associated with coronary heart disease and the C677T polymorphism in the *MTHFR* gene results in reduced *MTHFR* enzyme activity and reduced methylation of homocysteine to methioneine resulting in mild hyperhomocysteinemia[10]. Although several studies have examined the incidence of *APOE* and *MTHFR* polymorphisms[8, 11], these has been no large-scale study to determine the incidence of *APOE* and *MTHFR* polymorphisms and their association with lipoprotein profiles and homocysteine levels in the general Japanese population. In 2000, we conducted a lipid survey in the Japanese population, 12,839 people all over the country. In this survey, we examined *APOE* and *MTHFR* gene polymorphisms to determine the incidence of each and its relationship with lipid profiles and homocysteine levels in the Japanese.

Methods

Design and Data Collection

This work is part of Serum Lipid Level Survey 2000 from various parts of Japan. The Ethics committee, Graduate School and Faculty of Medicine, Kyoto University approved the study protocol and all subjects provided written informed consent for participation in the gene analysis. The handling of DNA samples followed the guidelines from the Ministry of Health, Labor, and Welfare. In Serum Lipid Survey 2000, a total of 12,839 subjects were recruited at 36 hospitals across the country. The subjects in the present study were participants in the survey at 9 hospitals from whom informed content for genotyping was sought. Of the 12,839 subjects, 2,267 (17.7%) with no lipid-altering medication were randomly selected for the present study. Among the 2,267 participants, we examined serum homocysteine levels and *MTHFR* gene polymorphisms in 505 participants.

Laboratory Methods

All serum and blood 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 TG levels were measured by enzymatic assay. HDL-cholesterol and LDL-cholesterol levels were measured enzymatically with 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[12]. The serum homocysteine level was assayed by high performance liquid chromatography with fluorescent detection as described by Ubbink *et al.*[13]. DNA was extracted with a QIAamp DNA blood kit (Qiagen, Hilden, Germany).

Detection of gene Mutations by Invader® Assay

We used the Invader® assay to screen for mutations of the *APOE* and *MTHFR* genes, as previously described. In brief, the probe/Invader®/MgCl2 mixture was prepared by combining 3 μL of primary probe/Invader® mix and 5 μL of 22.5 mM MgCl2 per reaction. The primary probes/Invader® mixture contained 3.5 μmol/L wild primary probe, 3.5 μmol/L mutant primary probe, 0.35 μmol/L Invader® oligonucleotide, and 10 mmol/L MOPS. Eight microliters of primary probe/Invader®/MgCl2 mixture was added per well of a 96-well plate. Seven microliters of 5 fmol/L synthetic target oligonucleotides, 10 μg/mL yeast tRNA (no target blank), and genomic DNA (15 ng/μL) were added, and denatured by incubation at 95℃ for 10 min. After 15 μL of mineral oil (Sigma, St. Louis, MO) was overlayed into all reaction wells, the plate was incubated isothermally at 63℃ for 4 h in a DNA thermalcycler (PTC-200; MJ Research, Watertown, MA) and then kept at 4℃ until fluorescence were measured. The intensity of the fluorescence was measured with a fluorescence microtiter plate reader (Cytoflour 4000; Applied Biosystems) with excitation at 485 nm/20 nm (Wavelength/Bandwidth) and emission at 530 nm/25 nm for FAM; and excitation at 560 nm/20 nm and emission at 620 nm/40 nm for RED. The genotyping was analyzed by calculating the ratio of net counts with wild primary probe to net counts with mutant primary probe. The probes used in this study were designed and synthesized by Third Wave Technologies, Inc (Madison, WI).

Data Analyses

Differences in means were evaluated with an analysis of variance. The analysis was performed with the statistical Package for Social Sciences (SPSS Japan Inc. ver. 11.5, Tokyo, Japan).

Results

We investigated the frequency and phenotypic association of *APOE* gene polymorphisms of 2,267 subjects. We found that the SNPs were in Hardy-Weinberg equilibrium. As previously described, the mean age, total cholesterol, TG, HDL-cholesterol, and LDL-cholesterol levels in this population were similar to the levels for all 12,839 patients in Serum Lipid Survey 2000[14]. We also found that the medians of total, LDL-, and HDL-cholesterol levels did not differ appreciably from the means, thereby excluding gross right-hand tailing of the distribution (data not shown). These data indicate that the participants in the gene analysis are representative of the general Japanese population.

The genotype and allelic frequency of *APOE* polymorphisms are presented in **Table 1**. The frequency of the $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$ alleles was 4.2, 85.3, and 10.5%, respectively. As in other studies, the genotypes $\varepsilon 2\varepsilon 2$, $\varepsilon 2\varepsilon 4$, and $\varepsilon 4\varepsilon 4$ were quite rare. High frequencies of the $\varepsilon 3$ allele are also found in Chinese, but the frequency is lower in Caucasians[15].

Table 1. Genotype and allele frequency of *APOE* gene in Japanese.

genotype	*n*	%	alleles	*n*	%
$\varepsilon 2/\varepsilon 2$	9	0.4	$\varepsilon 2$	192	4.2
$\varepsilon 2/\varepsilon 3$	155	6.8	$\varepsilon 3$	3,868	85.3
$\varepsilon 2/\varepsilon 4$	19	0.8	$\varepsilon 4$	474	10.5
$\varepsilon 3/\varepsilon 3$	1,653	72.9			
$\varepsilon 3/\varepsilon 4$	407	18.0			
$\varepsilon 4/\varepsilon 4$	24	1.1			

We next examined the association of the *APOE* genotype and lipid profiles in these participants. As shown in **Table 2**, all the lipid parameters and blood glucose differed significantly among these genotypes by ANOVA. Total cholesterol, triglyceride, HDL-cholesterol, LDL-cholesterol, and RLP-cholesterol levels were different among the groups. The *p* values are shown in the right column. According to the post-hoc analysis, the total cholesterol level was significantly lower for genotype $\varepsilon 2\varepsilon 2$ than $\varepsilon 4\varepsilon 4$ and genotype $\varepsilon 2\varepsilon 3$ than $\varepsilon 3\varepsilon 3$, $\varepsilon 2\varepsilon 4$, or $\varepsilon 4\varepsilon 4$. The HDL-cholesterol level was significantly higher for $\varepsilon 2\varepsilon 3$ than $\varepsilon 2\varepsilon 4$. The LDL-cholesterol level was significantly lower for genotypes $\varepsilon 2\varepsilon 2$ and $\varepsilon 2\varepsilon 3$ than for $\varepsilon 3\varepsilon 3$, $\varepsilon 3\varepsilon 4$, and $\varepsilon 4\varepsilon 4$. The RLP-cholesterol level was significantly higher for $\varepsilon 2\varepsilon 2$ than $\varepsilon 2\varepsilon 3$, $\varepsilon 3\varepsilon 3$, $\varepsilon 3\varepsilon 4$, or $\varepsilon 4\varepsilon 4$ and for genotype $\varepsilon 2\varepsilon 4$ than $\varepsilon 2\varepsilon 3$, $\varepsilon 3\varepsilon 3$, or $\varepsilon 3\varepsilon 4$, although there was no significant difference in triglyceride levels according to the post-hoc analysis. Blood glucose or age did not differ significantly among the groups.

We next examined the association of the *MTHFR* C667T polymorphism with serum homocysteine levels in 505 samples randomly selected from 2,267 samples. As shown in **Table 3**, the incidence of the CC, CT, and TT genotypes was 33.9, 46.1, and 20.0%, respectively. The TT genotype was significantly associated with higher homocysteine levels in men and women, and statistical significance was found between CC and TT and between CT and TT by a post-hoc analysis. However, the difference was more prominent in men.

Discussion

There, we have shown in a large-scale study, the

Table 2. Mean of serum lipid levels and blood glucose in each genotype of *APOE* in Japanese.

	$\varepsilon 2/\varepsilon 2$	$\varepsilon 2/\varepsilon 3$	$\varepsilon 2/\varepsilon 4$	$\varepsilon 3/\varepsilon 3$	$\varepsilon 3/\varepsilon 4$	$\varepsilon 4/\varepsilon 4$	total	*p* value
	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	mean ± SEM	
T-cho	165.0 ± 23.8	189.7 ± 3.00	202.9 ± 12.6	201.8 ± 0.92	206.8 ± 1.95	223.3 ± 9.18	202.1 ± 0.81	<0.0001
TG	171.4 ± 52.8	118.8 ± 8.55	189.0 ± 53.3	117.0 ± 2.41	128.0 ± 5.16	127.9 ± 18.9	119.8 ± 2.13	0.023
HDL-c	51.2 ± 9.51	63.6 ± 1.92	53.0 ± 3.07	59.8 ± 0.41	58.0 ± 0.82	61.9 ± 3.48	59.7 ± 0.36	0.007
LDL-c	70.5 ± 5.63	101.9 ± 2.75	117.2 ± 8.07	118.5 ± 0.91	120.5 ± 1.93	131.5 ± 7.97	117.7 ± 0.79	<0.0001
RLP-c	22.9 ± 1.15	4.4 ± 0.37	12.5 ± 7.59	4.7 ± 0.17	5.2 ± 0.33	4.1 ± 0.58	4.8 ± 0.15	<0.0001
FBS	121.3 ± 19.5	104.7 ± 3.27	110.6 ± 9.37	103.9 ± 0.94	103.3 ± 2.17	88.6 ± 2.54	103.9 ± 0.83	0.461
age	52.8 ± 10.1	49.5 ± 2.11	50.8 ± 53.2	46.7 ± 0.69	47.4 ± 1.30	43.2 ± 4.61	47.1 ± 0.58	0.659

T-cho: total cholesterol (mg/dL), TG: triglyceride (mg/dL), HDL-c: HDL-cholesterol (mg/dL), LDL-c: LDL-cholesterol (mg/dL), RLP-c: remnant-like particles cholesterol (mg/dL), FBS: fasting blood sugar (mg/dL), SEM: standard error of the mean

Table 3. Genotype frequency of the *MTHFR* gene and its association with serum homocysteine levels in Japanese.

total					
genotype	*n*	%	mean	SEM	
CC	171	33.9	10.9	0.3	$p<0.001$
CT	233	46.1	11.6	0.24	
TT	101	20.0	15.7	1.23	
total	505	100	12.2	0.29	
male					
genotype	*n*	%	mean	SEM	
CC	92	33.6	10.7	0.36	$p<0.001$
CT	132	48.2	12.9	0.35	
TT	50	18.2	19.8	2.41	
total	274	100	13.4	0.52	
female					
genotype	*n*	%	mean	SEM	
CC	79	34.2	10.2	0.43	$p=0.005$
CT	101	43.7	10.1	0.27	
TT	51	22.1	11.9	0.57	
total	231	100	10.5	0.23	

SEM: standard error of the mean

frequency of the *APOE* genotype in the Japanese and its association with serum lipid levels. Frequencies of *APOE* genotypes are highly heterogenous among various populations. Epidemiological data indicate that the frequency of the $\varepsilon 3$ allele is higher in Japanese and Chinese than in Caucasians, while the frequency of the $\varepsilon 4$ allele is lower in Asians than Caucasians[3, 16]. Our data indicate that the frequency of the $\varepsilon 3$ allele is quite consistent with previous reports in Japanese[8, 11, 16, 17], and is slightly higher than that of Icelandic and Hungarian populations and much higher than that in the Finnish population[15].

Our study confirmed that the $\varepsilon 4$ allele is associated with higher, and the $\varepsilon 2$ allele is associated with lower, LDL cholesterol levels. Although there was a trend for individuals with the genotypes $\varepsilon 2/\varepsilon 4$ and $\varepsilon 2/\varepsilon 2$ to have higher triglyceride levels, it was not statistically significant by a post-hoc analysis, probably because triglyceride levels are highly variable. However, individuals with $\varepsilon 2/\varepsilon 4$ and $\varepsilon 2/\varepsilon 2$ had significantly higher RLP-cholesterol levels than did those with the other genotypes, indicating that RLP-cholesterol might be better correlated with *APOE* genotype. Although in this study we could not compare the body mass index of $\varepsilon 2/\varepsilon 2$ homozygotes, it would be intriguing to know whether individuals with the $\varepsilon 2/\varepsilon 4$ and $\varepsilon 2/\varepsilon 2$ genotypes have metabolic abnormalities, such as abdominal obesity and insulin resistance, because they have higher triglyceride, RLP-cholesterol, and blood glucose levels.

Elevated levels of homocysteine have been considered a risk for cardiovascular disease. Our study is consistent with other studies that show higher homocysteine levels in people with the TT genotype. However, the relationship between the C677T *MTHFR* polymorphism and cardiovascular disease is still controversial. Because our study population is made up of healthy volunteers, a prospective study is necessary to determine which genotype is associated with cardiovascular risk.

In summary, we have provided the largest database of gene polymorphisms related to lipid metabolism and homocysteine in the general Japanese population. A prospective study is necessary to determine the contribution of these gene polymorphisms to cardiovascular risk in Japanese.

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Original Article

Prevalence of Metabolic Syndrome in the General Japanese Population in 2000

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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.

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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-

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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 ≧85 cm in men and ≧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 ≧150 mg/dL and/or HDL-cholesterol <40 mg/dL or under treatment for this type of dyslipidemia, 2, systolic blood pressure ≧130 and/or diastolic blood pressure ≧85, or under treatment for hypertension, 3, fasting glucose ≧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-

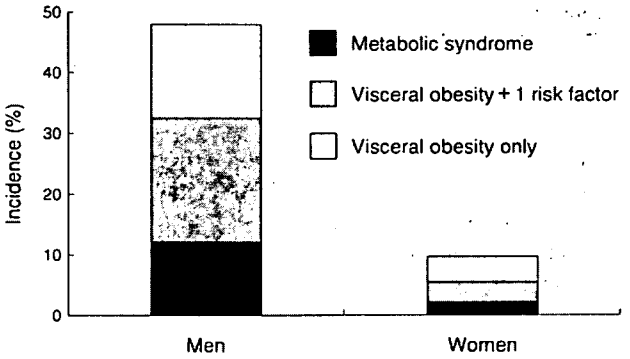

Fig. 1. Incidence of metabolic syndrome and visceral obesity in the lipid survey in 2000.

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

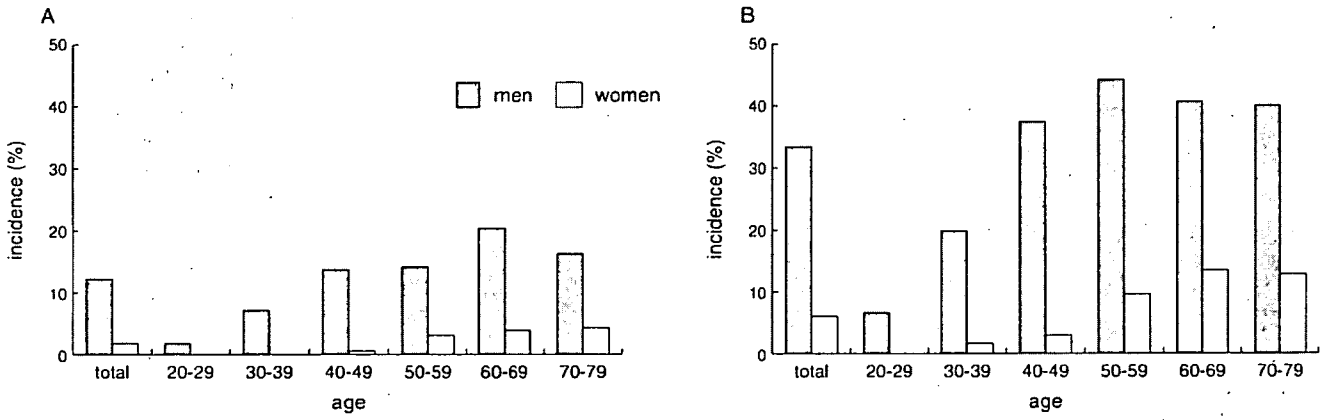

Fig. 2. Incidence of metabolic syndrome in each generation by Japanese and ATP III criteria.

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.

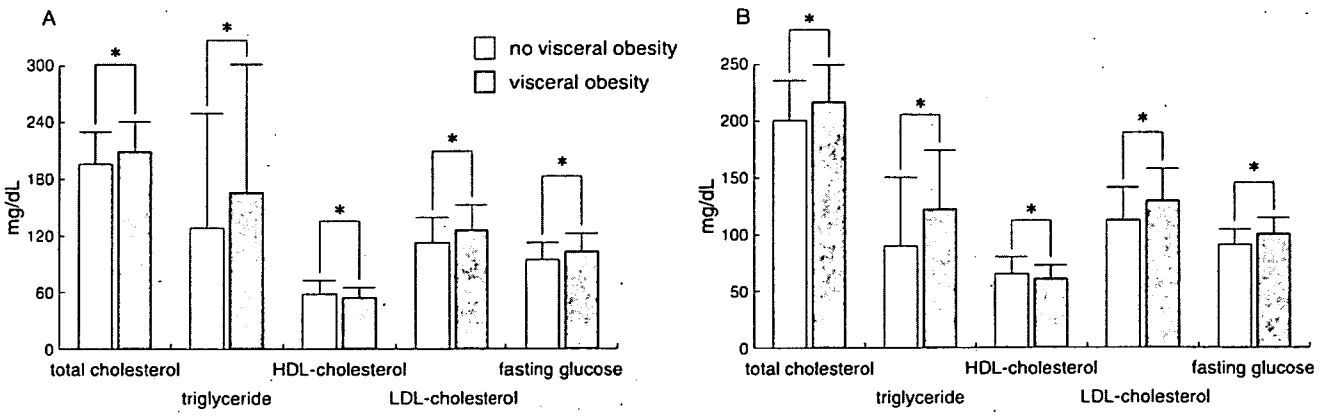

Fig. 3. Comparison of metabolic abnormalities with or without visceral obesity.

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). $^*p < 0.001$

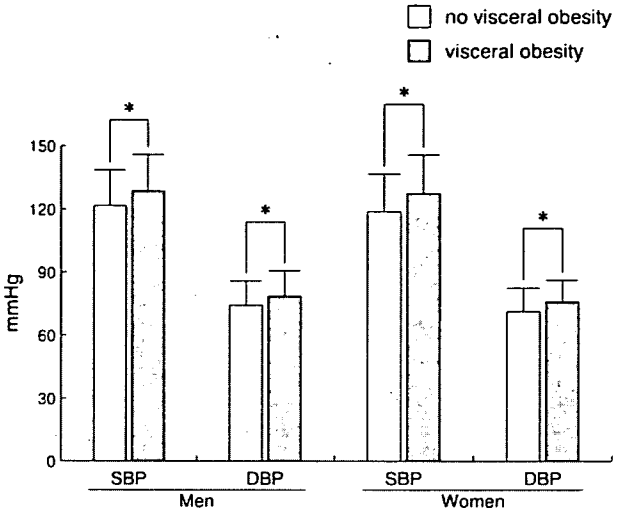

Fig. 4. Comparison of systolic and diastolic pressure with or without visceral obesity.

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

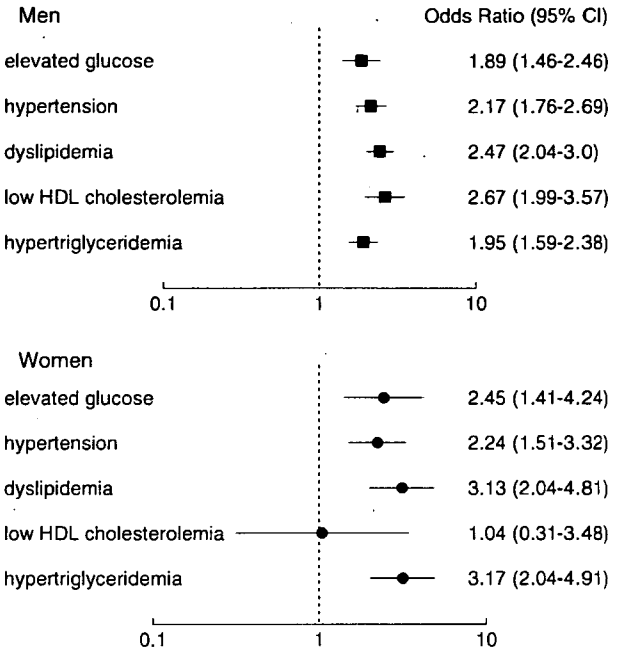

Fig. 5. Effect of visceral obesity on hypertriglyceridemia, low HDL cholesterolemia, dyslipidemia, hypertension, and glucose intolerance in men and women.

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 ($\geqq 5.5$) 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.

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Original Articles

Serum Lipid Survey and Its Recent Trend in the General Japanese Population in 2000

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To determine the recent serum lipid levels and other serum variables in the general Japanese population and trends in their changes over the past 40 years, a nationwide survey of serum lipid levels was conducted in 36 institutes from various districts around Japan in 2000. The total number of subjects was 12,839, aged 4 through 99 years. The mean total cholesterol level was 201 mg/dl; 202 mg/dl in men and 200 mg/dl in women. The mean HDL-cholesterol level was 59 mg/dl; 55 mg/dl in men and 65 mg/dl in women. The mean LDL-cholesterol level was 118 mg/dl; 121 mg/dl in men and 115 mg/dl in women. The mean triglyceride level was 118 mg/dl; 136 mg/dl in men and 92 mg/dl in women. The total cholesterol level slightly increased by 5 mg/dl in 10 years. Although the triglyceride level in women did not change, the triglyceride level in men increased over 10 years, especially in the 30s through 70s age bracket, indicating a possible increase in metabolic syndromes in the future. The present results will become the standard serum lipid level data for the Japanese people, and succeeding 10-year surveys will clarify the trends of lipid levels in this country. *J Atheroscler Thromb, 2005; 12: 98–106.*

Key words: Hyperlipidemia, Cholesterol, Triglyceride, Life style, Coronary heart disease

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Introduction

It has been well established that hyperlipidemia is a major risk factor for coronary heart disease (CHD) (1, 2). Numerous studies have shown that the reduction of serum lipid levels by dietary or drug treatment results in a

decrease in both the incidence of and the mortality from CHD (3–7). In contrast to the sharp decline in both serum cholesterol and mortality from CHD in the United States and Western Europe, remarkable increases in serum cholesterol levels as well as CHD mortality have been anticipated in the Asian-Pacific region, due to industrialization and modernization. Epidemiological studies indicate that changes in lifestyle have a great influence on the risk factors for atherosclerosis (8–10). Among the Asian-Pacific countries, Japan was found to have lower than average serum cholesterol values and a correspondingly lower incidence of CHD. Japanese in the 1960s consumed very little dietary fat, and both cholesterol levels and the incidence of CHD were low. Japanese who migrated to Hawaii and California, however, showed higher levels of serum cholesterol and a higher incidence of CHD than people in Japan (10). Thus, dietary habits and other environmental factors rather than genetic background affect serum cholesterol levels and CHD mortality in the population. In the United States, during the period of 1900 through 1991, many changes in nutritional lifestyle and medical therapeutic factors may have decreased serum total cholesterol levels among American adults (11). On the other hand, Japanese have adopted mixed dietary habits of a traditionally low fat and low cholesterol diet and a western style diet of relatively high fat and high cholesterol. As a result the serum cholesterol levels in the Japanese populations were found to have gradually increased over the 30 years from 1960 to 1990 according to 10-year-interval national surveys of serum cholesterol levels conducted in 1960, 1970, 1980, and 1990 (12–14). This study is the fifth survey and reveals the most recent serum lipid levels as well as fasting glucose, hemoglobin A1c (HbA1c), insulin, and uric acid levels in the general Japanese population, and the trends of serum lipid levels over the 40 years from 1960 to 2000.

Methods

Designs and data collection

The Research Group for Serum Lipid Level Survey 2000 in Japan co-ordinated members of 36 institutes from various areas in Japan. The project was designed to produce representative data of serum lipid, insulin, and uric acid plasma glucose and HbA1c levels in the civilian Japanese population. The subjects were people receiving annual health examinations in the general community, companies, and schools, and not patients visiting hospitals. The total number of subjects was 12,839, consisting of 7,658 men and 5,179 women (two of them were unknown for sex).

Laboratory methods

All serum and plasma samples were obtained in the fasting state except participants less than 20 years old, because it was hard to obtain permission to sample blood from children in a 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 by 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 (11). There were no differences between the data obtained by Zak-Henly's method in 1960 and 1970, and those by the enzymatic methods used in 1980 through 2000. Thus, the cholesterol levels in these five surveys appear to be comparable. In the present survey, we also measured remnant-like particles (RLP)-cholesterol with a kit from Japan Immunoresearch Laboratories (Gunma, Japan). 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 Laboratories, Abbot Park, IL, USA).

Data analyses

The statistical analyses of the present data were performed by SAS statistical. The study was designed by the Research group, which organized 36 institutions from various districts of Japan from the extreme North (Hokkaido) to the furthest South (Okinawa islands).

Results

Table 1 shows the age-specific means and standard deviations of serum total cholesterol levels by age group in all the participants as well as in men and women. The mean total cholesterol level in this survey was 201 mg/dl, which is 5 mg/dl higher than that in 1990. In men, the age-specific mean serum cholesterol levels gradually increased from 185 mg/dl in the 0- to 9-year-old age group to 207 mg/dl in the 50- to 59-year-old age group. There was a slight decrease after age 60. In women, the mean cholesterol levels gradually rose from 186 mg/dl in the 0- to 9-year-old age group to 218 mg/dl in the 50- to 69-year-old age groups, and fell to 208 mg/dl after age 80.

Table 2 shows the age-specific means and standard deviations of serum triglyceride levels in all the participants as well as in men and women. The mean triglyceride level in this survey was 118 mg/dl, which was 13 mg/dl higher than that in 1990. The age-specific mean triglyceride values were highest in 30- to 49-year-old age group in men. In contrast, in women, the age-specific mean triglyceride levels increased gradually from 59 mg/dl in the 0- to 9-year-old age group to 117 mg/dl in the 60- to 69-year-old age group, and then declined to 105 mg/dl above 80 years of age. Although the triglyceride

level in women did not change in ten years, the triglyceride level in men has markedly increased, especially 30- to 39-year-old to 70- to 79-year-old age groups over the last ten years.

Table 3 shows the age-specific means and standard deviations in serum HDL-cholesterol levels in all the participants as well as in men and women. The mean HDL-cholesterol level in this survey was 59 mg/dl, which is 5 mg/dl higher than that in 1990. The age-specific mean HDL-cholesterol levels in men gradually decreased from 70 mg/dl in the 0-to 9-year-old age group to 54 mg/dl in the 30- to 39-year-old age group, and remained at this level up to 89 years old age. The mean HDL-cholesterol levels in woman remained constant from the 0- to 9-year-old age group to the 50- to 59-year-old age group, and gradually decreased thereafter. Figure 1 summarizes the

Table 1. List of the institutes enrolled for this survey from each district around Japan.

Area	Name of Institute
Hokkaido	Sapporo Medical University
	Hokkaido University
	Asahikawa Red Cross Hospital
Tohoku	Yamagata University
	Hirosaki University
	Mizusawa General Hospital
Kantou	Tsukuba University
	Teikyo University
	St. Luka's International Hospital
	Chiba University
	National Defense Medical College
	Tokyo University
	Toranomon Hospital
	Nihon Medical School
	Nihon University
Hokuriku/Tokai	Hamamatsu Social Insurance Hospital
	Kanazawa University
	University of Fukui Faculty of Medical Sciences
	Himi Municipal Hospital
	Nagoya University
	Sugiyama Jogakuen University
	Nagoya City University
Kinki	National Cardiovascular Center
	Osaka University
	Kyoto Center for Preventive Medicine
	Kobe University
Chugoku/Shikoku	Egusa Clinic
	Yamaguchi University
	Chugoku Central Hospital
	Udajima Social Insurance Hospital
Kyushu/Okinawa	National Hospital Organization Kumamoto Medical Center
	Fukuoka University
	Saga University Faculty of Medicine
	Kagosima University
	Miyazaki Prefectural Nichinan Hospital
	University of Ryukyus

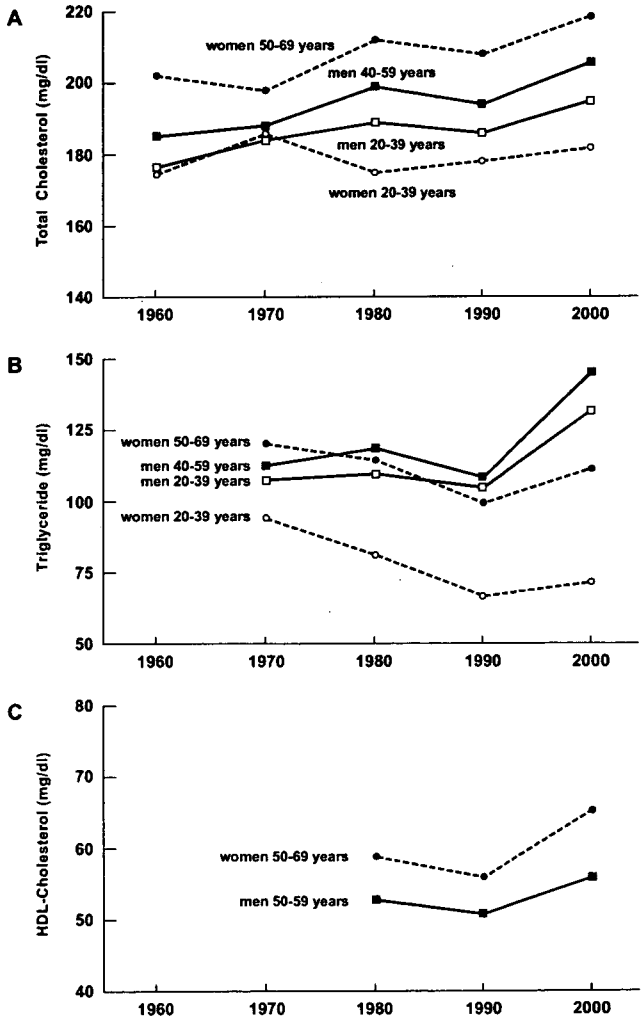

Fig. 1. Trends of serum lipid levels in Japanese in 40 years from 1960 to 2000. Results of the surveys carried out by the members of Japan Atherosclerosis Society. A. The mean cholesterol level in men and women of 20–39 years, men of 40–59 years, and women of 50–69 years from 1960 to 2000. B. The mean triglyceride level in men and women of 20–39 years, men of 40–59 years, and women of 50–69 years from 1970 to 2000. C. The mean HDL-cholesterol level in men and women of 50–59 years from 1980 to 2000.

Table 2. Serum total cholesterol (mg/dl) for each 10-year group in Japanese.

	All			Men			Women		
Age	*N*	Mean	S.D.	*N*	Mean	S.D.	*N*	Mean	S.D.
0–9	216	186	27	102	185	26	114	186	27
10–19	465	181	28	196	178	28	269	183	27
20–29	1,256	180	31	394	181	32	861	180	31
30–39	1,642	195	34	1,101	200	34	541	185	31
40–49	3,564	201	33	2,399	204	32	1,165	195	32
50–59	3,467	211	34	2,328	207	33	1,139	218	34
60–69	1,625	209	34	844	200	34	780	218	32
70–79	551	206	33	271	198	32	280	214	32
80–89	53	197	33	23	181	29	30	208	32
Total	12,839	201	34	7,658	202	34	5,179	200	35

Table 3. Serum triglyceride (mg/dl) for each 10-year group in Japanese.

	All			Men			Women		
Age	*N*	Mean	S.D.	*N*	Mean	S.D.	*N*	Mean	S.D.
0–9	216	56	30	102	53	30	114	59	30
10–19	465	67	36	196	66	39	269	68	33
20–29	1,256	83	65	394	105	74	861	73	58
30–39	1,642	118	109	1,101	142	123	541	70	42
40–49	3,564	129	103	2,399	150	112	1,165	87	63
50–59	3,467	129	102	2,328	139	115	1,139	108	66
60–69	1,625	123	83	844	128	98	780	117	64
70–79	551	118	63	271	123	67	280	113	59
80–89	53	100	44	23	93	38	30	105	47
Total	12,839	118	96	7,658	136	109	5,179	92	62

recent trend of the mean total cholesterol, triglyceride, and HDL-cholesterol levels in young and middle-aged men and women from 1960 to 2000. The trend indicates a gradual increase in the total cholesterol level in men and women in almost all generations over the last 40 years in Japan. The trend of the triglyceride level was somewhat different from that of the total cholesterol level. The triglyceride level in women, especially in young women, has tended to decrease over the last 30 years, while the level in men dramatically has increased in the last 10 years. The level of HDL-cholesterol increased both in men and women in the last 10 years.

Table 4 shows the age-specific means and standard deviations in serum LDL-cholesterol levels in all the participants as well as in men and women. LDL-cholesterol was measured directly, not by Friedewald equation. The mean LDL-cholesterol level in this survey was 118 mg/dl, which is almost the same as that in 1990. The age-specific mean LDL-cholesterol levels in men gradually increased from 101 mg/dl in the 0- to 19-year-old age group to 125 mg/dl in the 50- 59-year-old age group. The age-specific mean LDL-cholesterol level in women increased from 93 mg/dl in the 20- to 29-year-old age group to 135 mg/dl in the 60- to 69-year-old age group, and then decreased slightly thereafter.

In this survey we also measured RLP-cholesterol levels to assess the level of remnant particles. Table 5 shows the age-specific means and standard deviations in serum RLP-cholesterol levels in all the participants as well as in men and women. The mean RLP-cholesterol level in this survey was 4.5 mg/dl. The mean RLP-cholesterol level in men was significantly higher than that in women, and the age-specific mean RLP-cholesterol values were highest in 30- to 49-year-old age group in men as found in the triglyceride levels. The trends in age-specific means were similar to those of the triglyceride level. As expected, the RLP-cholesterol level correlated with the triglyceride level. (data not shown, $R = 0.878$, $p < 0.0001$).

Table 6 shows the age-specific means and standard deviations in plasma fasting glucose levels in all the par-

ticipants as well as in men and women. The mean fasting glucose level in this survey was 95 mg/dl. The mean glucose level was slightly higher in men than in women. The glucose level had a tendency to gradually increase according to age in both men and women. HbA1c levels also had a tendency to gradually increase according to age in both men and women. However, the mean HbA1c levels in men and women were almost the same in each

Table 4. Serum HDL-cholesterol (mg/dl) for each 10-year group in Japanese.

	All			Men			Women		
Age	*N*	Mean	S.D.	*N*	Mean	S.D.	*N*	Mean	S.D.
0–9	216	69	15	102	70	15	114	68	16
10–19	465	65	14	196	63	14	269	66	13
20–29	1,255	64	14	393	56	13	861	68	14
30–39	1,637	58	15	1,096	54	14	541	67	14
40–49	3,545	58	15	2,380	55	14	1,165	65	15
50–59	3,434	59	16	2,295	56	15	1,139	65	16
60–69	1,614	57	14	833	55	14	780	60	14
70–79	551	57	15	271	55	15	280	60	15
80–89	53	58	16	23	54	12	30	61	18
Total	12,770	59	15	7,589	55	14	5,179	65	15

Table 5. Serum LDL-cholesterol (mg/dl) for each 10-year group in Japanese.

	All			Men			Women		
Age	*N*	Mean	S.D.	*N*	Mean	S.D.	*N*	Mean	S.D.
0–9	154	104	22	70	101	22	84	106	22
10–19	162	103	24	51	101	21	111	104	25
20–29	713	97	24	240	105	26	472	93	22
30–39	751	112	29	484	119	29	267	101	25
40–49	1,179	121	30	750	124	31	429	116	29
50–59	1,243	127	30	733	125	30	510	130	30
60–69	726	129	31	387	124	30	338	135	29
70–79	246	126	28	117	120	27	129	130	28
80–89	32	123	29	10	113	27	22	127	30
Total	5,206	118	31	2,842	121	30	2,362	115	31

Table 6. Serum RLP-cholesterol (mg/dl) for each 10-year group in Japanese.

	All			Men			Women		
Age	*N*	Mean	S.D.	*N*	Mean	S.D.	*N*	Mean	S.D.
0–9	265	1.9	0.6	70	2.0	0.6	84	1.9	0.7
10–19	161	2.5	1.2	51	2.5	1.1	110	2.5	1.3
20–29	712	3.5	3.1	240	4.5	4.2	471	2.9	2.2
30–39	762	5.0	6.0	493	6.2	6.9	269	2.7	2.6
40–49	1,211	5.2	7.7	774	6.2	8.7	437	3.2	4.9
50–59	1,322	4.8	6.2	791	5.2	7.4	531	4.3	3.7
60–69	662	4.6	7.3	363	5.1	9.4	298	4.1	3.5
70–79	206	4.1	3.7	98	4.3	4.4	108	4.0	2.9
80–89	28	3.7	2.5	8	2.4	1.6	20	4.2	2.7
Total	5,218	4.5	6.2	2,888	5.4	7.6	2,328	3.4	3.5