intolerance in Hisayama was significantly increased at the examination in 1988 compared to those conducted in the 1960s and 1970s [7,15]. This trend is also likely to have played a role in leading to the positive association between HbA_{1C} and coronary atherosclerosis in men in the present study. The reason why there was no significant association between HbA_{1C} and coronary atherosclerosis in women is not well understood, but a similar gender difference in the relationship between HbA_{1C} and CHD has been reported in the recent EPIC (European Prospective Investigation into Cancer) study in Norfolk, in which the risk of CHD among men had already been significantly increased in those with an HbA_{1C} concentration of 5.0–5.4% compared to those with an HbA_{1C} concentration of less than 5.0%, but the coronary risk in women was significantly increased only at an HbA_{1C} concentration of 6% or greater [13].

WHR and obesity

A positive correlation between obesity and coronary atherosclerosis has been reported in Okumiya's Hisayama study and the PDAY studies, in which BMI was identified as a risk factor, but no investigation was done for WHR [17–19,24]. However, epidemiologic studies have demonstrated that indices of abdominal obesity, such as WHR and waist circumference, are more strongly correlated with CHD than BMI [4,27]. This is consistent with the result of multiple regression analysis in the present study, revealing the correlation of coronary atherosclerosis with WHR but not with BMI. As for gender difference, the epidemiologic studies performed in western countries show a correlation between WHR and CHD both in men and in women [4,27], but the correlation between WHR and coronary atherosclerosis was significant only in women in the present study. This difference may result from the difference in the baseline value of WHR between Japanese and Western populations. The mean WHR was generally smaller in men and larger in women in the present study (Table 1) than in Western studies. For example, the mean value of WHR is 0.94 for men and 0.79 for women in the EPIC study [25]. Abdominal obesity is closely related to metabolic syndrome. Many epidemiological studies, including Ninomiya's Hisayama study, demonstrated that metabolic syndrome is a risk factor for CHD [22]. However, the effects of metabolic syndrome on coronary atherosclerosis have not been thoroughly investigated yet and should be elucidated in the future.

Smoking

Evidence from many epidemiologic studies has definitively identified smoking as a strong risk factor for CHD [12,14,40]. On the other hand, there is no clear agreement on the correlation between smoking and coronary atherosclerosis. Some studies have shown a significant correlation between the two [1,19,28], but others, like the present study, have not [8,10,24,30]. The reason for this discrepancy between the clinical and autopsy findings is not thoroughly understood. However, factors other than atherogenesis, such as vasospasm [33], plaque erosion [2], and increased coagulability [20], must be considered when the correlation between smoking and CHD is evaluated. Particularly, vasospasm can be a very important risk factor for Japanese people, because Japanese people are more sensitive to vasospastic agents than Caucasians [26]. Examining angiographic data of Japanese CHD patients, Nobuyoshi et al. [23] found that smoking was strongly related to coronary vasospasm, but not to coronary stenosis. Thus, these results suggest that smoking is not significantly correlated with coronary atherosclerosis at least in Japanese people.

Conclusions

The present study revealed that aging, hypertension, hypercholesterolemia, obesity, and glucose intolerance are risk factors for coronary atherosclerosis in recent Japanese populations. As they are well-known risk factors for CHD as well, the results suggest that these factors may play a part in the development of CHD by affecting coronary atherosclerosis. However, smoking, another strong risk factor for CHD, was not found to be a risk for coronary atherosclerosis in the present study. Mechanism(s) other than atherosclerosis must be considered in evaluating the correlation between smoking and CHD. Finally, the significance of the metabolic risk factors for coronary atherosclerosis was different between men and women. The interaction of the various risk factors and underlying mechanisms for the observed gender differences should be elucidated in the future.

Acknowledgments

The authors gratefully acknowledge Hiroshi Fujii for his expert technical assistance. This study was supported by Grants-in-Aid for Scientific Research A (No. 18209024) and C (No. 20591063) from the Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan.

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Atherosclerosis 202 (2009) 573–581

ATHEROSCLEROSIS

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Overexpression of heme oxygenase-1 in coronary atherosclerosis of Japanese autopsies with diabetes mellitus: Hisayama study

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Received 24 December 2007; received in revised form 11 May 2008; accepted 18 May 2008
Available online 8 June 2008

Abstract

Few studies regarding the topographical expression of heme oxygenase-1 (HO-1) and its pathophysiological role in human coronary atherosclerotic lesions, particularly in relation to type 2 diabetes mellitus (DM) and intimal angiogenesis, have been reported. HO-1 expression was immunohistochemically examined in 312 tissue blocks of coronary arteries obtained from 53 Japanese autopsy cases in Hisayama cohort study that included 19 diabetic subjects and 34 age- and sex-matched non-diabetic subjects (56–93 years old, mean ± S.D.: 73 ± 10). The HO-1 was ubiquitously distributed in atherosclerotic intima, and was mainly expressed by macrophages and endothelial cells, and partly by smooth muscle cells. The prevalence of HO-1 expression increased as the lesion type (as classified by the American Heart Association (AHA) Committee) and stenotic grade progressed ($p < 0.0001$), and was significantly higher in diabetic than in non-diabetic subjects ($p < 0.01$). This HO-1 overexpression was associated with greater CD-68-positive macrophage infiltration ($p = 0.005$). Interestingly, the distribution of HO-1-positive cells was accentuated in coronary atherosclerotic lesions with intimal microvessels in diabetic subjects ($p < 0.05$), particularly those with hypercholesterolemia ($p < 0.05$), and was preferentially distributed in the shoulder region of atherosclerotic lesion type IV in the AHA classification ($p < 0.01$). In conclusion, HO-1 expression was distributed in overall human coronary atherosclerotic lesions, particularly in diabetic subjects, indicating that HO-1 expression is intimately associated with atherogenesis and may play an important role as an adaptive molecule in the inflammatory-repair process. The association of HO-1 overexpression with a greater extent of intraplaque angiogenesis suggests a multi-faceted role for HO-1 in modulating the progression of atherosclerosis.

Keywords: Heme oxygenase-1; Human coronary artery; Atherosclerosis; Diabetes mellitus; Immunohistochemistry

1. Introduction

Recent emerging evidence supports the hypothesis that atherosclerosis is a chronic inflammatory disease evoked and enhanced by multifactorial etiologies such as dyslipidemia, hypertension, diabetes mellitus (DM), smoking, adiposity and others [1]. All these risk factors are related to oxidative stress. DM in particular has become a worldwide epidemic with its global incidence and prevalence rapidly increasing in both developing and developed countries [2]. Atherosclerosis is the major macro-vascular complication of DM, and coronary heart disease is highly prevalent as a major cause of morbidity and mortality in diabetics [3].

Heme oxygenase (HO) is an initial and rate-limiting enzyme in the oxidative degradation of heme to equimolar quantities of bilirubin as an antioxidant, carbon monoxide (CO) as a vasodilator, and free iron that is promptly sequestered into ferritin [4]. Among HO isoforms, HO-1 can be transcriptionally induced by a variety of

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doi:10.1016/j.atherosclerosis.2008.05.057

pathophysiological conditions or substances in the cardiovascular system [4,5]. Furthermore, HO-1 has been assumed to have powerful cytoprotective effects on the vascular wall, particularly on endothelial cells (ECs), against oxidation stresses and inflammatory stimuli, mainly by decreasing reactive oxygen stresses [5]. Recent data obtained from animal models of atherosclerosis genetically or pharmacologically manipulated to suppress or overexpress HO-1 confirm that HO-1 is responsible for being atheroprotective [5,6]. In fact, HO-1 gene promoter polymorphism has been reported to relate to the susceptibility to cardiovascular disorders [7].

In addition, HO-1 has recently been assumed to intimately participate in angiogenesis in physiologic and pathologic conditions mainly via vascular endothelial growth factor (VEGF) function [8,9]. Newly formed blood vessels that are ubiquitously distributed in human atherosclerotic plaque [10–12] are assumed to play an important role not only in atherosclerotic plaque progression but also in the destabilization leading to plaque rupture [13–15]. Few studies regarding the relationship between HO-1 expression and plaque angiogenesis have been reported, and the pathophysiological role of HO-1 in human atherosclerotic lesions in diabetics remains unknown.

Wang et al. [16] reported that HO-1 was expressed mainly by macrophages and ECs, and partly by smooth muscle cells (SMCs) in atherosclerotic lesions, particularly fibrofatty lesions, in the human aorta. To our knowledge, however, little is known about the topographical expression of HO-1 and its pathophysiological role in the atherogenesis of human coronary arteries.

The purpose of the present study is to clarify the pathophysiological role of HO-1 in atherogenesis, particularly in diabetics. Thus, we morphometrically examined the relationship between HO-1 expression and macrophage infiltration and intimal neovascularization as morphological phenotypes of the inflammation-repair process in atherosclerotic lesions.

2. Materials and methods

2.1. Subjects and light microscopic examination

Since 1961, we have been conducting a long-term prospective cohort study of cardiovascular disease in the town of Hisayama, a suburb of Fukuoka City in Southern Japan. In 1988, a screening survey of 2742 residents over 40 years of age for the present study was performed in the town [17]. A total of 2742 residents over 40 years of age consented to participate in this survey examination (attendance rate: 80.9%). During the follow-up period from 1988 to July 31, 1996, 310 subjects died and 244 were autopsied (autopsy rate: 78.7%). Among them, 19 subjects with type 2 DM, i.e. with a HbA1c serum level more than 6.0%, and 34 age- and gender-matched NDM controls (56–93 years old, mean ± S.D.: 73 ± 10), who were autopsied within 16 h after death and in whom the histopathology of coronary arteries could be appropriately examined, were randomly selected for this study. Hypercholesterolemia corresponded to a serum level ≥200 mg/dl. Hypertension was defined as blood pressure ≥140/90 mmHg and/or current use of antihypertensie agents. Metabolic syndrome (MetS) was defined by using the criteria recommended in the National Cholesterol Education Program's adult Panel III guidelines [18] with a modification, abdominal obesity was assessed as a waist circumference >90 cm in men and >80 cm in women according to the International Obesity Task Force central obesity criteria for Asia [19]. The study was approved by the ethics committee of the Department of Pathology, Kyushu University, and was performed in accordance with the ethics standards laid down by the 5th revised Declaration of Helsinki, 2000.

The mean age, gender and variables that were selected as possible predictors for coronary atherosclerosis included serum levels of total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C) and glycohemoglobin (HbA1c), systolic and diastolic blood pressure

Table 1
Mean values or frequencies of cardiovascular risk factors in subjects examined

	Diabetes ($n=19$)	Non-diabetes ($n=34$)	p
Male (%)	73.7	64.7	0.50
Age (year)	69.8 (65.3–74.3)	74.4 (71.1–77.7)	0.12
Total cholesterol (mg/dl)	195.8 (174.1–217.5)	192.9 (176.5–209.3)	0.83
High-density lipoprotein cholesterol (mg/dl)	47.9 (42.6–53.2)	48.7 (44.2–53.2)	0.83
Triglycerides (mg/dl)	146.6 (103.3–189.9)	115.6 (94.4–136.8)	0.16
HbA1c (%)	7.0 (6.2–7.8)	5.4 (5.2–5.6)	<0.001
Systolic blood pressure (mmHg)	130.0 (124.6–135.4)	127.6 (121.9–133.2)	0.59
Diastolic blood pressure (mmHg)	72.5 (68.0–77.0)	72.8 (69.4–76.2)	0.92
Body mass index (kg/m^2)	22.2 (21.3–23.1)	20.9 (19.9–21.9)	0.08
Waist-to-hip ratio	0.94 (0.90–0.98)	0.90 (0.88–0.92)	0.08
Metabolic syndrome (%)	26.3	9.4	<0.001
Smokers (%)	63.2	61.8	0.92
Drinkers (%)	52.6	41.2	0.42
Sudden death (%)	5.3	8.8	0.64

All values except percentages are expressed as means and 95% confidence interval. Sudden death: death 1 h after clinical onset of symptoms.

(BP), body mass index (BMI), waist-to-hip ratio (WHR), the frequency of MetS, and smoking and drinking habits. These are shown in Table 1.

2.2. Histopathological examination

In all subjects, the heart was fixed with 10% formalin. Six specimens of coronary arteries were taken from each heart for histopathological examination. According to the American Heart Association (AHA) classification of coronary sections, these included segments 1, 2, 6, 7, 11 and 12. All specimens embedded in paraffin were cut into 3-μm-thick serial sections. Specimens with severe calcification were decalcified with acetic acid, and, therefore, the histological evaluation of the extent of calcium was based on the visualization of the calcified matrix and not on calcium per se. Histological sections were stained with hematoxylin and eosin (HE), Elastica-van Gieson (EVG) and Masson's trichrome (MT). The atherosclerotic lesion type of each specimen was carefully classified in accordance with the definitions proposed by the Committee on Vascular Lesions of the Council on Arteriosclerosis of AHA [20]. The atherosclerotic lesions were again classified into two categories, with lesion types I through III classified as early lesions and lesion types IV through VI classified as advanced lesions.

2.3. Immunohistochemistry

An anti-human HO-1 rabbit polyclonal antibody against recombinant N-terminal 15 amino-peptide of HO-1 (HC3001) and this recombinant HO-1 amino-peptide were purchased from Biomol International LP (PA, USA). The cell species-specific antibodies used were as follows: CD68 (KP-1, Dako A/S, Glostrup, Denmark) for the monocytes/macrophages, α-smooth muscle actin (α-SMA) for SMCs and CD34 (Novocastra, Newcastle upon Tyne, UK) mainly for the ECs.

An immunohistochemical examination was performed according to the standard two-step technique using polymeric conjugates as secondary antibodies (ChemMate EnVision, DAKO A/S) as previously reported [21]. In brief, the sections were deparaffinized, boiled in 10 mM citrate buffer (pH 6.0) in a pressure vessel to unmask the antigens, and then incubated with 3% skimmed milk-PBS solution to minimize the nonspecific binding of the primary antibody. The sections were incubated with the primary antibodies overnight at 4 °C in a moisture chamber, then incubated with the appropriate polymeric conjugate. To inhibit any endogenous peroxidase activity, the sections were incubated with % (wt/vol) H_2O_2–methanol solution. The visualization of a positive reaction was developed using a peroxidase substrate solution containing 3, 3′-diaminobenzidine tetrahydrochloride, and the sections were then lightly counterstained with hematoxylin.

To confirm the immunohistochemical specificity of each reaction, non-immune rabbit and mouse isotype IgGs were used as the negative controls instead of the respective primary antibody. Human liver and spleen tissue sections were used as the positive controls for HO-1 immunohistochemistry. In addition, the specificity of the anti-human HO-1 antibody was further confirmed with an antibody absorption experiment; namely, the primary anti-HO-1 antibody solution was incubated with a fivefold excess of recombinant human HO-1 at the molar ratio for 1 hr at room temperature, then centrifuged and the supernatant was used for immunohistochemical examination.

2.4. Morphometric study

By using an Olympus high-image color-camera OHD-200 and Scion Image Soft, the luminal stenosis of each coronary artery section was calculated as reported previously [11]. According to the Scion Image program instructions, the necrotic core size and calcified matrix area of the intima in each section were also measured, and the area percentage of each parameter per intimal area was then calculated.

The number of CD68-, α-SMA- or HO-1-positive cells in the intima at high-power field (HPF, 400×) were counted in more than 2 areas where the positive cells were mostly distributed, and was recorded as the number of positive cells. The immunohistochemically positive cell numbers/HPF for respective cell species were compared with those in matched serial sections separately stained with other immunohistochemical stains. The total number of intimal blood vessels, which were lined with CD34-positive ECs, was also counted under HPF [11]. To analyze topographical differences in the HO-1-positive cell distribution, the AHA-Type IV lesion (atheormatous plaque) was classified into the following four area categories depending on its location: plaque shoulder (S), fibrous cap (Fc), deep region of necrotic core (D), and other (O) [21]. The above-mentioned comparison was also made among these categories.

2.5. Statistical analysis

The results are presented as means ± S.D. unless otherwise stated. The data were statistically analyzed by means table ANOVA with Fisher's post hoc test and the chi-square test, and a comparison of the non-paired non-parametric data among more than three groups was made using the Kruskal–Wallis rank test followed by Mann–Whitney's *U*-test. The correlation between the HO-1-positive cell number/HPF and the intimal microvessel number was analyzed using Spearman's correlation analysis and j-statistic analysis. A value of $p < 0.05$ was considered statistically significant. Tests were performed using Statview software (SAS Institute, Cary, NC, USA).

3. Results

3.1. Clinical characteristics of subjects examined in DM and NDM groups

The clinical characteristics of the subjects in the diabetic and nondiabetic (DM and NDM, respectively) groups are shown in Table 1. The mean serum level of HbA1c and the frequency of MetS were statistically higher in the DM group than the NDM group, but other possible risk factors for cardiovascular disease were not, including serum levels of TC and HDL-C, systolic and diastolic BP, BMI, WHR current smokers and drinkers, and the incidence of sudden death.

3.2. Histopathology of coronary arteries

The mean heart weight was not statistically different between the DM and NDM groups (data not shown). Out of 318 coronary arterial specimens obtained from 53 subjects, 6 specimens were avoided due to marked artificial changes, and 312 coronary arterial specimens available for analysis were classified according to the AHA classification. Forty percent were classified as diffuse intimal thickening (DIT), 5% as type I, 10% as type II, 21% as type III, 4% as type IV, 18% as type V, and 2% as type VI (Supplementary data Fig. I). Therefore, the major histologic types consisting of about three-fourths in this study were DIT and early atherosclerotic lesions of types I–III.

To characterize the histopathological differences of atherosclerotic plaque in DM and NDM, the area percentages of necrotic core size, luminal stenosis and calcified matrix area were morphometrically assessed, and the occurrence of complex lesions such as thrombus formation, plaque rupture and intraplaque hemorrhage were counted (Supplementary data Table I). Necrotic core was apparent in 42 specimens (17 in DM and 25 in NDM), and 53 calcification foci (23 in DM and 30 in NDM) were noticed in atherosclerotic intimas. The area percentages of the necrotic core size and calcified matrix area were not statistically different between the two groups. As the coronary atherosclerotic lesions progressed from type I to type VI, the luminal stenosis correlatively progressed ($P < 0.0001$, data not shown), but was not significantly different between the DM and NDM groups. In addition, the mean percentage of luminal stenosis was higher in both the early (lesion types I–III) and advanced lesions (types IV–VI) in DM than it was in NDM, but this difference was not statistically significant. Among six sections with type VI lesions, thrombus formation without atheroma rupture was associated with DM in two sections; two sections showed atheroma rupture, one with DM and one with NDM; and two sections were complicated with intraplaque hemorrhage around the calcified area, one each in DM and NDM. However, the frequency of occurrence of these complicated lesions also shows no statistical significance between DM and NDM (Supplementary data Table I).

3.3. Immunohistochemical expression of HO-1 and HO-1-positive cell species in coronary artery

To characterize the topographical distribution of HO-1 in DIT and atherosclerotic plaques, all 312 blocks were immunohistochemically examined, and HO-1-positive cell numbers/HPF were calculated in each tissue type. Furthermore, to define the HO-1-positive cell species, the sequential sections of each block were immunohistochemically examined with CD68, α-SMA and CD34 for macrophages, SMCs and ECs, respectively. The expression of HO-1 was ubiquitously distributed in atherosclerotic lesions, but was extremely scarce in DIT, where HO-1 was expressed only by luminal ECs. The number of HO-1-positive cells/HPF increased as the atherosclerotic lesions progressed (data not shown, $p < 0.0001$ by Kruskal–Wallis rank test). In atherosclerotic lesions, HO-1 was mainly expressed by macrophages/monocytes (Figs. 1B and C, and 2B and C), ECs not only of the luminal surface (Fig. 1B and F) but also intimal newly formed blood vessels (Fig. 2B and D), and partly by SMCs, particularly in the fibrous cap of lesions types IV–VI (Fig. 1B, D, F and G). HO-1 reactivity was apparent not only in foamy macrophages around the atheroma (Fig. 1B and C) and within fatty streaks but also in non-foamy macrophages infiltrating around intimal microvessels (Fig. 2B and C). Interestingly, the macrophages scattered around the microvessels and ECs of these newly formed blood vessels were frequently and simultaneously positive for HO-1 (Fig. 2B and D). Lymphocytes and plasmacytes showed no apparent positive reaction to HO-1.

3.4. Topographical distribution of HO-1-positive cells, macrophages and SMCs in coronary atherosclerotic lesions with DM and NDM

To characterize the topographical distribution of HO-1-positive cells in coronary atherosclerotic lesions of the DM and NDM groups, all specimens except those with DIT were analyzed for the density of HO-1-positive cells as well as macrophages and SMCs. The mean of each positive cell density of six arterial specimens examined per patient was compared among the groups with atherosclerotic risk factors, including DM, hypercholesterolemia, hypertension and smoking. The mean number of HO-1-positive cells and CD-68-positive cells in coronary atherosclerotic lesions was significantly higher in DM than NDM (Table 2). Furthermore, in patients additionally with hypercholesterolemia and current smoking, the density of HO-1-positive cells and macrophages was also higher in DM than in NDM ($p < 0.05$). From these findings, DM seemed to correlate strongly with HO-1 expression and macrophage infiltration.

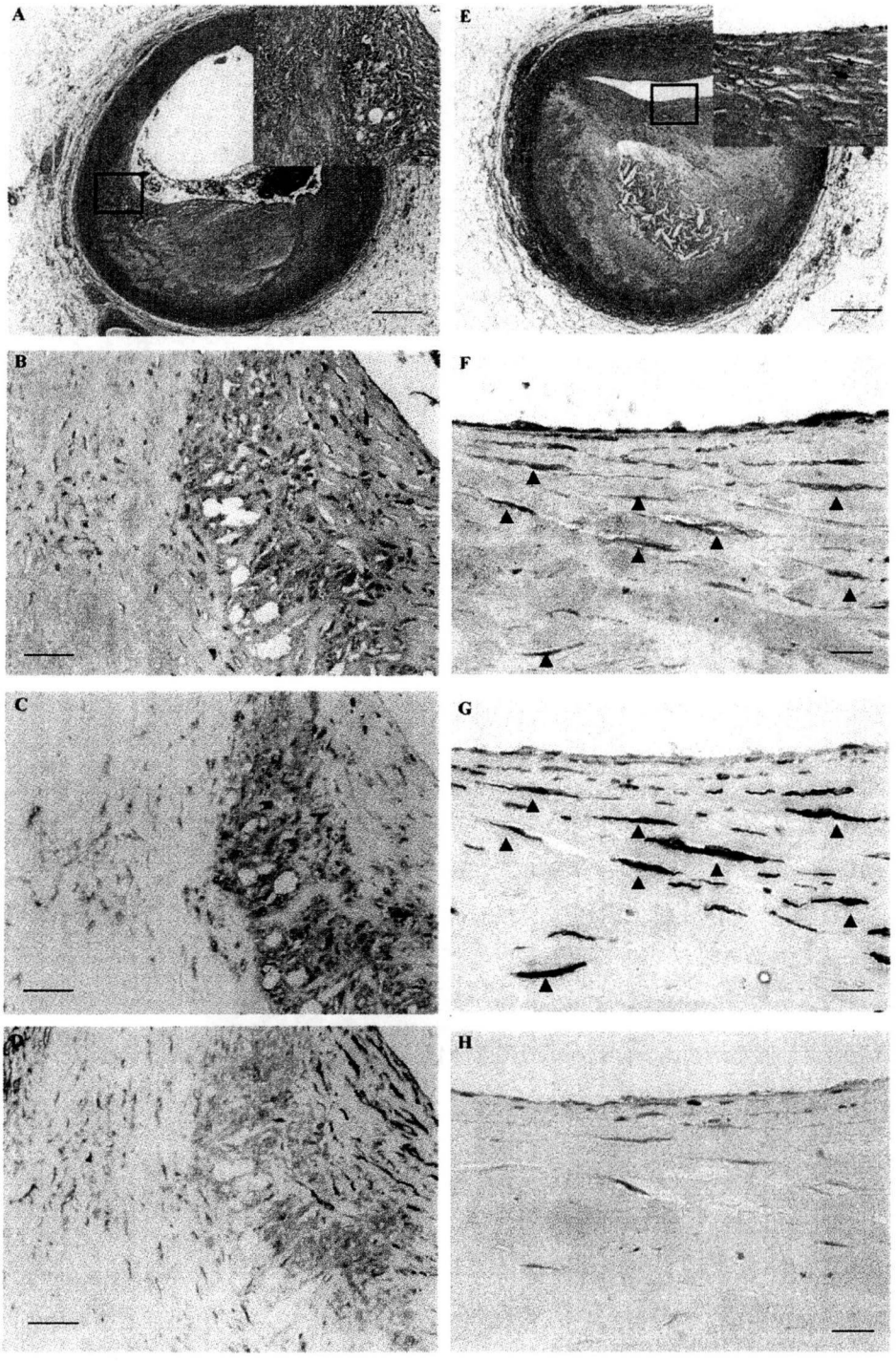

Fig. 1. Immunohistochemical expression of HO-1 in macrophages, endothelial cells and smooth muscle cells in atherosclerotic lesions, Serial sections were examined with HE staining (A and E) and immunohistochemically for HO-1 (B and F), CD68 (C and H), and (-SMA (D and G). The boxed square areas shown in the inserts, indicated in A and E, are demonstrated for the immunohistochemistry of B–D and F–H, respectively. HO-1 is apparently expressed in the macrophages/monocytes (B and C), SMCs (F and G) and luminal ECs of the coronary artery (B and F). The arrowheads in F and G indicate the cells to be positive for HO-1 (F) and (-SMA (G), but negative for CD68 (H). Scale bars represent 500 μm in A and E, 50 μm in B through D, and 20 μm in F through H.

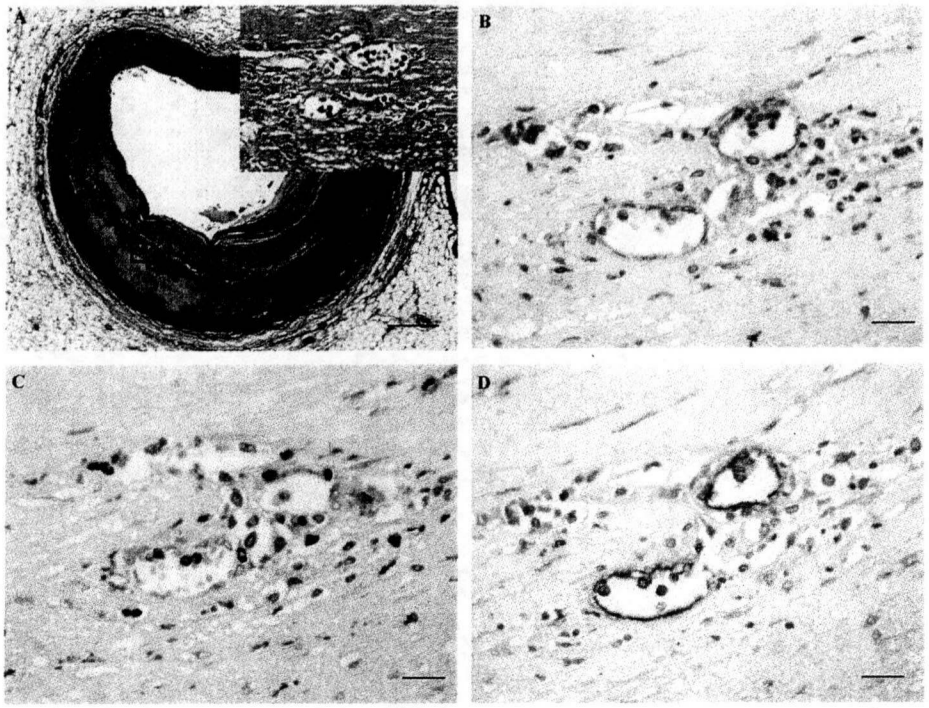

Fig. 2. Immunohistochemical expression of HO-1 in endothelial cells in atherosclerotic lesions, Serial sections were examined with Masson's trichrome (E), and immunohistochemically for HO-1 (B), CD68 (C) and CD34 (D). The boxed square area shown in the insert, indicated in A, demonstrates the immunohistochemistry of B–D. HO-1 is expressed by endothelial cells of newly formed microvessels (B and D), and macrophages/monocytes (B and C) within and around microvesels in atherosclerotic intima. Scale bars represent 500 μm in A and 50 μm in B through D.

3.5. HO-1 expression and intimal neovascularization in atherosclerotic lesions of DM and NDM

As HO-1-positive macrophages frequently infiltrated around newly formed blood vessels in atherosclerotic intimas (Fig. 2B and C), we next examined next the status of vascular density in atherosclerotic intimas in DM and NDM and its correlation with HO-1 expression and atherosclerosis risk factors including hypercholesterolemia. As shown in Table 2, the intimal vascular density per patient was significantly higher in DM than in NDM ($p < 0.05$). With the addition of hypercholesterolemia, the intimal vascular density was more accentuated in DM than NDM ($p < 0.05$). This intimal vascular density, however, was not affected by current smoking. For further confirming evidence regarding the role of HO-1 in intimal angiogenesis, we examined the correlation between the HO-1-positive cell number and the newly formed microvessel number in all subjects examined. Among

Table 2
Immunohistochemical characteristics of atherosclerotic lesions in diabetes and non-diabetes

	Diabetes	Non-diabetes	*p*
HO-1-positive cells ($n = 48$)	27.0 ± 11.3 (17)	19.2 ± 6.0 (31)	0.008
Hypercholesterolemia ($n = 23$)	26.9 ± 12.1 (7)	19.0 ± 6.1 (16)	0.045
Hypertension ($n = 16$)	22.1 ± 8.3 (4)	20.4 ± 8.6 (12)	0.705
Smoking ($n = 28$)	27.3 ± 12.5 (11)	18.9 ± 8.2 (17)	0.037
CD68-positive cells ($n = 48$)	25.2 ± 10.1 (17)	17.6 ± 7.5 (31)	0.005
Hypercholesterolemia ($n = 23$)	25.7 ± 11.8 (7)	17.9 ± 5.5 (16)	0.035
Hypertension ($n = 16$)	21.4 ± 7.9 (4)	18.0 ± 9.9 (12)	0.463
Smoking ($n = 28$)	25.5 ± 11.4 (11)	18.0 ± 8.1 (17)	0.042
Intimal microvessels ($n = 43$)	32.0 ± 20.8 (15)	21.6 ± 12.3 (28)	0.044
Hypercholesterolemia ($n = 23$)	41.0 ± 23.5 (8)	23.5 ± 12.9 (15)	0.030
Smoking ($n = 23$)	34.3 ± 23.0 (10)	20.0 ± 12.4 (13)	0.115

HO-1- and CD68-positive cell numbers were calculated from the mean of the 6 arterial specimens per patient. *n* in parentheses represents the number of patients examined in each group. Each value is expressed as mean ± S.D. Intimal microvessels: the number of microvessels lined with CD34-positive endothelial cells.

these patients the HO-1-positive cell number was well correlated with the microvessel number ($R = 0.675$, $p < 0.01$). This relationship was also statistically significant in both the DM ($R = 0.762$, $p < 0.01$) and NDM groups ($R = 0.564$, $p < 0.01$), but the correlation between the DM and NDM groups was not statistically significant.

3.6. Topographical characteristics of HO-1 expression in atheromatous plaque (lesion type IV) in DM and NDM

Fourteen tissue specimens of AHA-type IV lesion (5 of DM and 9 of NDM) were examined to confirm whether there were any differences in the degree of HO-1 expression among the topographically different areas of atheromatous plaque as described in Section 2. The number of HO-1-positive cells showed a significant variance ($p < 0.0001$, Kruskal–Wallis rank test) among the four location areas of AHA-type IV lesions of DM compared with NDM, and the HO-1-positive cell density was significantly higher in the S and Fc regions of DM than in those of NDM ($p < 0.01$ and $p < 0.05$, respectively), but not in the D and O regions (Supplementary data Fig. II).

4. Discussion

Clinical accumulating evidence indicates that DM increases the incidence of and accelerates atherosclerotic diseases [3]. In the present study, to analyze the pathophysiological role of HO-1 in coronary atherogenesis of diabetic subjects, HO-1 expression was immunohistochemically examined in the coronary arteries of 53 Japanese autopsied patients composed of 19 type 2 diabetics and 34 age- and sex-matched nondiabetic subjects, all of whom had been surveyed in the "Hisayama cohort study" [18,19]. We directly clarified the following key observations: (1) The HO-1 was ubiquitous in human coronary atherosclerotic lesions and was largely expressed by macrophages and ECs of both coronary arteries and intimal newly formed microvessels, and partly by SMCs in atherosclerotic intimas. (2) The extent of HO-1 expression and macrophage infiltration increased as the lesion type and stenotic grade progressed, and was significantly higher not only in early lesions but also in advanced lesions in the DM group compared with the NDM group. (3) Interestingly, the distribution of HO-1-positive cells was accentuated in coronary atherosclerotic lesions with newly formed microvessels in the DM group. These findings indicate that HO-1 expression is intimately associated with human coronary atherogenesis including intimal angiogenesis. Though HO-1 could play an anti-inflammatory role in atherosclerotic lesions, the possible participation of HO-1 in the intimal angiogenesis of atherosclerosis may also be responsible for the progression of atherosclerosis and plaque instability, particularly in diabetic subjects.

Few reports studying the detailed pathological characteristics of HO-1 expression in human atherosclerotic lesions are available [17]. To our best knowledge, this is the first report indicating that HO-1 expression is ubiquitously distributed in human coronary atherosclerotic lesions and is significantly enhanced in diabetic compared with nondiabetic subjects. The inflammatory process evoked via oxidative stress has been thought to intimately participate in the development and progression of atherosclerosis [1], and the oxidative stress induces antioxidatives including HO-1, which has been well established to function antiatherogenically in animal models of atherosclerosis [5,6]. It is, however, unclear whether atherogenic stimuli such as DM, dyslipidemia, hypertension and others can equally promote HO-1 expression in human atherosclerosis. The present study demonstrates that HO-1 expression is significantly enhanced in diabetic compared with nondiabetic subjects, with a greater macrophage content in atherosclerotic lesions. This enhancement of HO-1 expression is additionally upregulated by hypercholesterolemia and smoking, but not by hypertension. These findings support the contention that human atherogenesis is really multifactorial and suggest that DM, hypercholesterolemia and smoking among various types of atherogenic risk factors contribute greatly to HO-1 expression in human coronary atherosclerotic lesions. Recent studies indicate that the stress-responsive elements-Bach1-Nrf2 signal pathway [22] and PPARα and PPARγ/their ligands [23] would transcriptionally regulate HO-1 expression in vascular SMCs and ECs. These transcriptional pathways have been well known to contribute widely to the regulation of the inflammatory-proliferative process in the vasculature, evoked by several atherogenic stimuli including advanced glycation end products overexpressed in DM [24]. In addition, the heterogeneous expression of HO-1 may relate to the severity of human atherosclerosis and the incidence of such atherosclerotic diseases. In fact, a longer GT repeat in human HO-1 promoter has been suggested to result in the decrease of HO-1 transcriptional activity [7,23]. Together with these findings, the cumulative evidence favors therapeutic exploitation using an HO-1 induction strategy [6,25,26].

A few studies comparing the pathologic characteristics of human atherosclerotic lesions in diabetic and nondiabetic patients have been reported [27,28]. A diabetic or prediabetic state as indicated by elevated serum glycohemoglobin levels promotes coronary and aortic atherosclerosis not only in youths but also in adults. Burke et al. [28] reported that macrophage infiltration and necrotic core size play a greater role in the progression of atherosclerosis in diabetic adults who die suddenly. The current study indicates that diabetes is not associated with larger necrotic core size than in nondiabetic subjects, partly due to the small number of subjects examined. In addition, the fact that the major histologic types of more than about three-fourths of the Japanese subjects examined in this study consisted mainly of DIT and early atherosclerotic lesions of types I–III (Supplementary data Fig. I) may be relevant to the larger population of early atherosclerotic lesions and fibrous plaque in Japanese youths [29]. Macrophage infiltration was significantly fre-

quent in diabetic subjects, particularly those with advanced atherosclerotic lesions, in comparison to nondiabetic subjects, and was further accentuated by the co-existence of hypercholesterolemia and smoking. Furthermore, in our study the frequency of MetS was higher in diabetic subjects. The Hisayama cohort study suggested that MetS is a significant risk factor for the development of cardiovascular disease and found a possibility that the increased risk of MetS for cardiovascular disease resulted from the influence of diabetes [30]. These findings suggest that the association of DM with atherosclerosis in the Japanese population is particularly significant for the prevention programs highlighted in the recent Hisayama study [30].

Angiogenesis is an essential process not only physiologically in organ development and tissue regeneration but also pathologically in inflammation and cancer. Newly formed microvessels are ubiquitously distributed in atherosclerotic plaque [11–13], and this angiogenic process has been recently assumed to participate intimately in atherosclerotic progression [14] and in the occurrence of atherothrombosis [15,16]. Recent studies suggest that HO-1 is also involved in physiologic and pathologic angiogenesis [9,10], essentially via the functions of VEGF-A and gas mediators such as NO and CO. Bussolati et al. [9] proposed that HO-1 would play a bifunctional role during an inflammation-repair process, namely, anti-inflammatory action inhibiting leukocytic infiltration and the promotion of VEGF-driven noninflammatory angiogenesis, resulting in the facilitation of a sequential transition from active inflammation to noninflammatory tissue repair. However, the pathological function of HO-1 has not been fully clarified in the chronic inflammatory process including atherogenesis. The current study clearly demonstrated that the extent of newly formed microvessels is well correlated with the degree of HO-1 expression in atherosclerotic plaque, and that hypercholesterolemia in addition to DM accentuates the incidence of intimal angiogenesis. Furthermore, the topographical localization of HO-1 expressed by macrophages and ECs is closely distributed within and around intimal microvessels (Fig. 2). Devesa et al. [9] reported that upregulated HO-1 sustained chronic inflammation in an animal model of chronic arthritis by enhancing inducible nitric oxide synthase expression and VEGF-related angiogenesis. Together with these findings, angiogenesis may play bifunctional and reverse roles in pathological states, partly resulting in the outcome of angiogenic diseases including atherosclerosis and cancer. Though it remains undetermined whether the modulation of HO-1 expression accelerates or suppresses atherogenesis in an animal model of atherosclerosis associated with intimal angiogenesis similar to human lesions, further studies will be necessary to clarify the pathophysiological role of HO-1 in human atherogenesis and to assess the therapeutic effect of HO-1 on atherosclerosis-related disease.

In conclusion, our data demonstrate that HO-1 is ubiquitously upregulated in human coronary atherosclerotic lesions, particularly in diabetics, and that the extent of HO-1 expression is well correlated with the degrees of macrophage infiltration and angiogenesis in atherosclerotic plaque. Thus, HO-1 may participate intimately in the inflammatory-repair process during atherogenesis. Although it has not been determined how HO-1 function activates intraplaque angiogenesis, our findings suggest that HO-1 plays diverse roles in the progression of human coronary atherosclerosis.

Acknowledgments

The authors thank Hiroshi Fujii for his excellent technical assistance. This work was supported in part by a Grant-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan (#16209012 and 13307009).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2008.05.057.

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Impact of Glucose Tolerance Status on Development of Ischemic Stroke and Coronary Heart Disease in a General Japanese Population

The Hisayama Study

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Background and Purpose—Few studies have shown the association between glucose tolerance status defined by a 75-g oral glucose tolerance test and the development of different types of cardiovascular disease.

Methods—A total of 2421 community-dwelling Japanese subjects aged 40 to 79 years who underwent the oral glucose tolerance test were followed up for 14 years.

Results—In multivariable analysis, the risks of ischemic stroke in both sexes and coronary heart disease (CHD) in women were significantly higher in subjects with diabetes determined by the World Health Organization criteria than in those with normal glucose tolerance even after adjustment for other confounding factors, but such association was not seen for CHD in men (ischemic stroke: adjusted hazard ratio [HR]=2.54, P=0.002 in men; adjusted HR=2.02, P=0.03 in women; CHD: adjusted HR=1.26, P=0.47 in men; adjusted HR=3.46, P=0.002 in women). Similar associations were observed for fasting plasma glucose levels of ≥7.0 mmol/L (ischemic stroke: adjusted HR=2.15, P=0.03 in men; adjusted HR=2.10, P=0.045 in women; CHD: adjusted HR=1.29, P=0.47 in men; adjusted HR=3.83, P=0.003 in women) and for 2-hour postload glucose levels of ≥11.1 mmol/L (ischemic stroke: adjusted HR=2.71, P=0.003 in men; adjusted HR=2.19, P=0.03 in women; CHD: adjusted HR=1.58, P=0.16 in men; adjusted HR=4.44, P<0.001 in women). The age-adjusted incidences of ischemic stroke and CHD did not significantly increase in subjects with impaired fasting glycemia or impaired glucose tolerance in either sex.

Conclusions—Our findings suggest that diabetes is an independent risk factor for ischemic stroke in both sexes and CHD in women in the Japanese population. (***Stroke.* 2010;41:00-00.)**

Key Words: coronary heart disease ■ diabetes ■ ischemic stroke ■ oral glucose tolerance test ■ prospective study

Cardiovascular disease continues to be a major global public health concern. Investigations into glucose tolerance levels and cardiovascular disease have become increasingly important, because the impact of diabetes on cardiovascular disease is considered to be rising due to the rapid increase in the worldwide prevalence of diabetes mellitus in recent years. A number of epidemiological studies have demonstrated that Type 2 diabetic subjects have approximately 2.0 to 4.0 times higher risk of cardiovascular disease compared with nondiabetic subjects.[1–13] However, most of these studies had important limitations. In many cohort studies used to investigate this issue, the outcomes were evaluated using mortality data.[3–9,11,12] Because nonfatal events were not included in these studies, the results may not have represented the true association between glucose tolerance levels and cardiovascular disease. Thus, prospective studies using incidence data would provide further information for predicting cardiovascular disease. In addition, the methods used to define diabetes have varied among the epidemiological studies, ranging from administration of questionnaires to measurement of casual blood glucose levels or fasting plasma glucose (FPG) alone.[1,2,11,12] Furthermore, many investigators have evaluated cardiovascular generally, rather than by type, and did not separately evaluate sex, although it is well known that the effects of each risk factor are different for each type of cardiovascular disease and sex. Thus, there have been few cohort studies investigating the associations between glucose tolerance levels, defined by a 75-g glucose tolerance test (OGTT), and the risks of developing stroke and coronary heart disease (CHD) in each sex in Asian populations.

The purpose of the present study was to address the association between glucose tolerance levels and the devel-

Received August 4, 2009; final revision received October 14, 2009; accepted October 20, 2009.

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Stroke **is available at http://stroke.ahajournals.org** **DOI: 10.1161/STROKEAHA.109.564708**

opment of ischemic stroke and CHD in a prospective study of a defined community-dwelling Japanese population, all members of which underwent the OGTT.

Materials and Methods

Study Population

In 1988, a screening survey for the present study was performed in the town of Hisayama, a suburb of the Fukuoka metropolitan area in southern Japan.[14] Of a total 3227 residents aged 40 to 79 years on the town registry, 2587 (participation rate, 80.2%) consented to participate in the examination and underwent a comprehensive assessment. After excluding 82 subjects who had already had breakfast, 10 who were on insulin therapy and 15 due to nausea or general fatigue during the ingestion of glucose, a total of 2480 subjects completed the OGTT. From a total of 2490 subjects including 10 on insulin therapy, 68 who had a history of stroke or CHD based on questionnaires and medical records, and one who died before follow-up was started, were excluded. The remaining 2421 (1037 men and 1384 women) were enrolled in this study.

Follow-Up Survey

The subjects were followed up prospectively for 14 years, from December 1988 to November 2002, by repeated health examinations. The health status was checked yearly by mail or telephone for subjects who did not undergo a regular examination or who had moved from town. We also established a daily monitoring system among the study team, local physicians, and members of the town's health and welfare office. Using this system, we gathered information on new events of cardiovascular disease, including suspected cases. When stroke or CHD occurred or was suspected, physicians in the study team examined the subject and evaluated his or her detailed clinical information. The clinical diagnosis of stroke or CHD was based on the patient's history, physical and neurological examinations, and ancillary laboratory examinations. Additionally, when a subject died, an autopsy was performed at the Departments of Pathology of Kyushu University. During the follow-up period, one subject was lost to follow-up and 418 subjects died, of whom 312 (74.6%) underwent autopsy.

Definition of Cardiovascular Events

In principle, stroke was defined as a sudden onset of nonconvulsive and focal neurological deficit persisting for ≥24 hours. The diagnosis and classification of stroke were determined on the basis of clinical information, including brain CT and MRI, cerebral angiography, echocardiography, carotid duplex imaging, or autopsy findings. Ischemic stroke was classified as either lacunar or nonlacunar infarction based on the Classification of Cerebrovascular Disease III criteria proposed by the National Institute of Neurological Disorders and Stroke.[15] In brief, lacunar infarction was diagnosed as the presence of a relevant brain stem, basal ganglia, or subcortical hemispheric lesion with a diameter <1.5 cm demonstrated on brain imaging and no evidence of cerebral cortical or cerebellar impairment. Patients who had typical clinical findings of lacunar infarction and a negative imaging were also categorized as cases of lacunar infarction. The other ischemic strokes were defined as cases of nonlacunar infarction.

CHD included acute myocardial infarction, silent myocardial infarction, sudden cardiac death within 1 hour after the onset of acute illness, and coronary artery disease treated by coronary artery bypass surgery or angioplasty. Acute myocardial infarction was diagnosed when a subject met at least 2 of the following criteria: (1) typical symptoms, including prolonged severe anterior chest pain; (2) evolving diagnostic electrocardiographic changes; (3) cardiac enzyme levels more than twice the upper limit of normal range; and (4) morphological changes, including local asynergy of cardiac wall motion on echocardiography, persistent perfusion defect on cardiac scintigraphy, or myocardial necrosis or scars ≥1 cm long accompanied by coronary atherosclerosis at autopsy. Silent myocardial infarction was defined as myocardial scarring without any historical indication of clinical symptoms or abnormal cardiac enzyme changes.

During the follow-up, we identified 132 cases of ischemic stroke (for men, 61 total, or 27 lacunar and 34 nonlacunar infarctions; for women, 71 total, or 42 lacunar and 29 nonlacunar infarctions) and 112 CHD events (75 men and 37 women). All of the ischemic stroke cases underwent brain imaging.

Risk Factors

At the baseline examination, we performed the OGTT after at least a 12-hour overnight fast. Plasma glucose levels were determined by the glucose-oxidase method. FPG and 2-hour postload glucose (PG) levels were divided into 4 categories: for FPG: <5.6, 5.6 to 6.0, 6.1 to 6.9, and ≥7.0 mmol/L; for 2-hour PG: <6.7, 6.7 to 7.7, 7.8 to 11.0, and ≥11.1 mmol/L. Glucose tolerance status was also defined by the 1998 World Health Organization criteria[16]; namely, for normal glucose tolerance (NGT), FPG <6.1 and 2-hour PG <7.8; for hyperglycemia, FPG ≥6.1 and/or 2-hour PG ≥7.8; for impaired fasting glycemia (IFG), FPG 6.1 to 6.9 and 2-hour PG <7.8; for impaired glucose tolerance (IGT), FPG <7.0 and 2-hour PG 7.8 to 11.0; and for diabetes mellitus, FPG ≥7.0 mmol/L and/or 2-hour PG ≥11.1 mmol/L. Total and high-density lipoprotein cholesterol levels were determined enzymatically.

Blood pressure was measured 3 times using a sphygmomanometer after at least 5 minutes of rest; the average of 3 measurements was used for the analysis. Hypertension was defined as blood pressure levels of ≥140/90 mm Hg or current treatment with antihypertensive agents. Body mass index (kg/m^2) was used as an indicator of obesity. Electrocardiographic abnormalities were defined as left ventricular hypertrophy (Minnesota Code 3 to 1) or ST depression (4 to 1, 4 to 2, or 4 to 3). Each participant completed a self-administered questionnaire covering medical history, antidiabetic and antihypertensive treatments, smoking habits, alcohol intake, and leisure time activity. Smoking habits and alcohol intake were classified as either current use or not. Those subjects engaging in sports or other forms of exertion ≥3 times a week during their leisure time made up a regular exercise group.

Statistical Analysis

The SAS software package Version 9.2 (SAS Institute Inc, Cary, NC) was used to perform all statistical analyses. Incidence was calculated by a person-year method and was adjusted for age by the direct method using 10-year age groupings. The age- and multivariable-adjusted hazard ratios (HRs) and their 95% CIs were estimated using the Cox proportional hazards model.

Ethical Considerations

This study was conducted with the approval of the Ethics Committee of Kyushu University, and written informed consent was obtained from the participants.

Results

The baseline characteristics of the subjects are summarized by sex in Table 1. Mean values of age and body mass index did not differ between the sexes. The means of FPG, 2-hour PG, and systolic and diastolic blood pressures and frequencies of diabetes, hypertension, electrocardiographic abnormalities, smoking habits, alcohol intake, and regular exercise were higher in men than in women, whereas women had higher concentrations of total and high-density lipoprotein cholesterol.

The age-adjusted incidences and age-adjusted and multivariable-adjusted HRs of ischemic stroke and CHD according to FPG levels are shown in Table 2. The age-adjusted incidences of ischemic stroke and CHD did not differ between subjects with FPG levels of <5.6 mmol/L and those with FPG levels of 5.6 to 6.0 mmol/L in either sex. In women, the age-

Table 1. Characteristics of Subjects by Sex, 1988

	Men (n=1037)	Women (n=1384)
Age, years	57 (10)	58 (10)
Fasting plasma glucose, mmol/L	5.9 (1.3)	5.7 (1.3)
2-hour PG, mmol/L	7.7 (4.0)	7.4 (3.3)
Diabetes, %	15.1	9.7
Systolic blood pressure, mm Hg	134 (20)	131 (20)
Diastolic blood pressure, mm Hg	81 (11)	76 (11)
Hypertension, %*	43.3	34.8
Electrocardiographic abnormalities, %†	19.6	12.6
Body mass index, kg/m^2	22.9 (2.9)	23.0 (3.2)
Total cholesterol, mmol/L	5.07 (1.07)	5.51 (1.05)
High density lipoprotein cholesterol, mmol/L	1.25 (0.31)	1.33 (0.29)
Current smoking, %	50.1	6.7
Current alcohol use, %	62.2	9.0
Regular exercise, %	11.2	9.0

All values are given as the mean (SD) or as a percent.
*Blood pressure ≥140/90 mm Hg or current use of antihypertensive agents.
†Minnesota Codes 3–1, 4–1, 4–2, or 4–3.

adjusted incidence and HR of ischemic stroke were significantly higher in subjects with FPG levels of 6.1 to 6.9 mmol/L than in those with the FPG levels of <5.6 mmol/L; however, this association was attenuated after adjustment for the following confounding factors: age, systolic blood pressure, electrocardiographic abnormalities, body mass index, total and high-density lipoprotein cholesterol, smoking habits, alcohol intake, and regular exercise. An FPG level of ≥7.0 mmol/L was a significant risk factor for ischemic stroke in both sexes and for CHD in women, even after adjustment for the previously mentioned confounding factors (ischemic stroke: multivariable-adjusted HR=2.15, 95% CI, 1.07 to 4.31, P=0.03 in men; multivariable-adjusted HR=2.10, 95% CI, 1.02 to 4.35, P=0.045 in women; CHD: multivariable-adjusted HR=3.83, 95% CI, 1.59 to 9.25, P=0.003 in women).

Table 3 presents data of the analyses for ischemic stroke and CHD according to 2-hour PG levels. Compared with subjects with 2-hour PG levels of <6.7 mmol/L, the age-adjusted incidences and multivariable-adjusted HRs of ischemic stroke in both sexes and CHD in women were significantly higher in those with glucose levels of ≥11.1 mmol/L (ischemic stroke: multivariable-adjusted HR=2.71, 95% CI, 1.41 to 5.20, P=0.003 in men; multivariable-adjusted HR=2.19, 95% CI, 1.07 to 4.48, P=0.03 in women; CHD: multivariable-adjusted HR=4.44, 95% CI, 1.85 to 10.6, P<0.001 in women). Subjects with a prediabetic range of 2-hour PG levels did not have an increased risk of either ischemic stroke or CHD.

Finally, the relationships between glucose tolerance levels defined by the World Health Organization criteria and the risks of ischemic stroke and CHD are displayed in Table 4. Compared with those in women with NGT, the age-adjusted incidences and HRs of ischemic stroke and CHD were significantly increased in women with hyperglycemia, but these associations disappeared after adjustment for other confounding factors. In regard to subtypes of hyperglycemia, the age-adjusted incidences and HRs of ischemic stroke and CHD did not significantly increase in those with IFG or IGT

Table 2. Age-Adjusted Incidence and Age- and Multivariable-Adjusted HRs and Their 95% CIs for the Development of Cardiovascular Diseases According to FPG Levels

	FPG Level, mmol/L	Person-Years	No. of Events	Age-Adjusted Incidence per 1,000 Person-Years	Age-Adjusted HR (95% CI)	P	Multivariable-Adjusted HR (95% CI)	P
Ischemic stroke								
Men	<5.6	5391	26	5.4	1 (referent)		1 (referent)	
	5.6 to 6.0	3791	13	4.0	0.70 (0.36 to 1.36)	0.29	0.66 (0.33 to 1.29)	0.22
	6.1 to 6.9	1909	9	4.7	0.85 (0.40 to 1.82)	0.68	0.68 (0.30 to 1.54)	0.36
	≥7.0	1170	13	11.7	2.06 (1.06 to 4.00)	0.03	2.15 (1.07 to 4.31)	0.03
Women	<5.6	9707	28	3.4	1 (referent)		1 (referent)	
	5.6 to 6.0	4821	18	3.9	1.11 (0.61 to 2.00)	0.74	0.98 (0.54 to 1.79)	0.95
	6.1 to 6.9	1733	14	7.1	2.01 (1.05 to 3.84)	0.03	1.59 (0.80 to 3.13)	0.18
	≥7.0	1107	11	9.6	2.47 (1.22 to 4.97)	0.01	2.10 (1.02 to 4.35)	0.045
CHD								
Men	<5.6	5450	33	7.0	1 (referent)		1 (referent)	
	5.6 to 6.0	3808	16	4.7	0.68 (0.38 to 1.24)	0.21	0.67 (0.37 to 1.23)	0.20
	6.1 to 6.9	1942	14	7.3	1.01 (0.54 to 1.90)	0.97	0.80 (0.42 to 1.54)	0.50
	≥7.0	1195	12	9.9	1.50 (0.77 to 2.90)	0.23	1.29 (0.65 to 2.58)	0.47
Women	<5.6	9844	12	1.4	1 (referent)		1 (referent)	
	5.6 to 6.0	4893	9	1.8	1.31 (0.55 to 3.10)	0.55	1.13 (0.47 to 2.71)	0.78
	6.1 to 6.9	1815	6	2.5	1.99 (0.74 to 5.36)	0.17	1.36 (0.49 to 3.81)	0.56
	≥7.0	1138	10	7.0	5.30 (2.28 to 12.35)	<0.001	3.83 (1.59 to 9.25)	0.003

Multivariable adjustment was made for age, systolic blood pressure, electrocardiogram abnormalities, body mass index, total and high-density lipoprotein cholesterol, smoking habits, alcohol intake, and regular exercise.

Table 3. Age-Adjusted Incidence and Age- and Multivariable-Adjusted HRs and Their 95% CIs for the Development of Cardiovascular Diseases According to 2-Hour PG Levels

	Two-Hour PG Levels, mmol/L	Person-Years	No. of Events	Age-Adjusted Incidence per 1000 Person-Years	Age-Adjusted HR (95% CI)	*P*	Multivariable-Adjusted HR (95% CI)	*P*
Ischemic stroke								
Men	<6.7	6253	25	4.4	1 (referent)		1 (referent)	
	6.7 to 7.7	2246	7	3.5	0.81 (0.35 to 1.87)	0.61	0.84 (0.36 to 1.96)	0.68
	7.8 to 11.0	2363	13	5.5	1.22 (0.62 to 2.38)	0.57	1.05 (0.52 to 2.13)	0.89
	≥11.1	1399	16	10.9	2.66 (1.42 to 4.98)	0.002	2.71 (1.41 to 5.20)	0.003
Women	<6.7	8728	25	3.3	1 (referent)		1 (referent)	
	6.7 to 7.7	3982	17	5.3	1.51 (0.82 to 2.80)	0.19	1.29 (0.69 to 2.44)	0.43
	7.8 to 11.0	3374	15	3.8	1.18 (0.62 to 2.24)	0.62	0.99 (0.51 to 1.92)	0.96
	≥11.1	1284	14	10.3	2.80 (1.45 to 5.40)	0.002	2.19 (1.07 to 4.48)	0.03
CHD								
Men	<6.7	6239	33	6.0	1 (referent)		1 (referent)	
	6.7 to 7.7	2277	9	4.7	0.78 (0.37 to 1.63)	0.50	0.73 (0.34 to 1.55)	0.41
	7.8 to 11.0	2430	18	7.3	1.20 (0.67 to 2.13)	0.54	0.97 (0.53 to 1.77)	0.93
	≥11.1	1449	15	11.5	1.82 (0.99 to 3.34)	0.06	1.58 (0.83 to 3.00)	0.16
Women	<6.7	8858	11	1.4	1 (referent)		1 (referent)	
	6.7 to 7.7	4079	6	1.4	1.16 (0.43 to 3.15)	0.77	0.91 (0.33 to 2.52)	0.86
	7.8 to 11.0	3430	6	1.5	1.10 (0.40 to 2.97)	0.86	0.82 (0.29 to 2.29)	0.70
	≥11.1	1323	14	8.5	6.49 (2.93 to 14.36)	<0.001	4.44 (1.85 to 10.62)	<0.001

Multivariable adjustment was made for age, systolic blood pressure, electrocardiogram abnormalities, body mass index, total and high-density lipoprotein cholesterol, smoking habits, alcohol intake, and regular exercise.

in either sex. Diabetes was a significant risk factor for ischemic stroke in both sexes and for CHD in women. These significant associations also remained robust even after adjustment for the previously mentioned confounding factors (ischemic stroke: multivariable-adjusted HR=2.54, 95% CI, 1.40 to 4.63, P=0.002 in men; multivariable-adjusted HR=2.02, 95% CI, 1.07 to 3.81, P=0.03 in women; CHD: multivariable-adjusted HR=3.46, 95% CI, 1.59 to 7.54, P=0.002 in women). When ischemic stroke was classified as either lacunar or nonlacunar infarction, diabetes was an independent risk factor for lacunar infarction in women (multivariable-adjusted HR=2.65, 95% CI, 1.19 to 5.93, P=0.02) and nonlacunar infarction in men (HR=3.78, 95% CI, 1.74 to 8.19, P=0.001) after adjustment for other confounding factors (Table 5).

Discussion

Using data from a 14-year follow-up study of a defined general Japanese population, we demonstrated that diabetes defined by the OGTT is an independent risk factor for the development of ischemic stroke in both sexes and CHD in women after adjustment for other confounding factors. Furthermore, we found that diabetes significantly increased the risk of lacunar infarction in women and nonlacunar infarction in men. By contrast, an FPG level of 5.6 to 6.0 mmol/L, a newly extended range from the American Diabetes Association, was not associated with ischemic stroke or CHD in either sex. In women with the FPG levels of 6.1 to 6.9 mmol/L, the age-adjusted incidence of ischemic stroke increased significantly; however, this association was attenuated after multivariable adjustment.

Very few prospective studies have provided evidence of the associations between glucose tolerance levels defined by the OGTT and the incidence of stroke and CHD. Only investigators of the Strong Heart Study of American Indians have evaluated the association of glucose tolerance status defined by the 1998 World Health Organization criteria with the risk of developing stroke. The results showed that, compared with the subjects with NGT, subjects with diabetes had a 2-fold higher risk of stroke, but subjects with IFG or IGT did not have a higher risk.[19] In a follow-up examination of a Finnish population who was free of diabetes at baseline, diabetes that developed during the follow-up was a significant risk factor for CHD, but baseline IGT was not.[17] These findings are in accordance with those of the present study. In our study, diabetes was significantly associated with the development of ischemic stroke in both sexes as well as CHD in women, but such an association was not observed for CHD in men. Although the precise reasons for this sex difference in the CHD risk conferred by diabetes are unknown, the higher prevalence of smoking in men may be responsible for this phenomenon; a smoking habit, which is a major risk factor for CHD, is considered to increase the risk of CHD in subjects with normal glucose levels, which would weaken the association of diabetes with CHD in men. Several cohort studies indicated that elevated 2-hour PG levels of 7.8 to 11.0 mmol/L, a category of IGT, was associated with an increased mortality from cardiovascular disease.[6–8,18,19] However, there have been some epidemiological studies in which IGT was not a risk factor for cardiovascular death.[3,5,9] In the present study, IGT was not associated with the development of ischemic stroke or CHD. However, our previous study of

Table 4. Age-Adjusted Incidence and Age- and Multivariable-Adjusted HRs and Their 95% CIs for the Development of Cardiovascular Diseases According to Glucose Tolerance Levels Defined by the WHO Criteria

	WHO Criteria	Person-Years	No. of Events	Age-Adjusted Incidence per 1000 Person-Years	Age-Adjusted HR (95% CI)	P	Multivariable-Adjusted HR (95% CI)	P
Ischemic stroke								
Men	NGT	7397	29	4.6	1 (referent)		1 (referent)	
	Hyperglycemia	4863	32	6.6	1.47 (0.89 to 2.43)	0.14	1.32 (0.79 to 2.23)	0.29
	IFG	987	2	1.9	0.45 (0.11 to 1.89)	0.28	0.41 (0.10 to 1.74)	0.23
	IGT	2183	11	5.0	1.10 (0.55 to 2.21)	0.78	0.91 (0.44 to 1.89)	0.79
	Diabetes	1694	19	11.3	2.55 (1.43 to 4.55)	0.001	2.54 (1.40 to 4.63)	0.002
Women	NGT	11 769	35	3.6	1 (referent)		1 (referent)	
	Hyperglycemia	5600	36	5.7	1.60 (1.00 to 2.56)	0.049	1.34 (0.82 to 2.20)	0.25
	IFG	807	7	7.9	2.20 (0.98 to 4.97)	0.06	1.89 (0.82 to 4.34)	0.13
	IGT	3224	13	3.4	1.01 (0.53 to 1.92)	0.97	0.88 (0.46 to 1.70)	0.71
	Diabetes	1569	16	9.3	2.46 (1.36 to 4.46)	0.003	2.02 (1.07 to 3.81)	0.03
CHD								
Men	NGT	7415	37	5.9	1 (referent)		1 (referent)	
	Hyperglycemia	4979	38	7.8	1.31 (0.83 to 2.07)	0.24	1.10 (0.69 to 1.76)	0.69
	IFG	982	5	4.9	0.89 (0.35 to 2.27)	0.81	0.80 (0.31 to 2.05)	0.64
	IGT	2244	18	8.0	1.33 (0.76 to 2.35)	0.32	1.11 (0.62 to 2.00)	0.72
	Diabetes	1754	15	9.4	1.53 (0.84 to 2.78)	0.17	1.26 (0.67 to 2.35)	0.47
Women	NGT	11 932	16	1.5	1 (referent)		1 (referent)	
	Hyperglycemia	5759	21	3.1	2.07 (1.07 to 3.99)	0.03	1.52 (0.76 to 3.04)	0.23
	IFG	871	1	0.9	0.65 (0.09 to 4.88)	0.67	0.48 (0.06 to 3.76)	0.48
	IGT	3278	6	1.6	1.05 (0.41 to 2.70)	0.92	0.82 (0.31 to 2.15)	0.68
	Diabetes	1610	14	6.9	4.82 (2.34 to 9.94)	<0.001	3.46 (1.59 to 7.54)	0.002

Multivariable adjustment was made for age, systolic blood pressure, electrocardiogram abnormalities, body mass index, total and high-density lipoprotein cholesterol, smoking habits, alcohol intake, and regular exercise.

WHO indicates World Health Organization.

a 5-year follow-up of the same cohort showed that IGT was an independent risk factor for the occurrence of cardiovascular disease.[4] During a long follow-up period, a potential change in the glucose tolerance of participants may occur, which would induce some misclassification and weaken the relationship between 2-hour PG levels and cardiovascular disease. Thus, the association between the prediabetic range of 2-hour PG and cardiovascular events would attenuate over time.

The American Diabetes Association lowered the FPG cutoff point from 6.1 to 5.6 mmol/L in 2003.[20] This decision was prompted partly by population-based studies showing that the cutoff point of 5.6 mmol/L would increase the sensitivity of predicting future diabetes. In addition, this change was also intended to improve the selection of individuals at risk for cardiovascular diseases.[20] Two major organizations recently adopted the cutoff point of 5.6 mmol/L in the diagnostic criteria of metabolic syndrome.[21,22] Thus, it is very important to appropriately determine the FPG cutoff value for the prediction of cardiovascular disease. However, there is less evidence concerning the positive association between FPG levels of 5.6 to 6.0 mmol/L and the risk of cardiovascular disease. A recent study of a community-based medical center in the United States found that individuals with glucose of 5.6 to 6.0 mmol/L had lower prevalence of most CHD risk factors compared with individuals with glucose of 6.1 to 6.9 mg/dL.[23] Furthermore, some epidemiological studies have shown that the mortality and incidence of cardiovascular disease did not increase in those with FPG levels of 5.6 to 6.0 mmol/L.[11,12,19,24] These findings, together with those of the present study, suggest that FPG levels of 5.6 to 6.0 mmol/L are not associated with the risk of cardiovascular disease.

Conflicting data for FPG levels of 6.1 to 6.9 mmol/L as a risk factor for cardiovascular disease also exist. At least 4 studies have shown no significantly increased risk of cardiovascular disease in those with FPG levels of 6.1 to 6.9 mmol/L,[6,8,18,19] although others have found that this glucose range is a significant risk factor for cardiovascular disease.[7,11,12,24] In our study, the age-adjusted incidence of ischemic stroke was significantly higher in women with FPG levels of 6.1 to 6.9 mmol/L than in those with normal FPG levels, but after controlling for confounding risk factors, the risk was no longer statistically significant. Other known cardiovascular risk factors such as hypertension, obesity, and dyslipidemia tend to accumulate at this glucose level.[23] Thus, FPG levels of 6.1 to 6.9 mmol/L seem to have increased the risk of ischemic stroke through other coexisting risk factors in our population.

The strengths of our study include its longitudinal population-based design, long duration of follow-up, perfect follow-up of subjects, sufficient number of cardiovascular events, and accuracy of diagnosis of cardiovascular disease. One limitation of our study is that the diagnosis of glucose

Table 5. Age-Adjusted Incidence and Age- and Multivariable-Adjusted HRs and Their 95% CIs for the Development of Lacunar and Nonlacunar Infarctions According to Glucose Tolerance Levels Defined by the WHO Criteria

	WHO Criteria	Person-Years	No. of Events	Age-Adjusted Incidence per 1000 Person-Years	Age-Adjusted HR (95% CI)	*P*	Multivariable-Adjusted HR (95% CI)	*P*
Lacunar infarction								
Men	NGT	7397	14	2.3	1 (referent)		1 (referent)	
	Hyperglycemia	4863	13	2.7	1.19 (0.56 to 2.54)	0.65	0.99 (0.45 to 2.18)	0.99
	IFG	987	1	1.0	0.44 (0.06 to 3.38)	0.43	0.43 (0.06 to 3.28)	0.41
	IGT	2183	6	2.7	1.19 (0.46 to 3.11)	0.72	0.91 (0.32 to 2.57)	0.86
	Diabetes	1694	6	3.6	1.64 (0.63 to 4.28)	0.31	1.44 (0.54 to 3.86)	0.47
Women	NGT	11 769	19	2.0	1 (referent)		1 (referent)	
	Hyperglycemia	5600	23	3.8	1.97 (1.07 to 3.65)	0.03	1.62 (0.85 to 3.11)	0.14
	IFG	807	4	4.8	2.42 (0.82 to 7.13)	0.11	2.02 (0.67 to 6.09)	0.21
	IGT	3224	8	2.1	1.21 (0.53 to 2.78)	0.66	1.04 (0.44 to 2.43)	0.94
	Diabetes	1569	11	6.7	3.26 (1.54 to 6.89)	0.002	2.65 (1.19 to 5.93)	0.02
Nonlacunar infarction								
Men	NGT	7397	15	2.3	1 (referent)		1 (referent)	
	Hyperglycemia	4863	19	3.9	1.74 (0.88 to 3.42)	0.11	1.67 (0.83 to 3.37)	0.15
	IFG	987	1	0.9	0.45 (0.06 to 3.44)	0.44	0.41 (0.05 to 3.12)	0.39
	IGT	2183	5	2.3	1.00 (0.36 to 2.76)	1.00	0.91 (0.33 to 2.57)	0.87
	Diabetes	1694	13	7.7	3.44 (1.63 to 7.23)	0.001	3.78 (1.74 to 8.19)	0.001
Women	NGT	11 769	16	1.7	1 (referent)		1 (referent)	
	Hyperglycemia	5600	13	1.9	1.18 (0.57 to 2.47)	0.66	1.01 (0.46 to 2.20)	0.99
	IFG	807	3	3.1	1.94 (0.56 to 6.67)	0.29	1.78 (0.50 to 6.38)	0.38
	IGT	3224	5	1.3	0.80 (0.29 to 2.18)	0.66	0.70 (0.25 to 1.98)	0.51
	Diabetes	1569	5	2.6	1.58 (0.58 to 4.32)	0.37	1.26 (0.43 to 3.69)	0.67

Multivariable adjustment was made for age, systolic blood pressure, electrocardiogram abnormalities, body mass index, total and high-density lipoprotein cholesterol, smoking habits, alcohol intake, and regular exercise.

WHO indicates World Health Organization.

tolerance status was based on a single measurement of glucose levels at baseline as was the case in most other epidemiological studies. During the follow-up, risk factor levels were changed due to modifications in lifestyle or medication, and misclassification of glucose tolerance categories was possible. This could have weakened the association found in this study, biasing the results toward the null hypothesis. Therefore, the true association may be stronger than that shown in our study.

In conclusion, diabetes defined by an OGTT was an independent risk factor for cardiovascular disease, except for CHD in men. Notably, the new range in the 2003 American Diabetes Association criteria for IFG (FPG of 5.6 to 6.0 mmol/L) was not associated with ischemic stroke or CHD in either sex. The IFG category of the 1997 criteria (FPG of 6.1 to 6.9 mmol/L) increased the risk of ischemic stroke in women, although this association was not independent of other known risk factors. Because the risks of stroke and CHD and the prevalence of diabetes differ among races, further investigations are required to clarify the relationship between hyperglycemia and type of cardiovascular disease in other ethnic populations.

Sources of Funding

This study was supported in part by Grants-in-Aid for Scientific Research A (No. 18209024) and C (No. 20591063) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Health and Labour Sciences Research Grant of the Ministry of Health, Labour and Welfare of Japan (Comprehensive Research on Aging and Health; H20-Chouju-004).

Disclosures

None.

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ORIGINAL INVESTIGATIONS

Pathogenesis and Treatment of Kidney Disease

Association of Kidney Function With Coronary Atherosclerosis and Calcification in Autopsy Samples From Japanese Elders: The Hisayama Study

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Background: Chronic kidney disease (CKD) is associated with increased risk of coronary heart disease. However, information regarding the histopathologic characteristics of coronary atherosclerosis in individuals with CKD is scarce. This study investigated the relationship between CKD and severity of coronary atherosclerosis in population-based autopsy samples.

Study Design: Cross-sectional study.

Setting & Participants: 126 individuals randomly selected from 844 consecutive population-based autopsy samples.

Predictor: Estimated glomerular filtration rate (eGFR) calculated using the 6-variable Modification of Diet in Renal Disease (MDRD) Study equation.

Outcomes: Severity of atherosclerosis in 3 main coronary arteries, including atherosclerotic lesion types defined using the American Heart Association classification; stenosis rates; and coronary calcified lesions.

Measurements: The relationship between CKD and severity of coronary atherosclerosis was evaluated using generalized estimating equation methods.

Results: Frequencies of advanced atherosclerotic lesions increased gradually as eGFR decreased (33.6%, 41.7%, 52.3%, and 52.8% for eGFRs ≥60, 45-59, 30-44, and <30 mL/min/1.73 m^2, respectively; *P* for trend = 0.006). This relationship was substantially unchanged even after adjustment for potential confounding factors (ORs, 1.40 [95% CI, 0.76-2.55], 2.02 [95% CI, 0.99-4.15], and 3.02 [95% CI, 1.22-7.49] for eGFRs of 45-59, 30-44, and <30 mL/min/1.73 m^2, respectively). Frequencies of calcified lesions of coronary arteries also increased gradually with lower eGFRs (*P* for trend = 0.02). Hypertension and diabetes were associated with increased risk of advanced coronary atherosclerosis and calcification of coronary arteries in individuals with decreased eGFR.

Limitations: Cross-sectional study, absence of data for proteinuria, and extremely high proportion of aged people.

Conclusions: The autopsy findings presented here suggest that CKD is associated significantly with severity of coronary atherosclerosis. Patients with CKD should be considered a high-risk population for advanced coronary atherosclerosis.

Am J Kidney Dis 55:21-30.

INDEX WORDS: Chronic kidney disease; coronary atherosclerosis; population risk; coronary artery stenosis; glomerular filtration rate; coronary disease.

Editorial, p. 1

Chronic kidney disease (CKD) is a significant public health problem, affecting 10%-15% of the adult general population in developed countries.[1-3] CKD is associated with increased risks of cardiovascular disease and death.[4-7] A higher incidence rate of myocardial infarction and excessive cardiac mortality have been documented repeat-

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Received March 31, 2009. Accepted in revised form June 22, 2009. Originally published online as doi:10.1053/j.ajkd.2009.06.034 on September 22, 2009.

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0272-6386/09/5501-0007$36.00/0
doi:10.1053/j.ajkd.2009.06.034