studies had various limitations, including the use of body mass index (BMI) (*3, 4*), non-fasting triglyceride and glucose levels (*3, 4*), mortality (*4, 5*), or small sample size (*4–7*). In order to properly define MetS, it is essential to use data on waist circumference and on the levels of both fasting glucose and fasting triglycerides.

MetS has been defined in several ways by several groups, including the World Health Organization (*8*), the European Group for the Study of Insulin Resistance (*9*), the American Association of Clinical Endocrinologists, and the National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATPIII) (*10*). However, these definitions are aimed mainly at Western countries. The International Diabetes Foundation (IDF) (*11*) and the American Heart Association (*12*) have recently introduced alternative definitions that can be applied worldwide (*10*). Stroke incidence is relatively higher in Japan than in Western countries (*13*). It is uncertain whether these criteria can be applied well to Japanese populations. A MetS definition needs to be tailored to the epidemiological background of the area in question.

The Japanese Committee on the Criteria for MetS has recently proposed a definition of Japanese MetS (*14, 15*). Under both the IDF and the Japanese definitions, the presence of abdominal obesity is necessary for a diagnosis of MetS. However, no prospective study has examined the association between MetS based on the Japanese criteria and CVD, particularly in urban areas, where most Japanese live. Therefore, we undertook this study to examine the impact of MetS under the Japanese and modified NCEP-ATPIII criteria on CVD incidence in a general urban Japanese population.

Methods

Study Population

The Suita study (*16, 17*), an epidemiological survey of cerebrovascular disease and CVD, was based on a random sampling of 12,200 residents of Suita, a city of approximately 350,000 people in northern Osaka, Japan. As a baseline, in 1989, participants between the ages of 30 and 79 were arbitrarily selected from the municipality population registry and stratified into groups by sex and age in 10-year increments. Of these, 6,406 men and women participated in regular health checkups between September 1989 and March 1994. Since then, these participants have participated in regular health checkups at the National Cardiovascular Center every 2 years and answered health questionnaires every year.

Some cohort members in the study population were excluded from these analyses because they met one or more of the following criteria: past or present CVD illness at baseline (n=208), failure to fast for at least 10 h before venipuncture or missing data (n=170), or failure to follow up after their baseline examination (n=696). After these exclusions, 5,332 individuals remained for analysis.

Baseline Survey

We performed routine blood tests that measured fasting serum total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, and glucose levels. Physicians or nurses administered questionnaires covering the subjects' personal habits and any present illnesses. The subjects were classified as current smokers if they smoked at least one cigarette per day, as non-smokers if they had never smoked, and as past smokers if they had stopped smoking. Blood pressure was measured three times in a sitting position after at least 5 min of rest. Systolic and diastolic blood pressures (SBP and DBP) were taken to be the average of the second and third measurements that were recorded at least 1 min apart by well-trained doctors. Waist circumference was measured in a standing position at the umbilical level to the nearest 1 cm by well-trained technicians. Informed consent was obtained from all participants. This study was approved by the Institutional Review Board of the National Cardiovascular Center.

Definitions of Metabolic Syndrome

MetS was defined using two criteria. First, in accordance with NCEP-ATPIII (*18*) criteria, it was defined as the presence of three or more of the following five components: 1) abdominal obesity modified by the International Obesity Task Force central obesity criteria for Asia (waist circumference ≥90 cm in men and ≥80 cm in women) (*19*), 2) elevated blood pressure (SBP/DBP ≥130/85 mmHg and/or current use of antihypertensive medication), 3) hypertriglyceridemia (serum triglyceride levels ≥1.7 mmol/L [150 mg/dL] and/or current use of cholesterol-lowering medication), 4) low HDL cholesterol (serum HDL levels of ≤1.0 mmol/L [40 mg/dL] in men and of ≤1.3 mmol/L [50 mg/dL] in women), and 5) elevated blood glucose levels (fasting blood glucose ≥6.1 mmol/L [110 mg/dL] and/or current use of insulin or oral medication for diabetes).

Second, we used the definition of MetS recommended by the Japanese Committee on the Criteria for MetS (*14, 15*). MetS was defined by abdominal obesity (waist circumference ≥85 cm in men and ≥90 cm in women) (*20*) and least two of the following three components: 1) elevated blood pressure (SBP/DBP ≥130/85 mmHg), 2) hyperlipidemia (serum triglyceride levels ≥1.7 mmol/L [150 mg/dL] and/or HDL levels <1.0 mmol/L [40 mg/dL]), and 3) elevated blood glucose levels ≥6.1 mmol/L (110 mg/dL). Subjects taking medication for hypertension, hyperlipidemia, or diabetes were included as having that component.

Endpoint Determination

The endpoint of the follow-up period for each participant was whichever one of the following occurred first: 1) the date of the first myocardial infarction (MI) or stroke event, 2) the date of death, 3) the date the participant moved out of Suita,

Table 1. Baseline Distributions of Cardiovascular Disease Risk Factors According to Metabolic Syndrome under the NCEP-ATPIII Modified by Asian Obesity Definitions

	Men (n=2,492)			Women (n=2,840)		
	MetS(−) (n=2,043)	MetS(+) (n=449)	p*	MetS(−) (n=2,253)	MetS(+) (n=587)	p*
Age at baseline, years	55.4±13.3	58.1±11.5	<0.001	52.2±12.6	61.3±9.8	<0.001
Systolic blood pressure, mmHg	126±20	140±19	<0.001	120±20	141±20	<0.001
Diastolic blood pressure, mmHg	78±12	85±11	<0.001	73±11	83±12	<0.001
Total cholesterol, mg/dL	200±34	210±35	<0.001	210±38	227±38	<0.001
HDL cholesterol, mg/dL	51±13	40±10	<0.001	60±12	45±10	<0.001
Triglyceride, mg/dL†	121±73	241±156	<0.001	90±44	178±113	<0.001
Waist circumference, cm	81.0±7.3	89.7±7.0	<0.001	74.7±8.9	87.4±8.5	<0.001
Elevated blood pressure, %	41.8	85.8	<0.001	30.4	82.1	<0.001
Hypertriglyceridemia, %	21.6	82.9	<0.001	7.2	63.7	<0.001
Lower-HDL cholesterol, %	15.5	64.8	<0.001	18.7	80.1	<0.001
Hyperglycemia, %	8.9	43.9	<0.001	3.6	29.6	<0.001
Current smoker, %	50.5	47.6	0.278	11.9	11.8	0.958
Current drinker, %	75.5	72.6	0.207	34.6	25.4	<0.001

Elevated blood pressure: antihypertensive drug use or >130/85 mmHg; hypertriglyceridemia: antilipidemic drug use or triglyceride >150 mg/dL; lower-HDL cholesterol: HDL cholesterol <40 mg/dL. MetS, metabolic syndrome; HDL, high-density lipoprotein. *ANOVA or χ^2 tests were performed. †Log-transformed triglyceride was performed to statistical analysis.

Table 2. Age-Adjusted Hazard Ratios (Confidence Intervals) for Incidence of Cardiovascular Disease According to Abdominal Obesity at Baseline Examination

	Men				Women			
	Case, n	Person-year	HR (95% CI)	p	Case, n	Person-year	HR (95% CI)	p
Japanese criteria								
<85 cm (men)/<90 cm (women)	111	17,112	1		96	29,960	1	
≥85 cm (men)/≥90 cm (women)	77	11,247	0.97 (0.72–1.30)	0.844	33	3,890	1.64 (1.09–2.46)	0.019
Asian criteria								
<90 cm (men)/<80 cm (women)	145	23,136	1		53	21,139	1	
≥90 cm (men)/≥80 cm (women)	43	5,223	1.18 (0.84–1.67)	0.327	76	12,711	1.44 (1.00–2.07)	0.048
NCEP-ATPIII criteria								
<102 cm (men)/<88 cm (women)	182	27,976	1		91	28,730	1	
≥102 cm (men)/≥88 cm (women)	6	384	2.00 (0.88–4.54)	0.095	38	5,121	1.47 (1.00–2.17)	0.048

HR, hazard ratio; CI, confidence interval.

or 4) December 31, 2005 (censored). As a first-step survey to detect MI and stroke incidence, each participant's health status was checked during a clinical visit at the National Cardiovascular Center every 2 years. Furthermore, every year a health questionnaire was given to each participant *via* mail or telephone.

Confirmation of Strokes and Myocardial Infarctions

In total, five hospitals in this area were capable of performing computed tomographic scans and/or magnetic resonance imaging, and all were major hospitals that admitted acute stroke and MI patients. Medical records were reviewed by registered hospital physicians or research physicians who were blinded to the baseline information. Strokes and MI events were registered if they occurred after the date on which the baseline health examination was held and before January 1, 2006. Strokes were defined according to the National Survey of Stroke criteria (*21*). These criteria require the rapid onset of a constellation of neurological deficits lasting at least 24 h or until death. For each stroke subtype (cerebral infarction [thrombotic or embolic infarction], intracerebral hemorrhage, and subarachnoid hemorrhage), a definite diagnosis was established based on examination of computed tomographic scans, magnetic resonance images, or autopsy. Defi-

Table 3. Age-Adjusted Hazard Ratios (95% Confidence Intervals) for Incidence of Cardiovascular Disease, Myocardial Infarction, and All Strokes According to Metabolic Syndrome under the Japanese and NCEP-ATPIII Definitions

	Men			Women		
	MetS(−)	MetS(+)	*p* value	MetS(−)	MetS(+)	*p* value
Cardiovascular disease						
MetS Japanese definition						
Cases, *n*	140	48		110	19	
Person-year	23,542	4,817		32,325	1,526	
Age-adjusted	1	1.31 (0.94–1.82)	0.109	1	2.16 (1.31–3.54)	0.002
Multivariate-adjusted	1	1.34 (0.96–1.87)	0.080	1	2.20 (1.31–3.68)	0.003
<60 years old						
Cases, *n*	27	15		25	4	
Person-year	14,752	2,366		22,085	529	
Age-adjusted	1	2.76 (1.46–5.23)	0.002	1	5.39 (1.82–15.98)	0.002
Multivariate-adjusted	1	2.92 (1.54–5.55)	0.001	1	6.25 (2.08–18.79)	0.001
≥60 years old						
Cases, *n*	113	33		85	15	
Person-year	8,790	2,451		10,240	997	
Age-adjusted	1	1.04 (0.70–1.53)	0.841	1	1.83 (1.05–3.18)	0.033
Multivariate-adjusted	1	1.06 (0.71–1.57)	0.764	1	1.80 (1.01–3.20)	0.046
MetS NCEP-ATPIII (Asian) definition						
Cases, *n*	133	55		73	56	
Person-year	23,373	4,986		27,405	6,446	
Age-adjusted	1	1.70 (1.23–2.34)	0.001	1	1.93 (1.35–2.77)	<0.001
Multivariate-adjusted	1	1.75 (1.27–2.41)	<0.001	1	1.90 (1.31–2.77)	<0.001
<60 years old						
Cases, *n*	30	12		19	10	
Person-year	14,509	2,606		19,872	2,742	
Age-adjusted	1	1.79 (0.91–3.52)	0.089	1	2.72 (1.23–5.99)	0.013
Multivariate-adjusted	1	1.94 (0.98–3.82)	0.055	1	2.96 (1.34–6.57)	0.007
≥60 years old						
Cases, *n*	103	43		54	46	
Person-year	8,864	2,381		7,533	3,704	
Age-adjusted	1	1.67 (1.16–2.40)	0.005	1	1.78 (1.19–2.66)	0.005
Multivariate-adjusted	1	1.73 (1.20–2.48)	0.003	1	1.70 (1.12–2.59)	0.012
Myocardial infarction						
MetS Japanese definition						
Cases, *n*	56	22		32	7	
Person-year	22,962	4,663		31,697	1,457	
Age-adjusted	1	1.48 (0.90–2.44)	0.117	1	2.36 (1.02–5.46)	0.043
Multivariate-adjusted	1	1.51 (0.91–2.48)	0.105	1	2.70 (1.15–6.35)	0.023
MetS NCEP-ATPIII (Asian) definition						
Cases, *n*	52	26		18	21	
Person-year	22,833	4,795		26,944	6,211	
Age-adjusted	1	2.09 (1.30–3.37)	0.002	1	2.68 (1.41–5.10)	0.003
Multivariate-adjusted	1	2.12 (1.31–3.43)	0.002	1	2.77 (1.44–5.32)	0.002
All strokes						
MetS Japanese definition						
Cases, *n*	84	26		78	12	
Person-year	23,177	4,659		32,078	1,487	
Age-adjusted	1	1.21 (0.78–1.89)	0.381	1	2.09 (1.12–3.88)	0.019
Multivariate-adjusted	1	1.27 (0.81–1.97)	0.292	1	2.05 (1.07–3.92)	0.031
MetS NCEP-ATPIII (Asian) definition						
Cases, *n*	81	29		55	35	
Person-year	23,010	4,826		27,266	6,299	
Age-adjusted	1	1.52 (0.99–2.34)	0.053	1	1.70 (1.09–2.64)	0.018
Multivariate-adjusted	1	1.58 (1.02–2.43)	0.037	1	1.62 (1.02–2.58)	0.041

Multivariate adjusted for age, smoking and drinking status. MetS, metabolic syndrome.

nite and probable MI was defined according to the criteria set out by the MONICA (Monitoring Trends and Determinants of Cardiovascular Disease) project (22), which requires evidence from ECGs, cardiac enzymes, and/or autopsy. Sudden deaths of unknown origin were deaths that occurred within 24 h from onset and were included in MI. However, there was little difference in hazard ratios between the groups with and without sudden death from CVD, because sudden death constituted a small sample size (n=6).

To complete surveillance for fatal stroke and MI, we also systematically searched for death certificates, the purpose of which were permitted to use by the Ministry of Health, Labour and Welfare. We checked for possible stroke and MI using data from 1) the health examination and questionnaire for the stroke and MI registry, without informed consent for the medical records survey and 2) death certificates without registration of CVD incidence, which were defined as probable stroke or MI. CVD was defined as stroke and MI in this study. Informed consent to review in-hospital medical records was obtained from 86.2% of participants who were suspected of having any signs or information suggesting the incidence of stroke or MI. For 13.8% of subjects from whom informed consent was not obtained, final diagnoses of CVD were confirmed by physicians or epidemiologists who had been involved in the diagnostic process throughout the study, in order to avoid the misclassification of diagnoses.

Statistical Analysis

Analyses of variance and χ^2 tests were used to compare mean values and frequencies by sex, respectively, according to MetS based on the modified NCEP-ATPIII criteria. For each subject, the person-years of follow-up were calculated from September 1, 1989, to whichever came first: the first endpoint, MI or stroke event, death, emigration, or December 31, 2005. A Cox proportional hazards regression model was used to detect associations between abdominal obesity for Japanese (≥85 cm in men or ≥90 cm in women), Asian (≥90 cm in men or ≥80 cm in women), and American criteria (≥102 cm in men or ≥88 cm in women) and CVD during the follow-up period. The Cox proportional hazard regressions were fitted to the grouping (positive or negative MetS) after adjusting for age and the other potential confounding factors: baseline age, smoking status (never, ex-smoker, or current smoker), and drinking status (never, ex-drinker, or current drinker). Trend tests were conducted by assigning the number of MetS components to test the significance of these variables. All statistical analyses were conducted using the SAS statistical package (release version 8.2; SAS Institute Inc., Cary, USA).

Results

During the follow-up period (averaging 12.5 years), 200 strokes were documented (160 definite strokes and 40 probable strokes). These strokes comprised 130 cerebral infarctions, 31 intracerebral hemorrhages, 22 subarachnoid hemorrhages, and 17 unclassified strokes. In addition, 117 MIs were documented (61 definite MIs and 56 probable MIs or sudden cardiac deaths).

Table 1 shows the distribution of CVD risk factors at the baseline according to MetS as defined by the modified NCEP-ATPIII criteria. Compared with the non-MetS groups, men and women with MetS were more likely to be older and to have higher frequencies of each MetS component.

Table 2 presents the age-adjusted HRs (95% confidence intervals [CI]) for the incidence of CVD according to waist circumference by the NCEP-ATPIII, Japanese, and Asian obesity criteria. Regardless of the criteria set, abdominal obesity was associated with CVD only in women.

Table 3 shows the association of MetS by the Japanese and the modified NCEP-ATPIII criteria with CVD incidence according to age category and sex. Using the Japanese criteria, MetS was associated only in women with the incidence of CVD, MI, and all strokes (HR [95% CI]: 2.20 [1.31–3.68], 2.70 [1.15–6.35], and 2.05 [1.07–3.92], respectively), whereas in men overall MetS was not associated with the incidence of CVD or its subtypes. However, among men under 60 years old, MetS based on the Japanese criteria was associated with CVD incidence (HR=2.92, 95% CI: 1.54–5.55). Using the modified NCEP-ATPIII definition, MetS was associated with each CVD subtype in both men and women. Multivariate adjusted HRs of CVD incidence for MetS based on the NCEP-ATPIII criteria were 1.94 (0.98–3.82) and 1.73 (1.20–2.48) in men less than or equal to and over 60 years old, respectively.

Figure 1 shows that the frequency of MetS increased with age for men and women based on the NCEP-ATPIII (A) and Japanese (B) criteria, respectively. The frequency based on the NCEP-ATPIII modified by the Asian obesity criteria (25.1% for men and 14.3% for women) was higher than that based on the Japanese criteria (17.7% for men and 5.0% for women), especially in women.

The risk of CVD incidence increased according to the number of components combined in men and women with and without abdominal obesity (Fig. 2). In addition, compared with the non-abdominal obesity and non-component groups, the risks of CVD incidence were similar among participants who had the same numbers of components, regardless of the presence or absence of abdominal obesity in men and women combined.

Figure 3 shows the multivariate HRs for MetS based on the Japanese and NCEP-ATPIII definitions modified by the obesity criteria for waist circumference. When the Japanese definition was adopted and the risk of MetS was monitored through sequential waist circumference changes, the cut-off points for waist circumference, which conferred a risk of CVD in men and women, were 84 cm and 92 cm, respectively. When the definition of MetS-indicative waist circumference was higher than those values, the risk was not statistically significant. When the NCEP-ATPIII definition

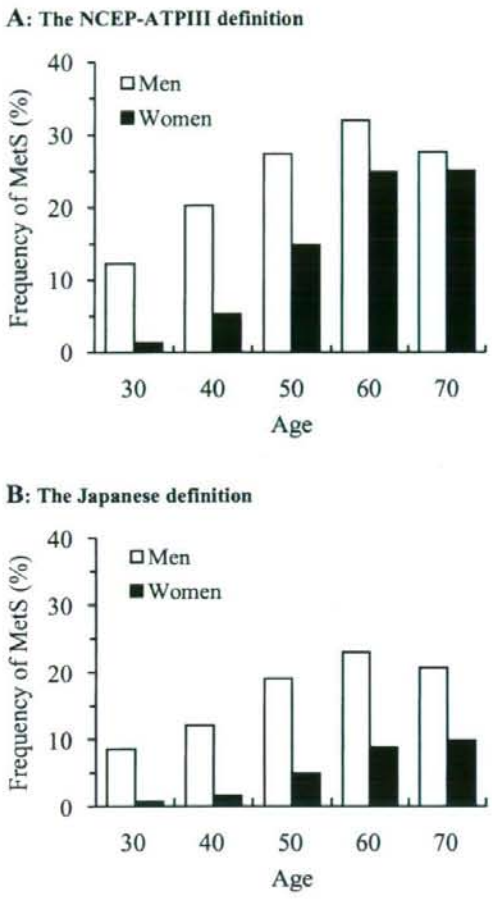

Fig. 1. *Frequencies of MetS components (A: the NCEP-ATPIII definition; and B: the Japanese definition, modified by the Asian waist circumference criteria) by sex. White and solid bars indicate men and women, respectively.*

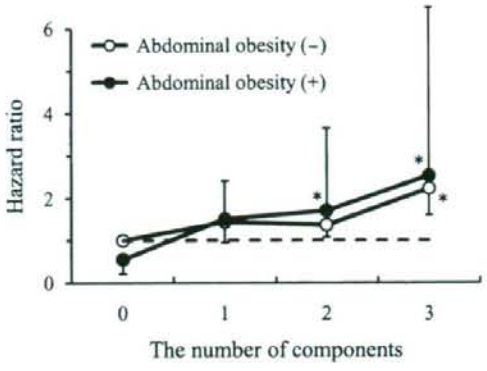

Fig. 2. *Multivariate HRs for the risks of CVD incidence according to the number of components based on the NCEP-ATPIII definition with and without abdominal obesity. White and solid circles indicate non-abdominal and abdominal obesity according to the Asian obesity criteria. *$p < 0.05$ compared to the reference of non-abdominal obesity and no-components. Bars show 95% CI for the HRs.*

was used, the value of waist circumference did not modify the risk of CVD, implying that the clustering of risk factors may be more important than waist circumference itself for determining CVD risk.

Discussion

In the current cohort study of a general urban Japanese population, the association between MetS and CVD was significant when the NCEP-ATPIII (modified by the Asian criteria) definition was applied. MetS based on the Japanese criteria was associated with CVD incidence in women, whereas in men the association was found only in those under 60 years old. In addition, the risk of CVD incidence was similar among participants who had the same numbers of components regardless of whether they were abdominally obese. To the best of our knowledge, this is the first study of an urban Japanese cohort.

Compared to the previous studies, this study has several methodological strengths. First, previous Japanese cohort studies associating MetS with CVD were based predominantly on BMI (*3*, *4*), non-fasting blood collection (*3*, *4*), and mortality as the endpoint (*4*, *5*). Our baseline subjects were observed in the fasting state, and we used waist circumference and a wide age range. Second, we evaluated a large prospective cohort of people randomly selected from a general Japanese population. A prospective study has little recall bias as well as results from a general population cohort that is more representative than occupational, hospital-based, or volunteer cohorts. Third, our sample size was relatively large for a cohort study and we could therefore perform sub-analysis by age and CVD subtypes. Fourth, our cohort population was selected at random from an urban population, in contrast to most of the other MetS cohort populations, which were selected from rural populations. Our study is the first of its kind in an urban area. Finally, our study examined the risk of CVD incidence, which is a more direct measure of CVD risk than the rate of CVD mortality, because the time to death from CVD is influenced by treatment.

Abdominal obesity induces inflammation in adiposities (*23*), endothelial dysfunction (*24*, *25*), and oxidative stress (*26*), thereby contributing to CVD development (*27*, *28*).

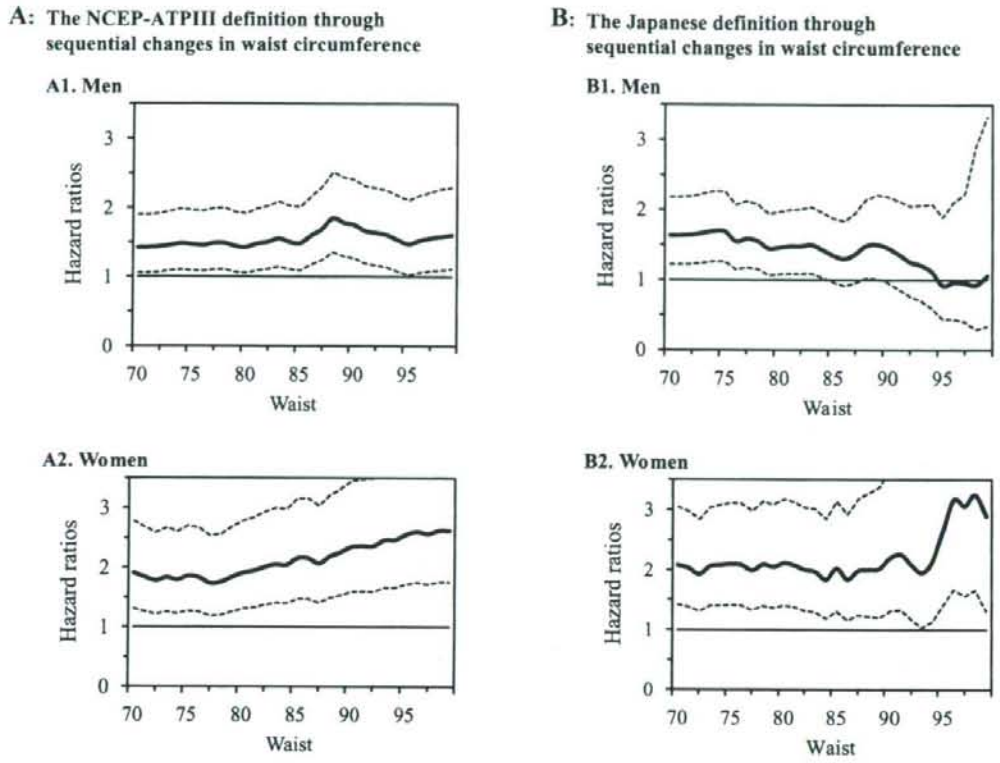

Fig. 3. *Multivariate HRs for MetS based on the NCEP-ATPIII (A) and Japanese (B) definitions through sequential changes in waist circumference by sex. Solid and dotted lines indicate HRs and 95% CI, respectively.*

Accumulating evidence suggests that MetS increases the risk of CVD (*29*). However, there has been a lack of convincing evidence (*29*) that MetS is associated with CVD in Japan. Iso *et al.* reported that MetS was associated with a risk for ischemic CVD in Japan (*3*), although they used BMI as well as non-fasting blood glucose and triglyceride levels to define MetS. Ninomiya *et al.* reported that MetS was a significant risk factor for CVD in a rural Japanese population (*6*). However, that study examined a rural population half the size of that in our study. Takeuchi *et al.* reported that MetS was a risk factor for cardiac disease in a rural cohort (*7*), but their data were based on a small sample that comprised only men. Kadota *et al.* reported that MetS, defined by BMI and non-fasting blood samples, was associated with CVD mortality (*4*).

We have shown that the components of MetS synergistically increase CVD risk. Abdominal obesity did not affect the association between the number of MetS components and the risk of CVD incidence. The risk of CVD was also not related to waist circumference when the NCEP-ATPIII definition was applied (data not shown), suggesting that the combination of risk factors *per se* is more important than abdominal obesity for conferring risk.

The definition of MetS may be reconsidered on the basis of age and sex. According to our results, lifestyle modifications may not be needed for older men who are free of cardiovascular risk factors even if they have abdominal obesity. Therefore, to prevent CVD, it is not adequate for only subjects with MetS to change their lifestyles; subjects with one or two MetS components, even without abdominal obesity, should modify their lifestyles.

When the waist-circumference threshholds were sequentially changed in the Japanese criteria for MetS, our data showed that the clustering of metabolic risk factors was statistically significant for CVD at waist circumferences less than 85 cm for men and 93 cm for women. When the definition of MetS-indicative waist circumferences was higher than those values, the risk clustering was not statistically significant for

CVD in men, and the 95% CI was much wider but still significant in women. Subjects with high risks and non-abdominal obesity with risk clustering aside from abdominal obesity will drop out when the waist-circumference definitions are raised.

Our study has several limitations. First, the annual emigration rate (1.5%) is relatively higher than that in rural areas. Second, about 10% of the subjects who underwent a baseline examination did not respond to our questionnaires afterward. We found no clinical background difference between participants and non-participants, because the main denial reason for participation in this study was not health problems. The frequencies of MetS according to NCEP-ATPIII modified by Asian criteria were 19% and 21% for participants and non-participants, respectively (χ^2 test $p=0.09$). In this study, the main reasons for emigration included job transfer, but not health problems.

In conclusion, the current prospective study for a general urban population showed that MetS, as defined by the Japanese criteria, was associated with CVD in women and middle-aged men; a stronger association was found when the NCEP-ATPIII definition modified by the Asian obesity criteria was applied. The number of MetS components may be more strongly associated with CVD incidence than the essential waist-circumference criteria.

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Pioglitazone treatment stimulates circulating CD34-positive cells in type 2 diabetes patients

Hisashi Makino[a,*], *Sadanori Okada*[a], *Ayako Nagumo*[a], *Takako Sugisawa*[a], *Yoshihiro Miyamoto*[a], *Ichiro Kishimoto*[a], *Taura K. Akie*[b], *Toshihiro Soma*[b], *Akihiko Taguchi*[c], *Yasunao Yoshimasa*[a]

[a] *National Cardiovascular Center, Department of Atherosclerosis and Diabetes, Suita, Osaka 565-8565, Japan*
[b] *National Hospital Organization Osaka Minami Medical Center, Department of Hematology, Osaka, Japan*
[c] *National Cardiovascular Center, Department of Cerebrovascular Disease, Suita, Osaka, Japan*

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ABSTRACT

Circulating bone marrow derived immature cells, including CD34-positive ($CD34^+$) cells, contribute to maintenance of the vasculature, not only as a pool of endothelial progenitor cells (EPCs), but also as a source of growth/angiogenesis factor. We hypothesized that the thiazolidineone compound pioglitazone could stimulate the circulating $CD34^+$ cells in diabetic patients. Thirty-four patients with type 2 diabetes received 15–30 mg pioglitazone for 24 weeks. The number of circulating $CD34^+$ cells significantly increased at 12 and continued this effect for 24 weeks (1.08 ± 0.39, 1.34 ± 0.34 and 1.32 ± 0.28 cells/μl at 0, 12 and 24 weeks, respectively). The change of $CD34^+$ cell levels ($\Delta CD34^+$ cells) between 0 and 12 weeks was significantly correlated with the change of high sensitive C reactive protein levels (Δhs-CRP) and change in adiponectin levels (Δadiponectin) ($r = -0.412$, $r = 0.359$, respectively). Our study demonstrated that pioglitazone treatment increased circulating $CD34^+$ cells, suggesting that this effect may at least partly contribute to the anti-atherosclerotic action of pioglitazone.

1. Introduction

Endothelial dysfunction plays a pivotal role in the progression of the atherosclerosis. Circulating EPCs contribute to the maintenance of vascular homeostasis and repair. They also play an important role in the maintenance of vascular endothelial function [1,2]. In diabetic patients, both a decrease in number and function of circulating EPCs are reported, suggesting that circulating EPCs participate in diabetic vascular complications [3].

Recent studies have identified circulating bone marrow derived immature cells, including $CD34^+$ cells, contribute to maintenance of the vasculature, not only as a pool of EPCs, but also as a source of growth/angiogenesis factor [4]. In fact, one recent report indicates that circulating $CD34^+$ cells are more strongly correlated with cardiovascular risk than circulating $CD34^+$/kinase insert domain receptor $(KDR)^+$ cells generally regarded as EPCs [5]. We have also reported that circulating $CD34^+$ cell levels are associated with cerebral infarction [6]. These findings indicate that persistent stimulation of $CD34^+$ cells may be a useful method to repair endothelial injury and microcirculation, and to suppress the progression of atherosclerotic disease at least theoretically. Recent experimental and clinical studies demonstrate that thiazolidinediones, peroxisome-proliferator-activated receptor γ (PPARγ) agonists, has the effects on the prevention of atherosclerosis including the maintenance of vascular endothelial function [7–9]. Therefore, we hypothesized that the thiazolidineone

* *Corresponding author.* Tel.: +81 6 6833 5012; fax: +81 6 6872 7486.
E-mail address: makinoh@hsp.ncvc.go.jp (H. Makino).
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compound pioglitazone could stimulate the circulating CD34⁺ cells in diabetic patients.

2. Methods

2.1. Study subjects

All subjects gave a written informed consent. The study was approved by the local ethics committee. Thirty-four patients with type 2 diabetes (age 60 ± 10, M/F; 18/16, HbA1c 9.3 ± 1.4%) received 15 or 30 mg pioglitazone for 24 weeks (15 mg; 31 patients, 30 mg; 3 patients). Other medications for diabetes, hypertension and hyperlipidemia were unchanged throughout the study. Insulin was given to 9 patients. Sulfonylurea was given to 15 patients. Biguanide was given to 21 patients. Alpha glucosidase inhibitor was given to 10 patients. Angiotensin converting enzyme inhibitor and/or angiotensin receptor blocker was given to 21 patients. Statin was given to 18 patients. Sixteen patients afflicted with cardiovascular diseases (CVD). Eighteen patients afflicted with nephropathy, 14 patients afflicted with retinopathy, and 15 patients afflicted with neuropathy.

2.2. Measurement of CD34⁺ cells

Three milliliters of heparinized peripheral blood were obtained after 12-h fasting and measured CD34⁺ cells. The precise number of circulating CD34⁺ cells was quantified as we described previously [10]. We evaluated circulating CD34⁺ cells with Stem-Kit™ (BeckmanCoulter, Marseille, France) according to manufacturers' protocols. These protocols are based on International Society of Hematotherapy and Graft Engineering (ISHAGE) Guidelines [11], and are frequently used for quantification of CD34⁺ cells mobilized into peripheral blood. To increase the reproducibility of CD34⁺ cell counts, the protocol of Stem-Kit was modified as follows: the blood sample volume, antibodies and lysing solution were doubled. After adding 30 μl of internal control (Stem count: BeckmanCoulter), samples were centrifuged for 5 min at 450 × g and 3860 μl of supernatant was removed carefully with a pipet. Samples were analyzed by Coulter CYTOMICS™ FC500 & XL-system II software (BeckmanCoulter) for 6 min each.

2.3. Other laboratory analysis

Blood samples were taken after 12-h fasting to measure adiponectin and, high sensitive C-reactive protein (hs-CRP) concentrations. Serum adiponectin and concentration was measured by enzyme-linked immunosorbent assay (SRL, Tokyo, Japan). Serum hs-CRP concentration was measured by latex nephelometry method (SRL, Tokyo, Japan). We also measured HbA1c, total cholesterol, HDL cholesterol and triglyceride levels.

2.4. Statistical analysis

Data was expressed using the mean ± S.D. The Student's t-test was used to compare parameter changes over time. The strength of correlation between variables was performed using Spearmann's correlation coefficient.

3. Results

3.1. Effects of pioglitazone on glucose and lipid metabolism

Treatment of pioglitazone significantly decreased HBA1c levels (9.3 ± 1.4, 7.4 ± 1.2 and 7.5 ± 1.7% at 0, 12 and 24 weeks, respectively). Systemic blood pressure levels did not change throughout the study period. BMI did not change throughout the study period (26.8 ± 3.2, 27.5 ± 3.0 and 27.9 ± 3.3 at 0, 12 and 24 weeks, respectively). Total cholesterol and triglyceride levels did not change throughout the study, whereas HDL cholesterol levels significantly increased at 12 and 24 weeks (1.08 ± 0.39, 1.34 ± 0.34 and 1.32 ± 0.28 mmol/l at 0, 12 and 24 weeks, respectively).

3.2. Effects of pioglitazone on adiponectin and inflammatory marker

The inflammatory marker, hs-CRP significantly decreased at 12 and 24 weeks (1518 ± 2350, 840 ± 975, and 838 ± 904 ng/ml at 0, 12, and 24 weeks, respectively). Serum adiponectin levels significantly increased at 12 and 24 weeks (5.0 ± 2.2, 13.5 ± 6.7 and 13.8 ± 8.4 μg/ml at 0, 12 and 24 weeks, respectively). The change in adiponectin levels between 0 and 12 weeks (Δadiponectin) of 30 mg pioglitazone was significantly larger than 15 mg of pioglitazone (15 mg; 7.9 ± 4.7 vs. 30 mg; 19.6 ± 2.5, $p < 0.05$), whereas there was no significant difference in the change in hs-CRP levels (Δhs-CRP) between 15 mg and 30 mg of pioglitazone (15 mg; 267 ± 322 vs. 30 mg; 480 ± 1883).

3.3. Effects of pioglitazone on circulating CD34⁺ cell level

The number of circulating CD34⁺ cells significantly increased at 12 and 24 weeks (0.90 ± 0.48, 1.10 ± 0.50, and 1.10 ± 0.57 cells/μl at 0, 12, and 24 weeks, respectively (Fig. 1). This effect was found in both patients with CVD and without CVD (patients with CVD; 0.81 ± 0.51, 1.05 ± 0.46 and 1.04 ± 0.50 cells/μl at 0, 12 and 24 weeks, respectively, $n = 16$, patients without CVD; 0.98 ± 0.41,

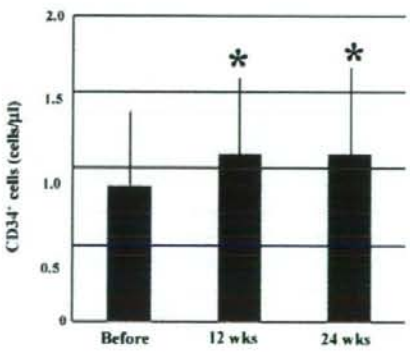

Fig. 1 – CD34+ cell level at 0, 12 and 24 weeks, *$p < 0.05$ vs. 0 week.

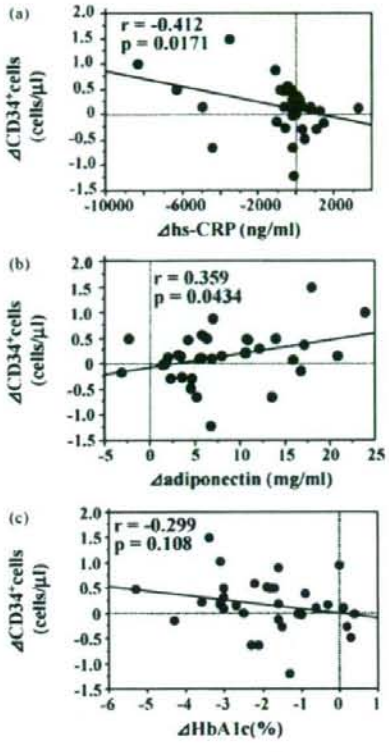

Fig. 2 – Correlation between ΔCD34⁺ cells and ΔhsCRP ($r = -0.412$, $p = 0.017$) (a), correlation between ΔCD34⁺ cells and Δadiponectin ($r = 0.359$, $p = 0.043$) (b), and correlation between ΔCD34⁺ cells and ΔHbA1c ($r = -0.299$, $p = 0.108$) (c).

1.15 ± 0.57 and 1.15 ± 0.65 cells/μl at 0, 12 and 24 weeks, respectively, $n = 18$). There was no significant difference in the change in CD34⁺ cell level (ΔCD34⁺ cells) between 15 mg and 30 mg of pioglitazone (15 mg; 0.07 ± 1.01 vs. 30 mg; 0.14 ± 0.32).

3.4. *Factors involved in the stimulation of CD34⁺ cells*

We next investigated which factors were correlated with the stimulation of CD34⁺ cells. ΔCD34⁺ cells were significantly correlated with Δhs-CRP in univariate analysis ($r = -0.412$, $p = 0.017$) (Fig. 2a). Further, Δadiponectin correlated with Δ CD34⁺ cells ($r = 0.359$, $p = 0.043$) (Fig. 2b). On the other hand, change in HbA1c levels (ΔHbA1c) ($r = -0.299$, $p = 0.108$) (Fig. 2c), change in HDL-C levels (ΔHDL-C) ($r = 0.253$, $p = 0.168$) and change in triglyceride levels (Δtriglycerides) ($r = 0.0072$, $p = 0.969$), were not significantly correlated to Δ CD34⁺ cells.

4. Discussion

Accumulating evidence shows that PPARγ agonists have anti-atherosclerotic actions other than their blood glucose level reduction effects [7,9]. One recent report showed that pioglitazone treatment could stimulate circulating EPCs in patients with coronary artery disease and normal glucose tolerance [12]. In this study, we demonstrated that pioglitazone treatment also increased circulating CD34⁺ cells and this effect continued for 24 weeks in type 2 diabetic patients. We studied the effects of pioglitazone on the stimulation of CD34⁺ cells but not CD34⁺/KDR⁺ cells regarded as EPCs. However, these circulating CD34⁺ cells have the capacity to participate in neovascularization of ischemic tissue. Indeed, their administration enhances the repair of ischemic tissue in ischemic stroke model [13] and improves myocardial circulation in myocardial infarction model [14]. Clinically, circulating CD34⁺ cell levels were reported to be correlated with cerebral blood flow in hypoperfusion area [6] and formation of collateral vessels in stroke patients [15]. These reports suggest that CD34⁺ cells may play a role in the maintenance of microcirculation. One recent clinical trial, PROactive Study, demonstrated that pioglitazone treatment could prevent cardiovascular events including stroke in type 2 diabetic patients [16]. Taken together, it is suggested that the stimulation of CD34⁺ cells may partly contribute to the preventive effects of pioglitazone on cardiovascular diseases. Our study also demonstrated that pioglitazone treatment increased circulating CD34⁺ cells in type 2 diabetic patients irrespective of with or without CVD, suggesting that pioglitazone treatment may be useful for primary prevention as well as secondary prevention of diabetic macroangiopathy.

It has been reported that the number of circulating EPCs is inversely correlated with HbA1c levels [3]. Since pioglitazone treatment significantly decreased HbA1c levels and this study did not have control group, we could not exclude the possibility that the stimulation of CD34⁺ cells was associated with the improvement of glycemic control. However, the results of this study suggest that pioglitazone may be capable of stimulating circulating CD34⁺ cells independently of glycemic control because ΔCD34⁺ cells was not positively correlated with ΔHbA1c at levels that achieved statistical significance.

Adipocyte derived factors and inflammation participate in atherogenesis of type 2 diabetic patients. Accumulating evidence show that adiponectin, one of adipocyte derived factors, has anti-atherogenic properties, and hypoadionectinemia was reported to be associated with endothelial dysfunction [17]. Pioglitazone treatment decreased hs-CRP levels and increased serum adiponectin levels in metabolic syndrome subjects [8], suggesting that these effects contribute to the anti-atherosclerotic action of pioglitazone. In this study, we also demonstrated that pioglitazone treatment decreased hs-CRP levels and increased serum adiponectin levels in type 2 diabetes patients. Interestingly, ΔCD34⁺ cells were significantly correlated with Δhs-CRP and Δadiponectin. An in vitro study showed that CRP impaired EPC migration and function [18]. In clinical study, it has been reported that circulating EPCs were inversely correlated to serum interleukin 6 levels [19]. These reports suggested that chronic inflammation may be involved in the regulation of EPCs. One recent clinical study showed that circulating EPCs were positively correlated to serum adiponectin levels in patients with coronary artery disease [20]. Another report showed that

adiponectin treatment increased EPC number and migration [12]. Taken together, it is suggested that the inhibitory effects on chronic inflammation and the effect on adiponectin regulation of pioglitazone may be directly or indirectly involved in the increase of CD34⁺ cells. However, further study is necessary to delineate this hypothesis.

In conclusion, our study demonstrated that pioglitazone treatment increased circulating CD34⁺ cells, suggesting that this effect may at least partly contribute to the anti-atherosclerotic action of pioglitazone.

Conflict of interest

There are no conflicts of interest.

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Impaired flow-mediated vasodilatation and insulin resistance in type 2 diabetic patients with albuminuria

Hisashi Makino, Kentaro Doi, Aki Hiuge, Ayako Nagumo, Sadanori Okada, Yoshihiro Miyamoto, Masaaki Suzuki, Yasunao Yoshimasa*

Department of Atherosclerosis and Diabetes, National Cardiovascular Center, 5-7-1 Fujishiro-dai, Suita City, Osaka 565-8565, Japan

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ABSTRACT

An elevated urinary albumin excretion is associated with an increased risk of cardiovascular disease due to atherosclerosis, but the pathophysiological mechanism underlying this association is poorly understood. We studied 217 diabetic patients, that is, 121 normoalbuminuric patients, 71 microalbuminuric patients, and 25 macroalbuminuric patients. We evaluated flow-mediated dilatation of brachial artery (%FMD, one endothelial function marker associated with endogenous NO production), von Willebrand factor (vWF, endothelial activation marker), high-sensitive CRP (hsCRP, a low-grade inflammation marker), asymmetric dimethyl arginine (ADMA, an endogenous inhibitor of NO synthesis), and insulin sensitivity by steady-state plasma glucose method. %FMD was apparently decreased in microalbuminuric and macroalbuminuric patients compared with normoalbuminuric patients ($p < 0.001$). Moreover, %FMD was significantly correlated with the degree of albuminuria ($r = -0.38$, $p < 0.05$). On the other hand, vWF and hsCRP did not show significant difference between normoalbuminuric patients and microalbuminuric patients. In diabetic patients with macroalbuminuria, ADMA was significantly elevated compared to those with normoalbuminuria. Insulin sensitivity was significantly associated with urinary albumin excretion rate. These results suggested that endothelial dysfunction which may be due to impaired NO production and insulin resistance underlie the association between diabetic nephropathy and atherosclerosis in diabetic patients.

1. Introduction

Elevated urinary albumin excretion rate (UAER) is strongly associated with an increased risk of cardiovascular diseases, which is independent of conventional risk factors including hypertension, hyperlipidemia, and smoking, among individuals with and without type 2 diabetes [1,2]. This suggests that elevated UAER may be associated with atherosclerosis by the unidentified mechanism.

The endothelium plays a crucial role in the maintenance of vascular tone and structure, and endothelial dysfunction is a key feature of atherosclerosis. Nitric oxide (NO) is one of the important endothelium-derived vasoactive mediators. NO is involved in a wide variety of regulatory mechanisms of cardiovascular system, including vascular tone and vascular structure [3].

Flow-mediated endothelium-dependent vasodilatation (FMD) method is based on the endothelial stimulus of increased shear stress (the tangential force on the vessel wall exerted by flowing blood). Increased shear stress is caused by post-ischemic hyperemia and elicits a slow Ca^{+}-independent two to threefold increase in NO production [4,5]. Indeed,

* *Corresponding author.* Tel.: +81 6 6833 5012; fax: +81 6 6833 9865.
E-mail address: makinoh@hsp.ncvc.go.jp (H. Makino).
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Celemajer et al. reported that flow mediate vasodilatation was mainly blocked by N-monomethyl-L-arginine (an inhibitor of endothelial NO synthetase) [6].

To clarify the contribution of impaired NO production in vascular endothelium to the association between atherosclerotic disease and diabetic nephropathy, we examined FMD by ultrasonography. In addition, we measured asymmetric dimethyl arginine (ADMA), an endogenous NO synthesis inhibitor [3]. Since low-grade inflammation is another key feature of the pathophysiology of atherosclerosis [7], we further examined high-sensitive CRP, which is an inflammation marker, to investigate whether this feature is involved in the association between atherosclerotic disease and diabetic nephropathy.

It has recently been indicated that microalbuminuria and atherosclerosis are closely associated with insulin resistance [8–10], implying that insulin resistance may underlie these pathophysiological conditions although the causative relationship remains unknown. In the present study, we further examined insulin sensitivity in the type 2 diabetic patients with different stage of albuminuria and analyzed the correlation between insulin sensitivity and FMD, to investigate whether elevated UAER and endothelial dysfunction may be associated with insulin resistance.

2. Methods

2.1. Study subjects

We studied 217 patients with type 2 diabetes who were <75 years of age. Patients with a current acute illness (including clinically significant infectious disease) were excluded from this study. Twenty-four-hour urine collections were performed for two consecutive days to determine the stage of diabetic nephropathy. Creatinine clearance (Ccr) was calculated from the 24-h urine sample and serum creatinine levels. The patients were divided into three groups according to the UAER, as follows: normoalbuminuria (UAER <30 mg/day), microalbuminuria (30 ≤ UAER < 100 mg/day) and macroalbuminuria (UAER ≥ 300 mg/day). To exclude diabetic patients with nondiabetic kidney disease, we excluded patients with hematuria or abnormal urinary sediments. This study was conducted with the approval of National Cardiovascular Center Trust Ethics Committee, and patients gave written informed consent before participation.

2.2. Brachial artery flow-mediated dilatation

Using ultrasonography, arterial endothelium and smooth muscle function were measured by examining brachial artery responses to endothelium-dependent and endothelium-independent stimuli. Ultrasoundsonographic measurements were carried out according to the method described by Celemajer et al. [6]. Brachial artery diameter was measured from B-mode ultrasound images using 10-MHz liner array transducer (ProSound SSD-5500; Aloka, Japan) while an ECG trace was simultaneously recorded. The right brachial artery was scanned in longitudinal sections 1–10 cm above elbow, after at least 15 min of rest in the supine position, the skin surface was marked and the arm was kept in the same position during the study.

Baseline measurements of the diameter were carried out. Endothelium-dependent vasodilatation (flow-mediated dilatation) was determined by scans during reactive hyperemia. A pneumatic cuff placed around the forearm was inflated to 220 mmHg and was deflated after 4.5 min. The diameter of the brachial artery was scanned and recorded after dilation. After 10 min rest, the second control scan of the diameter was recorded. Then, sublingual glyceryl trinitrate spray (300 μg) was administered and 3.5 min later a final scan of the diameter was recorded.

Measurements of the vessel diameter were taken from the anterior to posterior "m" line (interface between the media and adventitia) at end-diastole, coincident with the R wave on a continuously recorded ECG. The diameters at four cardiac cycles were measured for each scan, and these results were averaged. Determinations of the FMD were carried out 45–60 s after the cuff release to measure a maximal diameter. Vasodilatation by reactive hyperemia or glyceryl trinitrate (NTG) was expressed as the percent change in diameter compared with the baseline values.

2.3. Insulin sensitivity test

Glucose utilization in response to insulin was evaluated with a newly modified steady-state plasma glucose (SSPG) method with octreotide acetate (Sandostatin; Novartis) after an overnight fasting period of 12 h [11]. Sandostatin (9.8-pmol bolus followed by a constant infusion of 73.5 pmol/h) and Humulin R insulin (45 pmol/kg bolus followed by a constant infusion at a rate of 4.62 pmol/(kg min); Eli Lilly) were infused intravenously for 120 min. Glucose in a final 12% solution containing KCl (0.5 μmol/(kg min)) was infused at a rate of 0.033 mmol/(kg min) (6 mg/(kg min)) through an antecubital vein via a constant infusion pump. Blood samples were drawn routinely at 0 and 120 min (9:00 and 11:00 a.m.) for the determination of glucose, insulin, and lipids. The value of glucose at 120 min (SSPG) was used as a marker of insulin sensitivity to glucose utilization. High SSPG levels showed peripheral insulin resistance.

Another marker of insulin resistance (IR) was estimated by calculating homeostasis model assessment (HOMA-IR) index ((fasting serum insulin (μU/ml) × fasting plasma glucose (mmol/l))/22.5) [12].

2.4. Measurement of vWF, hsCRP, and ADMA

vWF was determined in citrated plasma using a homemade enzyme-linked immunosorbent assay. Data are given as the percentage of pooled human plasma (set at 100%). Serum hsCRP concentration was determined by latex nephelometry method (SRL, Tokyo, Japan). Serum ADMA concentration was determined by high-performance liquid chromatography method (SRL, Tokyo, Japan).

2.5. Statistical analysis

Values are expressed as means ± S.D. Statistical analysis was performed by use of ANOVA followed by Scheffes' test. The

Table 1 – Characteristics of diabetic patients with normoalbuminuria, microalbuminuria, and overt nephropathy

Parameter	Stage of nephropathy		
	Normoalbuminuria	Microalbuminuria	Macroalbuminuria
n	121	71	25
Age (years)	62 ± 9	65 ± 8	66 ± 7
Men/women	76/45	34/37	12/13
Duration of diabetes (years)	12 ± 8	14 ± 8	18 ± 8*
BMI (kg/m²)	25.0 ± 3.7	25.1 ± 3.7	25.1 ± 3.9
SBP (mmHg)	128 ± 13	133 ± 15	141 ± 19*
DBP (mmHg)	74 ± 10	73 ± 9	76 ± 10
FBS (mmol/l)	7.4 ± 1.4	7.5 ± 1.5	7.5 ± 1.9
HbA1c (%)	8.3 ± 1.5	8.9 ± 1.7*	8.8 ± 1.4
HOMA-IR	1.62 ± 0.98	1.71 ± 2.06	2.29 ± 1.47
Total cholesteroyl (mmol/l)	4.86 ± 0.90	4.86 ± 0.90	4.73 ± 0.75
Serum creatinine (μmol/l)	70 ± 20	60 ± 20	110 ± 40
Urinary albumin (mg/day)	10 ± 7	85 ± 79**	583 ± 576**
Creatinine clearance (ml/s)	1.43 ± 0.52	1.50 ± 0.63	0.73 ± 0.43**
ACEI or ARB (yes/no)	36/85	24/47	11/14*
Statin (yes/no)	45/76	25/46	10/15
Current smoker (yes/no)	11/110	7/64	6/19

*$p < 0.05$, **$p < 0.01$ vs. normoalbuminuria, mean ± S.D.

strength of correlation between variables was tested by linear correlation and multiple regression analysis. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. *Patients characteristics*

Table 1 shows the clinical characteristics of three groups. There was no significant difference in age, gender, BMI, FBS and total cholesterol among the three groups. HbA1c of diabetic patients with microalbuminuric patients was significantly higher than normoalbuminuric patients. Systolic blood pressure of macroalbuminuric patients was significantly higher than normo- and micro-albuminuric patients. Creatinine clearance was significantly decreased in macroalbuminuric patients compared with normo- and micro-albuminuric patients. There is no significant difference in rate of patients taking ACE/ARB between normo- and micro-albuminuric patients whereas the rate of patients taking ACE/ARB of macroalbuminuric patients were significantly large compared with other two groups. On the other hand, there is no significant difference in rate of patients taking statin among three groups.

3.2. *%FMD of diabetic patients*

We studied the endothelial function by FMD using brachial artery echography. %FMD (Δhyperemia) of diabetic patients with microalbuminuria (4.5 ± 3.7%) and macroalbuminuria (4.2 ± 2.4%) was apparently decreased compared with those of diabetic patients with normoalbuminuria (6.6 ± 3.7%) (Fig. 1A). Moreover, %FMD was significantly correlated with UAER in normo- and micro-albuminuric patients independent of age, HbA1c, and systolic blood pressure by multiple regression analysis ($r = -0.38$, $p < 0.05$) (Fig. 2). Dilatation of brachial artery by NTG (ΔNTG) showed no difference among three groups (Fig. 1B).

3.3. *vWF, hsCRP, and ADMA of diabetic patients*

We studied other atherosclerotic markers, that is, vWF, hsCRP, and ADMA. There was no significant difference of the levels of vWF and hsCRP between normoalbuminuric and microalbu-

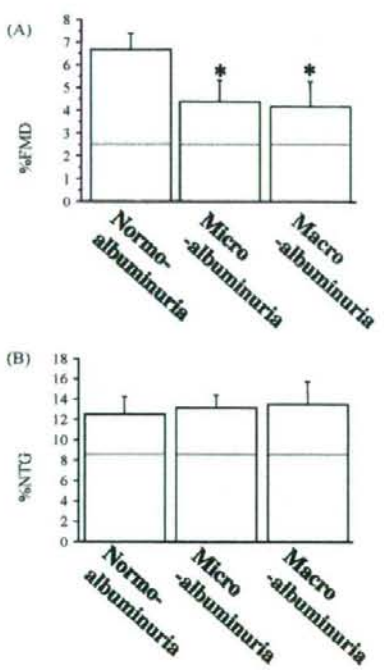

Fig. 1 – %FMD (A) and %NTG (B) in diabetic patients with normoalbuminuria, microalbuminuria and macroalbuminuria. Each value means (means ± S.D.), *$p < 0.001$.

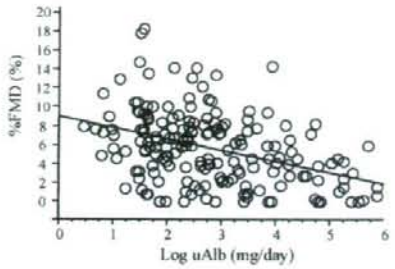

Fig. 2 – Correlation between degree of UAE and %FMD in normo- and micro-albuminuric diabetic patients. There was a significant correlation between both variables ($r = -0.38$, $p < 0.05$, $n = 192$).

minuric patients (Table 2). Although the levels of ADMA in microalbuminuric patients did not show significant difference compared with normoalbuminuric patients (Table 2), the levels of ADMA in macroalbuminuric patients were significantly elevated compared with normoalbuminuric patients (Table 2).

3.4. *Insulin sensitivity of diabetic patients*

We studied the insulin sensitivity by SSPG method. The levels of SSPG had weak but significant correlation with both %FMD ($r = -0.175$, $p < 0.05$) and UAER ($r = 0.181$, $p < 0.05$) independent of age, HbA1c, and systolic blood pressure (Fig. 3A, B).

4. Discussions

There were two main findings from this investigation in type 2 diabetic patients. First, diabetic micro- and macro-albuminuric patients showed significant reduction of %FMD compared with normoalbuminuric patients. This finding suggests that the endothelial dysfunction may account for the association between atherosclerosis and albuminuria in diabetic patients. Second, the level of SSPG was significantly associated with both UAER and %FMD. This finding suggests that insulin resistance may play a role in both atherosclerosis and nephropathy in type 2 diabetic patients.

In diabetic patients, %FMD is decreased compared with healthy control [13,14]. These reports indicated that diabetes mellitus is associated with endothelial dysfunction due to

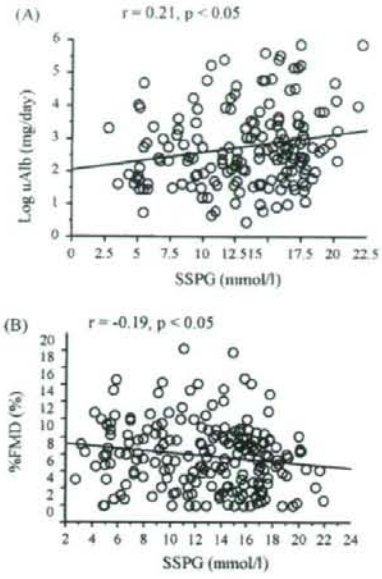

Fig. 3 – Correlation between SSPG and UAE (A), and correlation between SSPG and %FMD (B) in normo- and micro-albuminuric patients.

impaired NO production. However the involvement of endothelial dysfunction in diabetic nephropathy has been unclarified. We demonstrated that microalbuminuric and macroalbuminuric patients showed significant decreased %FMD compared with normoalbuminuric patients. In contrast, there was no significant difference of vWF between normoalbuminuric patients and microalbuminuric patients. vWF is a product of vascular endothelial cell, and induces coagulation and platelet aggregation [15]. These findings suggest that endothelial dysfunction due to impaired NO production is specifically induced in micro- and macroalbuminuric patients. One recent report showed that coronary endothelium-dependent dilatation was impaired in a rat model of spontaneous albuminuria [16] supporting this hypothesis. It has been reported that renal NO production was decreased in rodent diabetic model [17]. This report suggests that decrease of NO production may play a role in the

Table 2 – Parameters of atherosclerosis in diabetic patients with normoalbuminuria, microalbuminuria, and overt nephropathy

Parameter	Stage of nephropathy		
	Normoalbuminuria	Microalbuminuria	Macroalbuminuria
von Willebrand factor (%)	147 ± 44	146 ± 44	143 ± 41
High-sensitive CRP (ng/ml)	976 ± 1401	951 ± 1110	1113 ± 1187
ADMA (nmol/ml)	0.45 ± 0.06	0.47 ± 0.07	0.55 ± 0.11*

*$p < 0.001$ vs. normoalbuminuria, mean ± S.D.

progression of diabetic nephropathy as well as atherosclerosis. We investigated serum ADMA levels in diabetic patients. There was no significant difference of ADMA levels between normo- and micro-albuminuric patients, suggesting that the reduction of %FMD in microalbuminuric patients might not be resulted from the elevation of ADMA. However, in macroalbuminuric patients, ADMA level was significantly higher than normoalbuminuric patients. Vallance et al. reported that the level of ADMA was elevated in patients with chronic renal failure and suggested the involvement of this in coronary artery disease [18]. They indicate that the elevation of ADMA might be associated with atherosclerosis in patients with chronic renal disease [18]. Thus, this finding suggests that the elevation of ADMA might be associated with atherosclerotic change in diabetic patients with macroalbuminuria.

An association between chronic low-grade inflammation and development of atherosclerotic disease has been observed in basic and clinical studies [7,19–21]. Furthermore, diabetic patients have higher CRP levels than normal subjects, suggesting that chronic inflammation may contribute diabetic atherosclerotic complication [22]. An association between micro- and macro-albuminuria and inflammation has also been reported [23,24]. However, several other studies showed that inflammatory molecules were not associated with micro- and macro-albuminuria [25–27]. Thus the knowledge of this association is still controversial. Also we could not demonstrate the association between CRP and development of microalbuminuria in this study. Our data suggested that chronic low-grade inflammation might not be involved in the association between atherosclerosis and microalbuminuria. However, since this study was performed by cross-sectional analysis and other inflammatory marker was not measured, further study is necessary for demonstrating this hypothesis.

Insulin resistance has been reported to play an important role in the development and progression of atherosclerotic coronary disease [8,9]. Recently the association between insulin resistance and microalbuminuria was also reported [10]. Nakamura et al. demonstrated that administration of pioglitazone to diabetic patients attenuated UAER [28]. In this study, we showed that both the UAER and %FMD were significantly correlated to the level of SSPG. These findings suggest that insulin resistance may be involved in both the elevated urinary albumin excretion and endothelial dysfunction due to impaired NO production. However, HOMA-IR, another insulin sensitivity marker which reflects insulin sensitivity in both the liver and the periphery, did not show significant difference among three groups, suggesting that particularly peripheral insulin resistance may be important for the pathogenesis of atherosclerosis and diabetic nephropathy.

In summary, we showed that %FMD of micro- and macro-albuminuric patients was decreased compared with those of normoalbuminuric patients, without showing significant difference in other various atherosclerotic markers. Furthermore, the level of SSPG was significantly correlated to UAER and %FMD. These findings suggest that endothelial dysfunction which may be due to impaired NO production underlies the mechanism of association between elevated urinary albumin excretion and atherosclerosis in diabetic patients, and that peripheral insulin resistance might be possibly involved in both diabetic nephropathy and atherosclerosis.

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Association between insulin resistance and endothelial dysfunction in type 2 diabetes and the effects of pioglitazone

Masaaki Suzuki [a,*], Itaru Takamisawa [b], Yasunao Yoshimasa [b], Yutaka Harano [c]

[a] *Department of Diabetes and Metabolic Disease, Osaka General Medical Center, 3-1-56 Mandai-higashi, Sumiyoshi-ku, Osaka 558-8558, Japan*
[b] *Division of Diabetes and Atherosclerosis, Department of Medicine, National Cardiovascular Center, Japan*
[c] *Department of Nutrition, Koshien Univesity College of Nutrition, Japan*

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Abstract

Endothelial dysfunction is regarded as an early stage of atherosclerosis, and plays a role in the development of atherosclerotic diseases. Insulin resistance is related to the atherosclerotic process. In this study, we examined the association between endothelial function and insulin resistance in 48 subjects with type 2 diabetes. In addition, the effects of pioglitazone treatment on endothelial function and insulin resistance were investigated in a subgroup of subjects. Endothelial function of the brachial artery was non-invasively assessed using ultrasound technique. We measured flow-mediated endothelium-dependent vasodilation (FMD) and glyceryl trinitrate-induced endothelium-independent vasodilation (GTN). Insulin sensitivity was measured by the steady-state plasma glucose (SSPG) method. High SSPG levels indicate insulin resistance. There was a significant inverse correlation ($r = -0.462$, $p < 0.001$) between SSPG and FMD. Systolic blood pressure was inversely correlated with FMD ($r = -0.360$, $p < 0.013$). By multiple regression analysis, insulin resistance was the sole predictor of FMD. The effects of chronic treatment with pioglitazone were assessed in 10 subjects with type 2 diabetes. The increase in FMD significantly correlated with the decrease in SSPG. There is a significant association between vascular endothelial dysfunction and insulin resistance in type 2 diabetes. This result was supported by the effects of the insulin sensitizer, pioglitazone.

Keywords: Endothelial dysfunction; Insulin resistance; Pioglitazone

1. Introduction

Endothelial dysfunction is thought to be an important early feature in the development of atherosclerosis and occurs in subjects with type 2 diabetes mellitus [1–4]. Insulin resistance is also associated with atherosclerosis and is observed in subjects with type 2 diabetes [5,6]. We previously reported the association between endothelial dysfunction and insulin resistance in patients with essential hypertension [7]. However, the mechanisms responsible for endothelial dysfunction and insulin resistance in hypertension might be different from those of type 2 diabetes. Therefore, we evaluated the relationship between endothelial dysfunction and insulin resistance in patients with type 2 diabetes. Thiazolidinediones, an agonist for the peroxisome proliferator-activated receptor γ (PPARγ), improve insulin resistance. If there is a significant relationship

* Corresponding author. Tel.: +81 6 6692 1201; fax: +81 6 6606 7000.
E-mail address: masuzuki@gh.pref.osaka.jp (M. Suzuki).

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between endothelial dysfunction and insulin resistance, thiazolidinediones might influence endothelial function. Therefore, we examined the effects of pioglitazone on endothelial dysfunction and insulin resistance in a subgroup of subjects with type 2 diabetes to verify the relationship between endothelial dysfunction and insulin resistance.

The main purpose of this study was to investigate the relation between vascular endothelial dysfunction and insulin resistance in type 2 diabetes. In addition, the influence of pioglitazone treatment was examined.

2. Subjects and methods

2.1. Subjects

Forty-eight (30 males and 18 females) patients with type 2 diabetes were recruited in the Department of Diabetes and Atherosclerosis of the National Cardiovascular Center. The subjects did not have diabetic retinopathy or nephropathy. Subjects were included on the basis of the following criteria: age between 40 and 79 years, body mass index (BMI) between 17 and 35 kg/m^2, type 2 diabetes confirmed by American Diabetes Association criteria [8]. Subjects were excluded from participation if they had coronary heart, peripheral vascular, renal, hepatic or other endocrine diseases. Subjects were excluded if they had a resting seated blood pressure greater than 150 mmHg systolic or greater than 90 mmHg diastolic, or were taking anti-hypertensive drugs. Diabetes duration was 5.3 ± 1.9 years (3–7 years). Diabetes treatment regimens included diet alone (27 subjects), sulfonylures (18 subjects) and metformin (3 subjects).

The 48 subjects had an average age of 64 ± 1 years, with a mean BMI of 24.6 ± 0.3 kg/m^2, HbA_{1c} of 8.6 ± 0.2%, total cholesterol of 199 ± 5 mg/dl, HDL-cholesterol of 43 ± 2 mg/dl and triglycerides of 137 ± 14 mg/dl. Mean systolic and diastolic blood pressures were 131 ± 3 and 74 ± 2 mmHg, respectively.

Of the 48 diabetic subjects, 10 subjects were started on a single 15 or 30 mg-tablet of pioglitazone (Actos, Takeda Pharmaceuticals, Tokyo, Japan) by mouth each day. Inclusion criteria of the pioglitazone treatment were male, non-smoker, diet alone treatment and mild to severe insulin resistance (SSPG > 160 mg/dl). They received a mean dose of 25.5 ± 2.3 mg/day (30 mg/day: seven subjects and 15 mg/day: three subjects) of pioglitazone for 16.3 ± 1.6 weeks (10–20 weeks). The secondary assessments of endothelial function and insulin sensitivity were performed after the pioglitazone treatments.

The study protocol was approved by the ethics committee of the National Cardiovascular Center. The experiments were conducted with the understanding and the consent of each participant.

2.2. Methods

2.2.1. Assessment of endothelial function

Using the ultrasound method, arterial endothelium and smooth muscle function were measured by examining brachial artery responses to endothelium-dependent and endothelium-independent stimuli. Ultrasound measurements were carried out based on the method described by Celermajer et al. [9] and our method was reported previously [7]. The assessments were performed after an overnight fast in a quiet air-conditioned room (22–23 °C). The diameter of the brachial artery was measured on B-mode ultrasound images, with the use of a 10-MHz linear array transducer (ProSound SSD-5500, ALOKA, Tokyo, Japan). The right brachial artery was scanned in longitudinal sections 1–10 cm above the elbow, after at least 15 min of rest in the supine position. After the detection of the right transducer position, the skin surface was marked and the arm was kept in the same position during the study. All scans were recorded using a super-VHS videocassette recorder (SONY, SVO-9500MD), and analyzed later.

At first, baseline measurements of the diameter were carried out. Endothelium-dependent vasodilation (flow-mediated dilation) was determined by the scans during reactive hyperemia. Because flow-mediated vasodilation was mainly blocked by *N*-monomethyl-L-arginine (an inhibitor of endothelial nitric oxide synthase) this dilation was regarded as endothelium dependent [10]. A pneumatic cuff placed around the forearm was inflated to 220 mmHg and was deflated after 4.5 min. The diameter of the brachial artery was scanned and recorded after deflation. After 10–15 min rest, the second control scan of the diameter and the flow velocity was recorded. Then, sublingual glyceryl trinitrate spray (300 μg) was administered and 3.5–4 min later a final scan of the diameter was recorded.

Measurements of the vessel diameter were taken from the anterior to the posterior 'm' line (interface between the media and adventitia) at endo-diastole, coincident with the R wave on a continuously recorded electrocardiogram. The diameters at four cardiac cycles were measured for each scan, and these results were averaged. Determinations of the flow-mediated dilation were carried out 45–60 s after the cuff release to measure a maximum diameter. Vasodilation by reactive hyperemia (flow-mediated dilation, FMD) or glyceryl trinitrate (GTN) was expressed as the percent change in diameter compared to the baseline values.

2.2.2. Insulin sensitivity test

Glucose utilization in response to insulin was evaluated by a modified steady state plasma glucose (SSPG) method [6,7,11] using Sandostatin (octreotide acetate; Novartis, Basel, Switzerland) after an overnight fasting for at least 12 h. Sandostatin (9.8 pmol in bolus followed by a constant infusion of 73.5 pmol/h) and Novolin R insulin (Novo Nordisk S/A, Tokyo, Japan, 45 pmol/kg [7.5 mU/kg] in a bolus followed by a constant infusion at a rate of 4.62 pmol/kg/min [0.77 mU/kg/min]) were infused intravenously for 120 min.