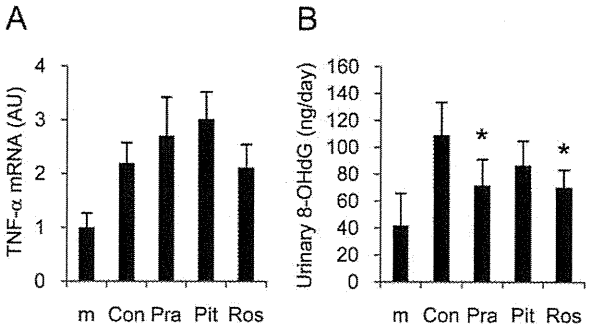

Figure 3. Effect of statins on renal inflammation and oxidative stress in db/db mice. (A) Expression of TNF-α mRNA in whole kidney and (B) urinary 8-OHdG levels in db/m mice (m), non-treated (Con), pravastatin-treated (Pra), pitavastatin-treated (Pit) and rosuvastatin-treated (Ros) db/db mice. Results are expressed as mean ± SD. *P<0.05 vs. non-treated db/db mice (n=6 in each group).

nephropathy along with albuminuria. Therefore, we assessed the glomerular hypertrophy in db/db mice and the effect of statins by measuring the glomerular surface area. Mean glomerular surface area size in db/db mice was increased compared with db/m mice. Pitavastatin and rosuvastatin treatment, but not pravastatin treatment, suppressed the glomerular hypertrophy as well as urinary excretion of albumin in db/db mice (Fig. 4).

Discussion

In the present study, we showed that pitavastatin and rosuvastatin treatment improved albuminuria and suppressed glomerular hypertrophy, independent of its lipid-lowering and anti-oxidative effect in db/db mice.

In CKD patients, there is an increase in total cholesterol and LDL levels (17). The level of cholesterol is directly correlated with the degree of albuminuria (18), suggesting that hyperlipidemia is associated with the development of CKD such as diabetic nephropathy. In fact, lipid-lowering therapy by statin has been successful to the amelioration of renal function in patients with diabetic nephropathy (19,20). However, the present study and other animal studies showed that statin treatment significantly improved renal function without affecting the plasma lipid profile (5,8,21). Therefore, the renoprotective effect of statins may be mainly caused by its pleiotropic action rather than their lipid-lowering action.

Insulin resistance is associated with the development of renal dysfunction in type 2 diabetes. It has been shown that insulin resistance correlates with the onset of microalbuminuria in patients with type 2 diabetes as well as in nondiabetic subjects (14). Several studies showed that amelioration of insulin resistance resulted in a restoration of renal function (22-24). Statin also has an ability to ameliorate insulin resistance. Takagi *et al* (25) reported that pravastatin treatment improved insulin resistance through the increase in plasma adiponectin levels in db/db mice. In the present study, we also observed that all statin treatment improved insulin resistance detected by the reduction of HOMA-IR, while adiponectin was not altered by statin treatment in db/db mice. However, this amelioration was not consistent with the renoprotective effects of statins in db/db mice.

Oxidative stress and inflammation are also far more prevalent in CKD patients than in normal subjects (26). In the present study, we also observed the elevation of oxidative stress and inflammation in the kidneys of db/db mice compared with that of lean control mice. Renal disease is associated with a graded increase in oxidative stress markers even in early CKD (27). This oxidative stress can accelerate renal injury progression. In addition, inflammatory markers such as C reactive protein and cytokines increase with renal function deterioration suggesting that CKD is a low-grade inflammatory process (28). Therefore, the agents which have anti-oxidative and anti-inflammatory action have been attracted as a therapeutic strategy for renal dysfunction (29). Anti-oxidative and anti-inflammatory actions are also major pleiotropic effects of statins (12). Several reports have shown that these actions of statins contribute to their renoprotective effects (5,30,31). In the present study, we also observed that pravastatin and rosuvastatin suppressed oxidative stress in db/db mice as well as these reports, whereas we could not detect the anti-inflammatory effect of statins in the kidneys of db/db mice. Pitavastatin had no effect on oxidative stress, despite the presence of the restored renal function in db/db mice. This result suggests that the anti-oxidant action of statins is not primarily responsible for their renoprotective effect.

In the present study, we observed a correlation between the renoprotective effects of statins and their suppressive effect

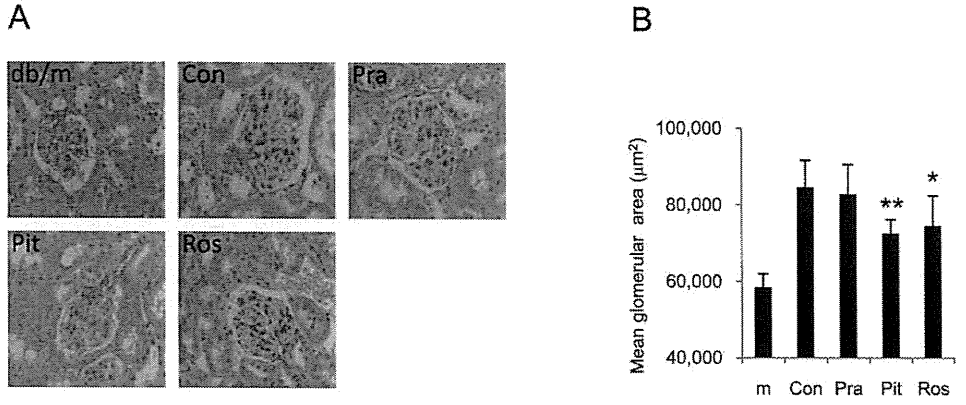

Figure 4. Effects of statins on the glomerular hypertrophy in db/db mice. (A) H&E staining of glomeruli (magnification, x200) and (B) mean glomerular surface area of db/m mice (m), non-treated (Con), pravastatin-treated (Pra), pitavastatin-treated (Pit) and rosuvastatin-treated (Ros) db/db mice. The mean area of fifty glomeruli per mouse was analyzed. Results are expressed as mean ± SD. *P<0.05, **P<0.01 vs. non-treated db/db mice (n=6 in each group).

of glomerular hypertrophy in db/db mice. The glomerular morphological changes in diabetic nephropathy are characterized primarily by mesangial expansion and glomerular based membrane (GBM) thickening. It has been reported that the dysregulated cell cycle by the increased inhibitor of cyclin dependent kinase (such as p21 and p27) contributes to these morphological changes and renal dysfunction (32,33). Pleiotropic effects of statins on the cell cycle are well known (12). Furthermore, Danesh *et al* (34) reported that statin treatment normalized the cell cycle through the suppression of p21 expression in high glucose-stimulated mesangial cells. In the present study, pleiotropic effects of statin on the cell cycle thus might improve glomerular hypertrophy and albuminuria. However, further study is required to clarify the effect of statins in glomerular hypertrophy and renal dysfunction.

In conclusion, we have shown the effects of various statins on diabetic nephropathy in db/db mice. Our study suggests that its renoprotective effect is mainly dependent on suppressing the glomerular hypertrophy, independent of its lipid-lowering or anti-oxidative effects, and there may be differences in the renoprotective ability between various statins.

Acknowledgements

We thank Dr Takeshi Matsubara (Kyoto University) for helpful discussion and critical reading of our manuscript. We also thank Nami Sawada (Kyoto University) for excellent technical assistance. This study was supported by the Takeda Science Foundation.

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Original Article

Risk Stratification Based on Metabolic Syndrome as well as Non-Metabolic Risk Factors in the Assessment of Carotid Atherosclerosis

Hiroyuki Noda[1], Hiroyasu Iso[1], Shizuya Yamashita[2], Hikaru Ueno[3], Masayuki Yokode[4], Nobuhiro Yamada[5], and Yasuyoshi Ouchi[6] for Defining Vascular Disease (DVD) Research Group

[1]Public Health, Department of Social and Environmental Medicine, Osaka University, Graduate School of Medicine, Osaka, Japan
[2]Department of Cardiovascular Medicine, Osaka University, Graduate School of Medicine, Osaka, Japan
[3]Department of Biochemistry and Molecular Pathophysiology, University of Occupational and Environmental Health, Japan, School of Medicine, Fukuoka, Japan
[4]Department of Clinical Innovative Medicine, Translational Research Center, Kyoto University Hospital, Kyoto, Japan
[5]Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, Ibaraki, Japan
[6]Department of Geriatric Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

Aim: We aimed to develop a new approach to risk stratification using metabolic syndrome as well as traditional non-metabolic risk factors, and to examine its validity in carotid atherosclerosis.
Methods: A total of 1,189 men and women aged 21-93 years old were stratified according to the absence or presence of metabolic syndrome defined by Japanese criteria, non-metabolic risk factors, and a past history of coronary heart disease. The risk stratification was as follows: (S-1) persons without a past history, non-metabolic risk factors and metabolic syndrome, (S-2a) those with metabolic syndrome only, (S-2b) those with non-metabolic risk factors only, (S-3) those with non-metabolic risk factors and metabolic syndrome but no past history, and (S-4) those with a past history. Carotid atherosclerosis was defined as maximum intima-media thickness ≥1.1 mm of the far wall of the common carotid artery.
Results: Compared with individuals without these three risk components (S-1), the odds ratio was 7.2 (2.8-18.6) for a past history (S-4), 4.3 (1.7-10.9) for non-metabolic risk factors plus metabolic syndrome but no past history (S-3), 2.6 (1.1-6.4) for non-metabolic risk factors only (S-2b) and 0.5 (0.0-5.7) for metabolic syndrome only (S-2a). Net reclassification improvement from metabolic syndrome only (presence versus absence) to our risk stratification (≥S-3 versus <S-3) was 16.4% ($p<0.0001$), suggesting that our risk stratification improved the classification of atherosclerosis in comparison to metabolic syndrome only.
Conclusion: Risk stratification based on traditional non-metabolic risk factors plus metabolic syndrome rather than metabolic syndrome only appears to be more useful for the clinical assessment of atherosclerosis, and probably in the prevention and control of atherosclerotic disease.

J Atheroscler Thromb, 2011; 18:000-000.

Key words; Metabolic syndrome, Risk factor, Carotid atherosclerosis, Risk stratification

Introduction

Metabolic syndrome, which has become a major worldwide disease management target[1-8], is a constellation of cardiovascular risk factors associated with an increased risk of cardiovascular disease[9-15], and the Japanese government started a nationwide screening and intervention strategy for metabolic syndrome since April 2008[16]. However, recent epidemiological studies have shown that the emphasis on metabolic syndrome may dismiss some high-risk individuals, especially in the non-obese population[14, 15, 17]. Therefore, we need further classification of the population

Address for correspondence: Hiroyasu Iso, Public Health, Department of Social and Environmental Medicine, Osaka University, Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871 Japan
E-mail: iso@pbhel.med.osaka-u.ac.jp
Received: October 7, 2010
Accepted for publication: January 7, 2011

with and without metabolic syndrome to reduce misclassified high-risk patients in general clinical practice.

The Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III), Final Report[3] concludes that individuals with a past history of cardiovascular disease have a substantially higher risk of coronary heart disease than those without, and that current smoking, older age, and a family history of cardiovascular disease are independent risk factors for coronary heart disease. Because these risk factors are easy to identify in general clinical practice, reclassification using this information as well as metabolic syndrome may be more useful for the clinical assessment of atherosclerosis.

For identification in clinical practice of groups at high risk of atherosclerotic disease, we attempted to develop a new method of risk stratification based on the combination of metabolic syndrome and traditional non-metabolic risk factors. We also examined the validity of this new stratification method in terms of intima-media thickness (IMT) of carotid arteries.

Materials and Methods

Study Population

We, the Defining Vascular Disease (DVD) group, conducted a cross-sectional study of 41 collaborating clinical centers in 2004. Healthy individuals and patients with cardiovascular disease, who had clinical records of risk factors and a history of cardiovascular disease, were recruited as study subjects from the institutes. They consisted of 3,415 individuals (2,034 men and 1,381 women) aged 16 to 97 years. We recruited the participants at health check-ups and from clinical outpatients at each clinical institute. The average number of participants was 88 with 631 maximum, and the percentage of individuals with a past history of coronary heart disease was 0% to 17% among the 41 institutes. Informed consent was obtained to conduct an epidemiological study based on guidelines of the Council for International Organizations of Medical Science[18]. The study protocol was approved by each institute's human ethics review committee.

We excluded 1,422 individuals who did not undergo carotid ultrasound examination and 804 without data of a past and/or family history and/or waist circumference. We did not exclude patients with familial hypercholesterolemia, because we did not collect that information; however, none of the subjects had serum total cholesterol levels ≥500 mg/dL. Therefore, 1,189 individuals (581 men and 608 women), 21 to 93 years old, from 18 clinical centers were enrolled in this study.

Cardiovascular Risk Factors

The cardiovascular risk factor data included age, height and weight, waist, circumference systolic and diastolic blood pressure, serum total cholesterol, HDL-cholesterol, triglycerides and glucose at fasting, hs-CRP, use of medication for hypertension, hyperlipidemia and diabetes mellitus, smoking status (never smoker, ex-smoker, and current smoker), alcohol intake category (never drinker, ex-drinker, and current drinker), past history of coronary heart disease, past history of other vascular diseases (transient ischemic attack, stroke, arteriosclerosis thrombangiitis obliterans, and/or aortic aneurysm), and a family history of coronary heart disease. We calculated body mass index (BMI) as weight (kg) divided by the square of height in meters (m^2), LDL-cholesterol with the Friedewald formula[19] as LDL-cholesterol (mg/dL) = total cholesterol (mg/dL) − HDL-cholesterol (mg/dL) − 0.2*triglycerides (mg/dL), and the LDL/HDL ratio as LDL-cholesterol (mg/dL)/HDL-cholesterol (mg/dL). Only two individuals had severely high levels of triglycerides (≥800 mg/dL), and we treated then as missing LDL-cholesterol, because the estimated LDL-cholesterol may have been biased.

Identification of Carotid Atherosclerosis

Carotid arteries were evaluated with high-resolution B-mode ultrasonography. We adopted the same ultrasonography protocol used in one of the largest population-based studies of carotid atherosclerosis conducted among elderly Americans, i.e., the Cardiovascular Health Study[20]. The imaging protocol involved obtaining a single longitudinal lateral view of the distal 10 mm of the right and left common carotid arteries (CCAs). To quantify the degree of thickening of the carotid artery walls, we assessed the maximum IMT of CCA, which was defined as the thickest section of either the far right or left wall of the CCA. Carotid atherosclerosis was measured at each clinical center. The carotid atherosclerosis measurement was not standardized, but we assumed that the maximum IMT of CCA is frequently measured in clinical practice and may be reliable. Carotid atherosclerosis was defined as maximum IMT of CCA ≥1.1 mm.

Risk Stratification Algorithm

For risk stratification, we used the presence or absence of 1) a past history of coronary heart disease, 2) non-metabolic risk factors and 3) metabolic syndrome, data which are easily obtained in medical prac-

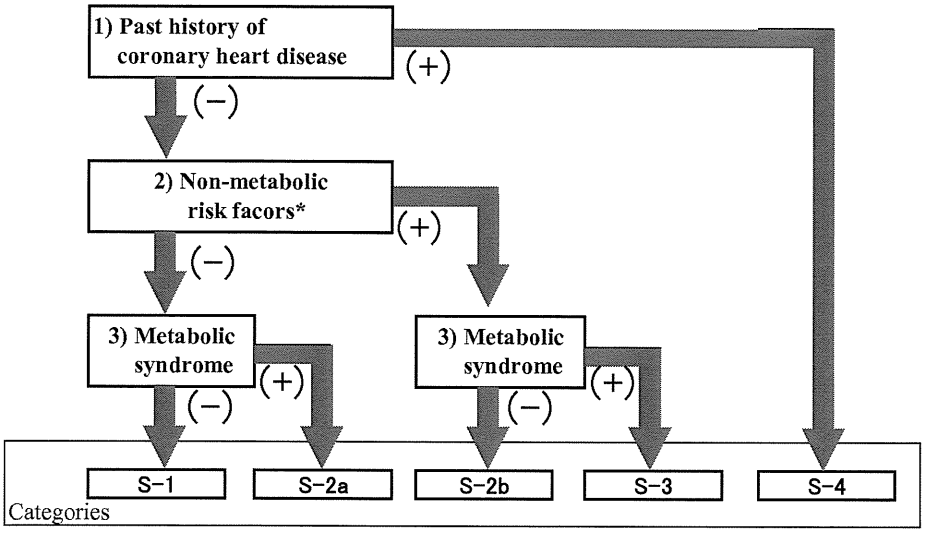

Fig. 1. Algorithm of risk stratification according to a past history of coronary heart disease, non-metabolic risk factors, and metabolic syndrome.

*Non-metabolic risk factors include older age, current smoking, family history of coronary heart disease, and a past history of other vascular diseases.

tice.

The definition by the Japanese Committee to Evaluate Diagnostic Standards for Metabolic Syndrome[6, 7] was used for the diagnosis of metabolic syndrome. This definition is based on abdominal obesity (waist ≥85 cm for men and ≥90 cm for women) plus two or more components of metabolic risk factors, namely, 1) high blood pressure: ≥130/85 mmHg; 2) high glucose: fasting glucose ≥6.1 mmol/L (110 mg/dL); 3) dyslipidemia: HDL cholesterol <1.03 mmol/L (40 mg/dL) and/or triglycerides ≥1.69 mmol/L (150 mg/dL).

We stratified the participants into five categories (S-1, S-2a, S-2b, S-3, and S-4) based on the absence or presence of 1) a past history of coronary heart disease, 2) non-metabolic risk factors, and 3) metabolic syndrome (**Fig. 1**). Non-metabolic risk factors were: 2-1) older age: ≥45 years for men and ≥55 years for women, 2-2) current smoker, 2-3) family history of coronary heart disease, and 2-4) past history of other vascular diseases (transitory ischemic attack, stroke, arteriosclerosis obliterans, and/or aortic aneurysm). Although LDL-cholesterol levels or novel risk factors such as hs-CRP were not used for our risk stratification, they were used as adjustment variables.

Statistical Analysis

Student's *t* test and the chi square test were used to compare the characteristics of subjects with and without carotid atherosclerosis. A logistic regression model including the random effect of clinical-center levels was used to calculate crude and multivariable odds ratios (ORs) and 95% confidence intervals (95%CIs) for carotid atherosclerosis according to risk stratification. Tertiles of hs-CRP and the LDL/HDL ratio were used for multivariable adjustment as potential confounding factors, because the distribution of hs-CRP and the LDL/HDL ratio were skewed.

To assess the improvement of misclassification using our risk stratification, we calculated net reclassification improvement[21], which focuses on reclassification tables constructed separately for participants with and without incidences and quantifies the correct movement in categories.

All statistical tests were two-sided and $p<0.05$ was regarded as significant. SAS, version 9.13 (SAS Institute, Inc., Cary, NC, USA) was used for all statistical analyses.

Results

Risk Factors between Subjects with and without Carotid Atherosclerosis

Compared with subjects without carotid atherosclerosis, those with carotid atherosclerosis were older, more likely to smoke, to use medication for hypertension and hyperlipidemia, and to have a past history of coronary heart disease and other vascular diseases, and were less likely to drink (**Table 1**). They also had higher mean values of weight, body mass index, waist

Table 1. Characteristics of cardiovascular risk factors stratified by the absence and presence of carotid atherosclerosis

Mean ± SD/Percentage	Total			Men			Women		
	Carotid atherosclerosis			Carotid atherosclerosis			Carotid atherosclerosis		
	(−)	(+)	*p*-value	(−)	(+)	*p*-value	(−)	(+)	*p*-value
	(*n*=784)	(*n*=405)		(*n*=353)	(*n*=228)		(*n*=431)	(*n*=177)	
Men, %	45.0	56.3	0.0002						
Age, year	59.1±11.0	66.3±9.9	<0.0001	57.8±11.7	64.9±10.3	<0.0001	60.3±10.3	68.0±9.2	<0.0001
Current smoker, %	35.7	47.7	<0.0001	65.4	73.7	0.04	11.4	14.1	0.34
Current drinker, %	45.0	29.7	<0.0001	63.7	43.2	<0.0001	30.1	12.3	<0.0001
Past history of coronary heart disease, %	7.9	32.1	<0.0001	10.5	38.2	<0.0001	5.8	24.3	<0.0001
Past history of other vascular disease, %	4.5	9.1	0.002	4.5	9.6	0.02	4.4	8.5	0.05
Family history of coronary heart disease, %	15.7	18.5	0.22	13.0	20.2	0.03	17.9	16.4	0.72
Body mass index, kg/m^2	23.3±3.6	24.1±3.8	0.0007	24.0±3.3	24.4±3.4	0.24	22.8±3.8	23.8±4.2	0.004
Waist, cm	83.1±10.0	85.4±9.7	0.0001	85.7±8.5	86.5±8.6	0.24	80.9±10.6	83.9±10.7	0.002
Systolic blood pressure, mmHg	126.6±18.0	135.9±19.0	<0.0001	127.4±16.7	135.4±18.1	<0.0001	125.9±19.1	136.4±20.1	<0.0001
Diastolic blood pressure, mmHg	73.0±10.4	74.5±11.4	0.02	74.6±10.4	76.1±11.1	0.09	71.6±10.3	72.4±11.4	0.39
Fasting blood glucose, mg/dL	103.7±23.1	114.3±35.6	<0.0001*	107.7±25.7	114.6±34.1	<0.0001*	100.4±20.2	114.0±37.6	0.01*
Total cholesterol, mg/dL	212±37	209±42	0.37	204±36	203±41	0.72	218±37	218±43	0.98
LDL-cholesterol, mg/dL	130±33	132±39	0.26	125±31	128±36	0.30	134±34	138±41	0.21
HDL-cholesterol, mg/dL	59±16	52±15	<0.0001	53±15	48±14	<0.0001	64±16	57±14	<0.0001
LDL/HDL ratio	2.35±0.89	2.70±0.98	<0.0001	2.50±0.91	2.84±1.04	<0.0001	2.23±0.86	2.53±0.86	0.0001
Triglycerides, mg/dL	118±86	125±65	0.002*	137±105	135±69	0.01*	103±61	112±57	0.30*
Hs-CRP, mg/L	1.1±2.1	1.2±2.1	0.23*	1.2±2.3	1.4±2.4	0.45*	1.0±2.0	1.0±1.7	0.21*
Medication use for hypertension, %	67.9	85.4	<0.0001	69.5	87.5	0.001	66.4	82.7	0.01
Medication use for diabetes, %	68.6	78.9	0.07	61.4	76.2	0.05	79.2	82.4	0.81
Medication use for hyperlipidemia, %	40.6	57.6	<0.0001	34.1	54.3	0.001	45.0	61.2	0.01
Metabolic syndrome and its components									
Metabolic syndrome, %	26.1	48.6	<0.0001	35.7	55.7	<0.0001	18.3	39.5	<0.0001
Abdominal obesity, %	36.6	47.4	0.0004	55.0	59.6	0.30	21.6	31.6	0.01
High blood pressure, %	53.8	77.0	<0.0001	56.4	76.8	<0.0001	51.7	77.4	<0.0001
High glucose, %	26.8	48.4	<0.0001	34.6	50.0	0.0003	20.4	46.3	<0.0001
Dyslipidemia, %	51.0	72.3	<0.0001	51.8	71.9	<0.0001	50.3	72.9	<0.0001

*: Student's *t*-test using log-transformed values because of skewed distributions.

circumference, systolic and diastolic blood pressure, fasting blood glucose, LDL/HDL ratio, and triglycerides, and lower mean values of HDL-cholesterol, and to have metabolic syndrome. These results did not change substantially after stratification for men and women; therefore, further analyses were conducted for men and women combined, adjusted for sex.

Odds Ratios of carotid Atherosclerosis According to Risk Factors

A significantly higher prevalence of carotid atherosclerosis was observed in association with each of the components except current smoking, a past history of other vascular diseases and a family history of coronary heart disease (**Table 2**). The multivariable odds ratios (95% confidence intervals) for carotid atherosclerosis were 2.5 (1.6-3.9; $p=0.0004$) for the presence versus absence of a past history, 3.8 (1.7-8.8; $p=0.003$) for the presence versus absence of non-metabolic risk factors, and 1.4 (1.0-2.0; $p=0.04$) for the presence versus absence of metabolic syndrome. These results were similar for men and women (not shown in the table). Among the components of metabolic syndrome, high blood pressure and then high glucose were strongly associated with the prevalence of carotid atherosclerosis.

Risk Stratification Algorism and Odds Ratio of Carotid Atherosclerosis

After risk stratification (**Table 3** and **Fig. 2**), we

Table 2. Crude and multivariable odds ratios (OR) and 95% confidence intervals (95%CI) of carotid atherosclerosis according to cardiovascular risk factors for men and women combined

	No. at risk	No. of cases	Crude OR (95%CI)	Multivariable* OR (95%CI)
Past history of coronary heart disease	192	130	3.0 (2.0-4.5)	2.5 (1.6-3.9)
Non-metabolic risk factors	1,096	396	4.1 (1.8-9.3)	3.8 (1.7-8.8)
Older age	1,015	384	3.9 (2.2-6.7)	3.8 (2.2-6.8)
Current smoking	473	193	1.5 (1.1-2.0)	1.3 (0.9-1.9)
Family history of coronary heart disease	198	75	1.0 (0.7-1.5)	1.0 (0.7-1.5)
Past history of other vascular diseases	72	37	1.5 (0.8-2.7)	1.4 (0.8-2.6)
Metabolic syndrome	324	148	1.7 (1.3-2.4)	1.4 (1.0-2.0)
Abdominal obesity	479	192	1.6 (1.2-2.2)	1.4 (1.0-1.9)
High blood pressure	734	312	2.4 (1.7-3.3)	2.2 (1.6-3.1)
High glucose	406	196	2.1 (1.5-3.0)	1.9 (1.4-2.7)
Dyslipidemia	693	293	1.7 (1.3-2.4)	1.4 (1.0-2.0)

*: Adjusted for sex, drinking status, hs-CRP (tertile), and LDL/HDL ratio (tertile).

observed the higher prevalence of carotid atherosclerosis in high-risk categories (S-2b, S-3, and S-4), compared with the reference category (S-1). Adjustment for potential confounding factors, i.e., sex, drinking status, hs-CRP, and the LDL/HDL ratio, did not result in a substantial change in these associations. The multivariable odds ratios (95%CI) for the study population compared to subjects without a past history, non-metabolic risk factors and metabolic syndrome (S-1) were 7.2 (2.8-18.6) for subjects with a past history (S-4), 4.3 (1.7-10.9) for those with non-metabolic risk factors and metabolic syndrome but no past history (S-3), 2.6 (1.1-6.4) for those with non-metabolic risk factors but no metabolic syndrome and no past history (S-2b), and 0.5 (0.0-5.7) for those with metabolic syndrome but no other two risk components (S-2a). Net reclassification improvement from metabolic syndrome only (presence versus absence) to our risk stratification (≥S-3 versus <S-3) was 16.4% ($p<0.0001$), suggesting that our risk stratification improved the classification of atherosclerosis in comparison to metabolic syndrome only.

The odds ratios of potential confounding factors was 1.2 (0.8-1.6) for sex (men versus women), 0.8 (0.6-1.1) for drinking status (current versus never drinkers), 1.2 (0.8-1.6) for hs-CRP (the highest versus lowest categories), and 1.9 (1.3-2.8) for LDL/HDL ratio (the highest versus lowest categories).

When subjects in S-1 were further divided into those without any metabolic risk factors (S-1a) and those with metabolic risk factors (S-1b), these was only one case of carotid atherosclerosis in S-1a and seven in S-1b (not shown in Table). The respective multivariable odds ratio of carotid atherosclerosis with reference to S-1a was 2.8 (0.3-30.3) for S-1b, 1.1 (0.1-25.6) for S-2a, 5.8 (0.7-51.6) for S-2b, 9.5 (1.1-85.8) for S-3, and 15.9 (1.7-146.5) for S-4.

Odds Ratio According to Risk Factors Stratified by Abdominal Obesity

Of 996 subjects with metabolic risk factors, 552 (55%) had no abdominal obesity but had a similarly high prevalence of a past history for coronary heart disease (17.9% versus 19.1%) and of non-metabolic risk factors (93.1% versus 96.6%), as did those with abdominal obesity (not shown in Table). As shown in **Table 4**, we observed a higher prevalence of carotid atherosclerosis in subjects with the higher number of metabolic risk factors, irrespective of abdominal obesity. Subjects with abdominal obesity but no other metabolic risk factors had higher age- and sex-adjusted triglyceride levels (67.1 mg/dL versus 89.6 mg/dL; $p=0.001$) and lower HDL-cholesterol levels (64.8 mg/dL versus 57.7 mg/dL; $p=0.009$) than those without abdominal obesity or other metabolic risk factors (not shown in Table). There were no differences in the mean blood pressure, glucose and LDL-cholesterol levels between them. The excess prevalence of carotid atherosclerosis was similarly observed for subjects with each metabolic risk factor, i.e. high blood pressure, high glucose and dyslipidemia, irrespective of abdominal obesity (**Table 4**).

Discussion

In this large cross-sectional study of Japanese men and women, we developed a new risk stratification for prevention and control of atherosclerotic dis-

Table 3. Crude and multivariable odds ratio (95% confidence interval) for subjects with ≥1.1 mm of IMT(intima-media thickness)-Cmax-far wall according to risk stratification using a past history of coronary heart disease, non-metabolic risk factors and metabolic syndrome for men and women combined.

Past history of coronary heart disease	[---------- Absence ----------]								Presence
Non-metabolic risk factors	[------ Absence ------]		1	2	3-4	1	2	3-4	
Metabolic syndrome	Absence	Presence	[---------- Absence ----------]			[---------- Presence ----------]			Absence/ Presence
Names of categories	S-1	S-2a	[---------- S-2b ----------]			[---------- S-3 ----------]			S-4
No. at risk	82	10	374	244	43	113	110	21	192
No. of cases	8	1	84	71	11	42	45	13	130
Crude OR (95%CI)	1.0	0.8 (0.1-8.7)	2.7 (1.1-6.7)	3.4 (1.4-8.4)	2.7 (0.9-8.6)	4.7 (1.8-12.5)	6.0 (2.3-15.6)	12.1 (3.2-45.3)	9.1 (3.5-23.8)
Crude OR (95%CI)	1.0	0.8 (0.1-8.5)	[----------	3.0 (1.2-7.1)	----------]	[----------	5.8 (2.3-14.4)	----------]	9.1 (3.5-23.7)
Multivariable OR (95%CI)*	1.0	0.6 (0.1-6.6)	2.6 (1.1-6.5)	3.1 (1.2-8.0)	2.5 (0.8-8.2)	4.0 (1.5-11.2)	4.9 (1.8-13.4)	10.0 (2.6-38.7)	7.9 (3.0-21.1)
Multivariable OR (95%CI)*	1.0	0.5 (0.0-5.7)	[----------	2.6 (1.1-6.4)	----------]	[----------	4.3 (1.7-10.9)	----------]	7.2 (2.8-18.6)

*: Adjusted for sex, drinking status, hs-CRP (tertile), and LDL/HDL ratio (tertile).

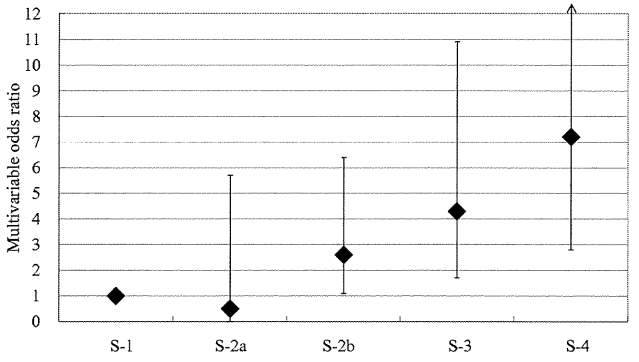

Fig. 2. Multivariable odds ratio (95% confidence interval) for subjects with ≥1.1 mm IMT (intima-media thickness) according to risk stratification using a past history of coronary heart disease, non-metabolic risk factors and metabolic syndrome.

ease based on non-metabolic risk factors (past history of coronary heart disease, older age, current smoker, family history of coronary heart disease, past history of other vascular diseases) and metabolic syndrome, which we can easily obtain in general clinical practice. We also examined the validity of this risk stratification in relation to intima-media thickness (IMT) of common carotid arteries as an indicator of carotid atherosclerosis. Our risk stratification may improve the detection of carotid atherosclerosis, compared with that using metabolic syndrome alone, since the net reclassification improvement from metabolic syndrome only to our risk stratification was large (16.4%, $p<0.0001$).

The advantage of our risk stratification is its ease of application because we used general information from medical interviews and metabolic risk factors. Previous frames for risk stratifications required the measurement of serum total cholesterol[22, 23], creatinine, aspartate transaminase, alanine transaminase and urinary protein[22], total cholesterol[23] and LDL-cholesterol[24], but some risk factors (e.g. total cholesterol) and creatinine are no longer measured in the Japanese nationwide screening and intervention program for metabolic syndrome[16].

Subjects with both non-metabolic risk factors plus metabolic syndrome (S-3) had a 4.3 times higher risk of atherosclerotic disease than the reference group (S-1), while the risk for subjects with non-metabolic risk factors only (S-2b) was still 2.6 times higher. This result suggests the importance of non-metabolic risk factors in the risk stratification of high-risk individuals, as described in a previous study[24].

On the other hand, the presence of metabolic syndrome was associated with a higher risk of atherosclerotic disease among subjects with non-metabolic risk factors. Subjects with non-metabolic risk factors plus metabolic syndrome (S-3) had a 1.7 higher prevalence of carotid atherosclerosis than those with non-metabolic risk factors only (S-2b); therefore, our results suggest the importance of both metabolic syndrome and non-metabolic risk factors for the detection of atherosclerotic disease.

It also should be mentioned that subjects without metabolic syndrome included high-risk individuals, such as those with high blood pressure, high glucose, or dyslipidemia but not abdominal obesity, when we used the Japanese criteria for metabolic syndrome

Table 4. Multivariable odds ratios (OR) and 95% confidence intervals (95%CI) of carotid atherosclerosis according to metabolic risk factors stratified by abdominal obesity for men and women combined

Abdominal obesity	[---------- Absence ----------]			[---------- Presence ----------]		
Number of metabolic risk factors	0	1	2-3	0	1	2-3
No. at risk	158	239	313	35	120	324
No. of cases	8	56	149	7	37	148
Multivariable OR (95%CI)*	1.0	4.3 (1.8-10.3)	8.5 (3.7-20.0)	3.9 (1.1-13.3)	5.9 (2.3-14.7)	8.0 (3.4-18.6)
High blood pressure	(−)	(+)		(−)	(+)	
No. at risk	332	378		123	356	
No. of cases with carotid atherosclerosis	61	152		32	160	
Multivariable OR (95%CI)*	1.0	2.3 (1.5-3.5)		1.3 (0.7-2.3)	2.5 (1.7-3.9)	
High glucose	(−)	(+)		(−)	(+)	
No. at risk	516	194		267	212	
No. of cases	108	105		101	91	
Multivariable OR (95%CI)*	1.0	3.3 (2.0-5.2)		2.0 (1.3-3.0)	2.2 (1.4-3.5)	
Dyslipidemia	(−)	(+)		(−)	(+)	
No. at risk	339	371		157	322	
No. of cases	67	146		45	147	
Multivariable OR (95%CI)*	1.0	1.6 (1.1-2.5)		1.7 (1.0-2.8)	2.0 (1.3-3.2)	

*: Adjusted for sex, drinking status, hs-CRP (tertile), and LDL/HDL ratio (tertile).

where abdominal obesity as an essential component. In fact, 55% of subjects with metabolic risk factors had no abdominal obesity but had a similar high prevalence of a past history of coronary heart disease and non-metabolic risk factors, as did those with abdominal obesity. Subjects with and without abdominal obesity also had a similar high prevalence of carotid atherosclerosis. Our finding correlates with the results from recent cohort studies that non-overweight individuals with metabolic risk factors had a similar excess risk of cardiovascular disease to overweight individuals with metabolic risk factors[14, 15, 17].

There are a few limitations to our study. First, the epidemiological data were obtained from a cross-sectional study. A causal inference could thus not be assessed. However, evidence from previous cohort studies and clinical trials supports the causality of metabolic syndrome and non-metabolic risk factors in the development of atherosclerosis. Second, our study participants were recruited from medical centers, which may have caused a selection bias. In fact, the prevalence of metabolic syndrome (28.1% for men and 25.7% for women) was higher than in the national survey (23.0% for men and 8.9% for women), especially for women[25]. Risk prediction in our study may thus have been underestimated. Third, carotid atherosclerosis was measured at each clinical center, and was not centralized; however, previous studies showed that the assessment of maximum IMT of CCA ≥1.1 mm had high reliability and was of use for the prediction of coronary heart disease events[20, 26, 27]. Fourth, we did not measure some potential cardiovascular risk factors (e.g. socioeconomic status and psychosocial factors), which may have led to residual confounding. Fifth, in our primary analysis, we did not divide S-1 into those without any metabolic risk factors (S-1a) and those with metabolic risk factors (S-1b) due to the relatively small sample size of cases in S-1; however, as discussed above, subjects with high blood pressure, high glucose or dyslipidemia, but not abdominal obesity were also likely to be at high risk. Thus, we need to pay attention to these patients in the prevention and control of atherosclerotic disease. Finally, we recruited participants with a wide range of health status (i.e. health check-ups and clinical outpatients), and excluded 2,226 subjects from our analyses due to missing data. These selections may have led to potential bias; therefore, further studies are necessary to confirm the generalizability of our risk stratification.

In summary, the study presented here provides epidemiological evidence that risk stratification based on metabolic syndrome as well as non-metabolic risk

factors is useful for the clinical assessment of atherosclerosis and probably in the prevention and control of atherosclerotic disease. We also need to pay attention to high-risk individuals without abdominal obesity, but with high blood pressure, high glucose or dyslipidemia.

Conflict of Interest Statement

None declared.

Acknowledgements

We thank the participating Defining Vascular Disease (DVD) Research Group Members listed in Appendix 1 for helping us to obtain the data. This study was jointly conducted by the DVD Research Group, AstraZeneca PLC and SHIONOGI & CO., LTD.

Appendix 1

The following individuals were Defining Vascular Disease (DVD) Research Group Members: A Kitamura, H Daida, T Shoji, T Mannami, T Murohara, K Kukiyama, M Masutani, K Kitagawa, T Hiro, A Kawaguchi, M Kuroki, M Kinoshita, S Ishibashi, M Eto, H Kotake, T Hayashi, K Shimada, Y Kumon, T Miura, H Bujo, E Nomura, T Gotohda, N Yoshioka, Y Ishigaki, S Koba, K Hirata, M Akishita, H Ogawa, S Sugiyama, K Ishiwata, K Kozaki, Y Sato, K Shirai, M Yoshida, T Hirano, K Mizuno, K Node.

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Distinct Characteristics of Circulating Vascular Endothelial Growth Factor-A and C Levels in Human Subjects

Hiromichi Wada[1]*, Shuichi Ura[1], Shuji Kitaoka[2], Noriko Satoh-Asahara[3], Takahiro Horie[4,5], Koh Ono[1,5], Tomohide Takaya[1,5], Rieko Takanabe-Mori[1], Masaharu Akao[1,6], Mitsuru Abe[1,6], Tatsuya Morimoto[1,7], Toshinori Murayama[4], Masayuki Yokode[4], Masatoshi Fujita[8], Akira Shimatsu[9], Koji Hasegawa[1]

1 Division of Translational Research, National Hospital Organization Kyoto Medical Center, Kyoto, Japan, **2** Health Evaluation Center, National Hospital Organization Kyoto Medical Center, Kyoto, Japan, **3** Division of Diabetic Research, National Hospital Organization Kyoto Medical Center, Kyoto, Japan, **4** Department of Clinical Innovative Medicine, Translational Research Center, Kyoto University Hospital, Kyoto, Japan, **5** Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **6** Department of Cardiology, National Hospital Organization Kyoto Medical Center, Kyoto, Japan, **7** Division of Molecular Medicine, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan, **8** Department of Human Health Sciences, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **9** Clinical Research Institute, National Hospital Organization Kyoto Medical Center, Kyoto, Japan

Abstract

The mechanisms that lead from obesity to atherosclerotic disease are not fully understood. Obesity involves angiogenesis in which vascular endothelial growth factor-A (VEGF-A) plays a key role. On the other hand, vascular endothelial growth factor-C (VEGF-C) plays a pivotal role in lymphangiogenesis. Circulating levels of VEGF-A and VEGF-C are elevated in sera from obese subjects. However, relationships of VEGF-C with atherosclerotic risk factors and atherosclerosis are unknown. We determined circulating levels of VEGF-A and VEGF-C in 423 consecutive subjects not receiving any drugs at the Health Evaluation Center. After adjusting for age and gender, VEGF-A levels were significantly and more strongly correlated with the body mass index (BMI) and waist circumference than VEGF-C. Conversely, VEGF-C levels were significantly and more closely correlated with metabolic (e.g., fasting plasma glucose, hemoglobin A1c, immunoreactive insulin, and the homeostasis model assessment of insulin resistance) and lipid parameters (e.g., triglycerides, total cholesterol (TC), low-density-lipoprotein cholesterol (LDL-C), and non-high-density-lipoprotein cholesterol (non-HDL-C)) than VEGF-A. Stepwise regression analyses revealed that independent determinants of VEGF-A were the BMI and age, whereas strong independent determinants of VEGF-C were age, triglycerides, and non-HDL-C. In apolipoprotein E-deficient mice fed a high-fat-diet (HFD) or normal chow (NC) for 16 weeks, levels of VEGF-A were not significantly different between the two groups. However, levels of VEGF-C were significantly higher in HFD mice with advanced atherosclerosis and marked hypercholesterolemia than NC mice. Furthermore, immunohistochemistry revealed that the expression of VEGF-C in atheromatous plaque of the aortic sinus was significantly intensified by feeding HFD compared to NC, while that of VEGF-A was not. In conclusion, these findings demonstrate that VEGF-C, rather than VEGF-A, is closely related to dyslipidemia and atherosclerosis.

Citation: Wada H, Ura S, Kitaoka S, Satoh-Asahara N, Horie T, et al. (2011) Distinct Characteristics of Circulating Vascular Endothelial Growth Factor-A and C Levels in Human Subjects. PLoS ONE 6(12): e29351. doi:10.1371/journal.pone.0029351

Editor: Marc Tjwa, University of Frankfurt-University Hospital Frankfurt, Germany

Received April 22, 2011; **Accepted** November 27, 2011; **Published** December 20, 2011

Funding: This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, Japan Research Foundation for Clinical Pharmacology, and the Smoking Research Foundation (to HW). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: hwada@kuhp.kyoto-u.ac.jp

Introduction

Obesity plays a major role in the development of dyslipidemia, hypertension and many other sub-clinical abnormalities that contribute to the atherosclerotic process and onset of cardiovascular events [1,2]. However, the mechanisms that lead from obesity to atherosclerosis and cardiovascular events are not fully understood.

It is widely accepted that adipose tissue development involves adipogenesis and angiogenesis [3]. Vascular endothelial growth factor-A (VEGF-A) signaling through VEGF receptor-2 (VEGFR-2) is the main angiogenic pathway [4]. It has been reported that VEGF-A accounts for much of the angiogenic activity of adipose tissue [5]. In addition, the administration of anti-VEGF-A antibody inhibited not only angiogenesis but also adipogenesis, which provides direct evidence that angiogenesis is essential for adipogenesis in obesity [6]. Circulating levels of VEGF-A are elevated in overweight and obese subjects [7]. Levels of VEGF-A is positively correlated with body mass index (BMI), and this correlation is apparently disconnected from insulin sensitivity [8]. However, a population-based cross-sectional study revealed that circulating VEGF-A levels have only a minor impact on the development of atherosclerosis [9].

Vascular endothelial growth factor-C (VEGF-C), a homologue of VEGF-A, plays a key role in lymphangiogenesis via VEGF receptor-3 (VEGFR-3). Deletion of *Vegfc* in mice leads to a complete absence of lymph vessels and embryonic lethality [10]. Overexpression of VEGF-C in the skin of transgenic mice induces

selective hyperplasia of the lymphatic vasculature [11]. In the clinical setting, serum levels of VEGF-C are increased in patients with some cancers and are suggested to be associated with lymph node and distant metastases, as well as a poor prognosis [12–16]. Serum levels of VEGF-C are also elevated in overweight and obese subjects [7]. However, precise relationships of serum VEGF-C levels with clinical, lipid, and metabolic profiles and atherosclerosis are unknown.

Therefore, in the present study, we examined: 1) circulating levels of VEGF-A and VEGF-C in subjects not receiving any medications and examined their association with clinical, lipid, and metabolic parameters in comparison with those of VEGF-A, and 2) serum levels of VEGF-A and VEGF-C as well as their expression levels in the aortic sinus including atheromatous plaque in apolipoprotein E (apoE)-deficient mice fed a high-fat-diet in comparison with those fed normal chow.

Methods

Subjects

A cross-sectional study was carried out during a specified period from April 2008 to March 2011. A total of 423 Japanese subjects not receiving any medications were recruited in the Health Evaluation Center of Kyoto Medical Center. All participants provided written informed consent. The study protocol was approved by the Institutional Ethics Committee of Kyoto Medical Center.

Data collection

Details are described elsewhere [17]. Briefly, blood was taken from the antecubital vein from 9 to 10 in the morning after a 12-h fast. Plasma levels of glucose and hemoglobin A1c (HbA1c), and serum levels of triglycerides, high-density-lipoprotein cholesterol (HDL-C), total cholesterol (TC), and low-density-lipoprotein cholesterol (LDL-C) were measured according to standard procedures. Non-high-density-lipoprotein cholesterol (nonHDL-C) was calculated employing the following formula: Non-HDL-C = TC−HDL-C. Immunoreactive insulin was measured using an enzyme immunoassay with a commercially available kit (Tosoh, Tokyo, Japan). The serum and plasma obtained were divided into aliquots and stored at −80°C until being assayed for VEGF-A and VEGF-C. Their serum (VEGF-C) or plasma (VEGF-A) concentrations were measured employing specific, commercially available, enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions (Quantikine, R&D Systems, Minneapolis, Minnesota, USA). The sensitivities of the assays for VEGF-C and VEGF-A were 4.6 and 5.0 pg/ml, respectively. Inter-/intra-assay coefficients of variation of ELISA for VEGF-C and VEGF-A were 7.2/3.5 and 7.0/4.5%, respectively. These assays were performed by an investigator blinded to the sources of the samples.

Experimental atherosclerosis

ApoE-deficient mice (129Ola × C57BL/6 mixed background) were a generous gift from Edward M. Rubin (University of California at Berkeley, Berkeley, California, USA) [18]. They were mated with C57BL/6 mice to produce F1 hybrids. The F1 apo E+/− mice were then backcrossed with C57BL/6 mice for 10 generations. Mice homogeneous for the apoE-null allele on a C57BL/6 background were subsequently generated. Male mice were used in the subsequent experiments. They were kept in a temperature-controlled facility under a 12-h light–dark cycle with free access to food and water. After being weaned at 4 weeks of age, mice were fed a normal chow diet (NC, Oriental Yeast Co., Ltd., Tokyo, Japan) until 6 weeks of age, when they were divided into an NC group and a high-fat-diet (HFD) group containing 40% fat and 0.15% cholesterol (Oriental Yeast). The experimental protocols were approved by the Ethics Committee for Animal Experiments of Kyoto University.

Serum samples from mice

At the age of 22 weeks, blood was drawn from the inferior vena cava of anesthetized mice and serum was separated by centrifugation at 4°C and stored at −80°C. Serum levels of total cholesterol, LDL-C, HDL-C, and triglycerides were measured using the standard methods (Nagahama Life Science Laboratory, Shiga, Japan). Those of VEGF-A and VEGF-C were measured employing specific ELISA kits according to the manufacturers' instructions (Quantikine, R&D Systems, Minneapolis, Minnesota, USA for VEGF-A, Cusabio Biotech Co., Ltd., Newark, Delaware, USA for VEGF-C).

Preparation of tissue and quantification of atherosclerosis in mice

After anesthesia, the mice were euthanized at 22 weeks of age, and their proximal aortas were excised, fixed in 4% paraformaldehyde (Nacalai Tesque, Inc, Kyoto, Japan), washed in sucrose, embedded in OCT compound (Tissue-Tek, Sakura Finetechnical Co., Ltd., Tokyo, Japan), frozen on dry ice, and then stored at −80°C until sectioning. The OCT-embedded aortas were sectioned with a cryostat, and 6-μm sections were obtained sequentially, beginning at the aortic valve. Eight sections obtained every 24 μm from the aortic sinus were stained with oil red O and used for quantification of the lesion areas. The total and atherosclerotic areas of each aorta were measured with image analysis (ImageJ), and the ratio of the atherosclerotic area to the total area was calculated.

Immunohistochemistry

The frozen sections were washed in phosphate-buffered saline (PBS) and endogenous peroxidase activity was blocked by 0.3% H_2O_2 in methyl alcohol for 30 min. The sections were washed in PBS (6 times, 5 each min) and mounted with 1% normal goat serum in PBS for 30 min. Subsequently, primary antibody (rat anti-mouse VEGF-A antibody (1:100), Biolegend, San Diego California, USA; rabbit anti-rat VEGF-C antibody (1:200) (also reacts with mouse and human VEGF-C), Abcam plc., Tokyo, Japan) was applied overnight at 4°C. After washing in PBS (6 times, 5 min), they were incubated with peroxidase-labeled secondary antibody polymer (Histofine Simple Stain Mouse MAX-PO (Rat or Rabbit), Nichirei Biosciences Inc., Tokyo, Japan) for 30 min. After washing in PBS (6 times, 5 min), a coloring reaction was carried out with diaminobenzidine (Wako Pure Chemical Industries, Osaka, Japan) and nuclei were counterstained with hematoxylin. The numbers of VEGF-A-positive and VEGF-C-positive cells were counted in a cross-section of the aortic sinus including atheromatous plaque in each mouse.

Statistical analysis

All statistical analyses were performed using Stat View version 5.0 for Windows (SAS Institute Inc., Cary, North Carolina, USA.). The Mann-Whitney U test was employed for comparisons of values between the two groups. Relationships between either of VEGF-A or VEGF-C and other parameters were analyzed by age- and gender-adjusted correlations and a stepwise linear regression. Stepwise regression was performed in a forward direction with F for the entry set to 4. Because triglycerides, fasting glucose,

immunoreactive insulin, homeostasis model assessment of insulin resistance (HOMA-IR), adiponectin, high-sensitivity C-reactive protein (hsCRP), and VEGF-A were normally distributed after logarithmic transformation, the logarithms of these parameters were used in the analyses. Data are expressed as the means ± SD or the medians and inter-quartile ranges, as appropriate. Values of $P<0.05$ were considered significant.

Results

Differential Association of Circulating Levels of VEGF-C and VEGF-A with Clinical, Lipid, and Metabolic Parameters

The clinical characteristics of subjects are shown in Table 1. The prevalence of obesity (defined as a body mass index >25 kg/m^2), hypertension (defined as systolic blood pressure >= 140 or diastolic blood pressure >= 90 mmHg), dyslipidemia (defined as LDL-C >= 140 mg/dL, HDL-C<40 mg/dL, or triglycerides >= 150 mg/dL) was 26, 13, and 42%, respectively. That of metabolic syndrome (defined as the presence of any 3 of the following 5 criteria: 1) increased waist circumference (>= 85 cm in men or >= 90 cm in women), 2) elevated triglycerides (>= 150 mg/dL), 3) reduced concentration of high-density lipoprotein cholesterol (HDL-C) (<40 mg/dL in men or <50 mg/dL in women), 4) elevated blood pressure (systolic blood pressure >= 130 or diastolic blood pressure >= 85 mmHg), and 5) elevated fasting glucose (>= 100 mg/dL)) was 16%. Thus, they were not necessarily healthy, but had yet to receive any medications. Distribution of circulating levels of VEGF-A were skewed, while levels of VEGF-C were almost normally distributed. Therefore, values of VEGF-A levels were log-transformed for subsequent analyses.

Table 1. Demographic Data of Human Subjects.

Number of patients, n	423
(Male/Female, n)	(281/142)
Age, y	45±9
Male gender,%	66±47
Body mass index, kg/m^2	22.9±3.1
Waist circumference, cm	83±9
Systolic blood pressure, mmHg	117±16
Diastolic blood pressure, mmHg	74±11
Fasting plasma glucose, mg/dL	95 [90–101]
Hemoglobin A1c,%	5.19±0.29
Immunoreactive insulin, mU/L	5.0 [4.0–8.0]
HOMA-IR	1.3 [0.8–1.8]
Triglycerides, mg/dL	96 [66–138]
HDL-C, mg/dL	69±18
Total cholesterol, mg/dL	209±32
LDL-C, mg/dL	126±31
Non-HDL-C, mg/dL	139±36
hsCRP, μg/mL	0.15 [0.10–0.30]
Adiponectin, μg/mL	7.7 [5.7–10.9]
VEGF-A, pg/mL	278 [163–434]
VEGF-C, pg/mL	6135±1409

Data are expressed as the mean ± SD, median [25–75 percentile], or number of patients. HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; Non-HDL-C: non-high-density lipoprotein cholesterol; HOMA-IR: homeostasis model assessment of insulin resistance; hsCRP: high-sensitivity C-reactive protein; VEGF-A: vascular endothelial growth factor-A, VEGF-C: vascular endothelial growth factor-C.
doi:10.1371/journal.pone.0029351.t001

Then, we examined the association of circulating VEGF-A and VEGF-C levels with clinical, lipid, and metabolic parameters after adjusting for age and gender (Table 2). Levels of VEGF-A were significantly and more strongly correlated with the body mass index, waist circumference, and adiponectin levels than VEGF-C. Conversely, levels of VEGF-C were significantly and more closely correlated with lipid (e.g., triglyceride, TC, LDL-C, and non-HDL-C) and metabolic parameters (e.g., fasting plasma glucose, hemoglobin A1c, immunoreactive insulin, and HOMA-IR).

Independent Determinants of VEGF-A and VEGF-C levels

To identify independent determinants of VEGF-A and VEGF-C levels, stepwise multiple regression analyses were performed. The body mass index and age were independent determinants of VEGF-A levels (Table 3). In contrast, independent determinants of VEGF-C were age, triglycerides, non-HDL-C, and hemoglobin A1c (Table 3). These findings suggest that VEGF-A is associated with overweightness itself, whereas VEGF-C is closely associated with lipid and metabolic disorders. The correlation between VEGF-A and the body mass index and correlations of VEGF-C with triglycerides and non-HDL-C are shown in Figures 1A, B, and C, respectively.

Serum and expression levels in atheromatous plaque of VEGF-A and VEGF-C in apoE-deficient mice

To examine the relationship between VEGF-C and atherosclerosis with dyslipidemia, apoE-deficient mice, one of the most popular animal models of dyslipidemia and atherosclerosis, were fed a HFD (n = 3) or NC (n = 3) for 16 weeks. Thereafter, body weight (42±1 vs. 31±1 g, respectively, $P<0.05$) and serum levels of total cholesterol (1137±237 vs. 685±125 mg/dL, respectively, $P<0.05$) and LDL-C (393±78 vs. 162±21 mg/dL, respectively, $P<0.05$), but not HDL-C (28±5 vs. 20±3 mg/dL, respectively) and triglycerides (122±37 vs. 127±60 mg/dL, respectively), were significantly higher in the HFD than NC group. Atheromatous plaque in proximal aortas quantified by oil red O staining was markedly greater in the HFD than NC group (Figure 2A). Immunohistochemistry revealed that the number of VEGF-C-positive cells, but not that of VEGF-A, was significantly greater in HFD with advanced atherosclerosis than NC mice with minimal atherosclerosis (Figures 2B–F). Interestingly, serum levels of VEGF-C, but not those of VEGF-A, were significantly higher in HFD than NC mice (Figures 2G and H). These findings indicate that VEGF-C, rather than VEGF-A, is closely related to advanced atherosclerosis with marked hypercholesterolemia induced by HFD in apoE-deficient mice.

Discussion

The present study demonstrated that circulating levels of VEGF-C are closely associated with dyslipidemia in marked contrast to the fact that the strongest independent determinants of VEGF-A was the body mass index. These findings suggest that VEGF-A increases in association with overweightness itself; however, VEGF-C increases in association with dyslipidemia rather than overweightness per se. To our knowledge, this is the first study to report an association between VEGF-C and dyslipidemia.

Loebig et al. demonstrated that a positive correlation between VEGF-A and body mass index and that the relationship is

Table 2. Correlations of Vascular Endothelial Growth Factor-A (VEGF-A) and Vascular Endothelial Growth Factor–C (VEGF-C) with Other Parameters.

	VEGF-A		VEGF-C	
	r	*P*	*r*	*P*
Body mass index , kg/m^2	0.21	<0.0001	0.13	0.008
Waist circumference, cm	0.19	0.0001	0.13	0.007
Systolic blood pressure , mmHg	0.09	0.06	0.08	0.09
Diastolic blood pressure , mmHg	0.16	0.001	0.08	0.09
Fasting plasma glucose, mg/dL [a]	0.09	0.06	0.11	0.03
Hemoglobin A1c,%	0.11	0.02	0.13	0.007
Immunoreactive insulin, mU/L [a]	0.08	0.1	0.17	0.0006
HOMA-IR [a]	0.09	0.07	0.18	0.0003
Triglycerides, mg/dL [a]	0.10	0.04	0.23	<0.0001
HDL-C, mg/dL	−0.09	0.054	−0.08	0.1
Total cholesterol, mg/dL	0.07	0.2	0.18	0.0002
LDL-C, mg/dL	0.07	0.1	0.17	0.0004
Non-HDL-C, mg/dL	0.11	0.03	0.20	<0.0001
hsCRP, ng/mL [a]	0.10	0.04	0.12	0.01
Adiponectin, μg/mL [a]	−0.14	0.003	−0.08	0.1
VEGF-A, pg/mL [a]	-	-	0.11	0.03
VEGF-C, pg/mL	0.11	0.03	-	-

Abbreviations used in this table are the same as in Table 1.
[a]Log-transformed to obtain normal distributions. Values were adjusted for age and gender.
doi:10.1371/journal.pone.0029351.t002

apparently disconnected from insulin sensitivity [8]. Sandhofer et al. have shown that circulating VEGF-A levels have only a minor impact on the development of atherosclerosis [9]. However, VEGF-C is tightly associated with dyslipidemia, a potent risk factor as well as a therapeutic target of cardiovascular disease. In addition, we demonstrated that serum levels and expression levels in atheromatous plaque of VEGF-C, but not VEGF-A, were significantly increased in HFD-fed apoE-deficient mice with advanced atherosclerosis, suggesting that VEGF-C was more closely related to atherosclerosis with dyslipidemia than VEGF-A.

Table 3. Independent determinants of VEGF-A and VEGF-C levels.

	VEGF-A			VEGF-C		
	β	SEM	*F*	*β*	SEM	*F*
Body mass index, kg/m^2	0.16	3.4	11			
Age, y	0.11	0.1	5	−0.18	8.1	13.1
Triglycerides, mg/dL [a]				0.14	1.3	6.2
Non-HDL-C, mg/dL				0.14	2.2	5.7
Hemoglobin A1c, %				0.11	251	4.3

Abbreviations used in this table are the same as in Table 1. These models include data on the age, a male gender, body mass index, waist circumference, systolic and diastolic blood pressures, fasting plasma glucose, hemoglobin A1c, immunoreactive insulin, HOMA-IR, triglycerides, HDL-C, total cholesterol, LDL-C, non-HDL-C, hsCRP, and adiponectin.
doi:10.1371/journal.pone.0029351.t003

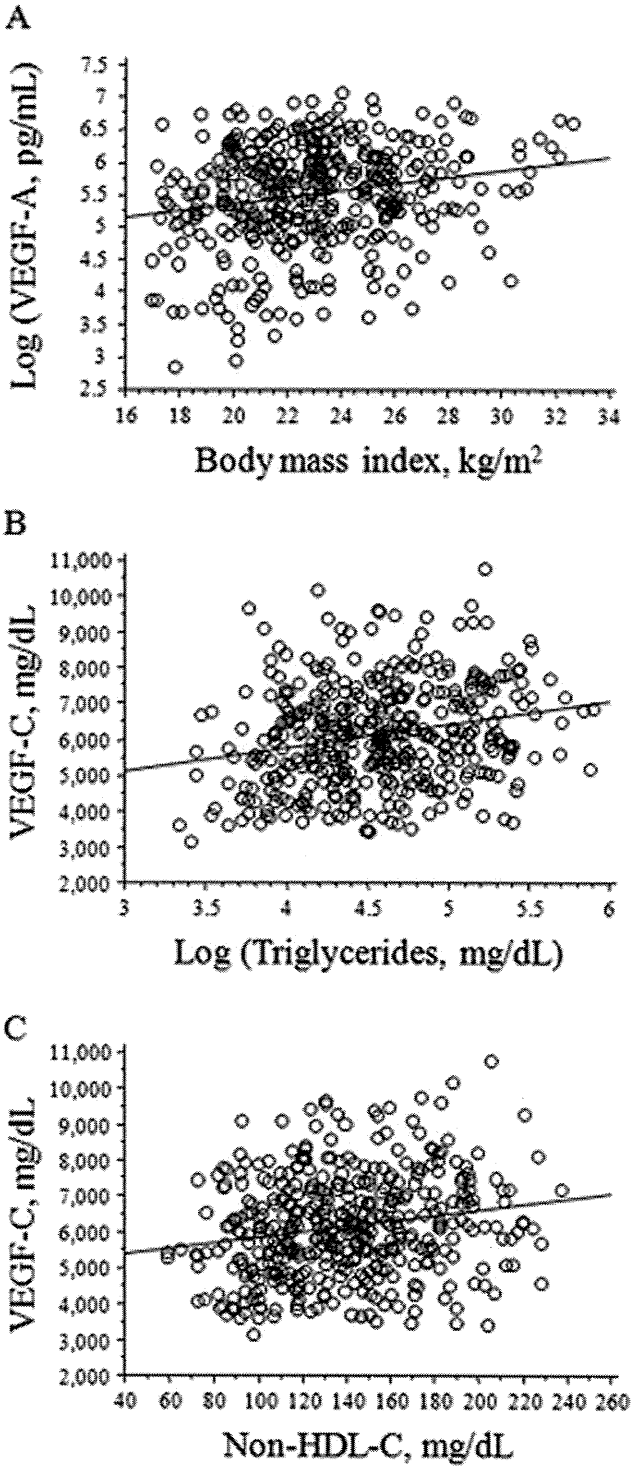

Figure 1. The correlation of circulating vascular endothelial growth factor-A (VEGF-A) or C (VEGF-C) levels with their independent determinants. A. The correlation between circulating VEGF-A levels and the body mass index. B. The correlation between those of VEGF-C and those of triglycerides. C. The correlation between those of VEGF-C and those of non-high-density-lipoprotein cholesterol (nonHDL-C).
doi:10.1371/journal.pone.0029351.g001

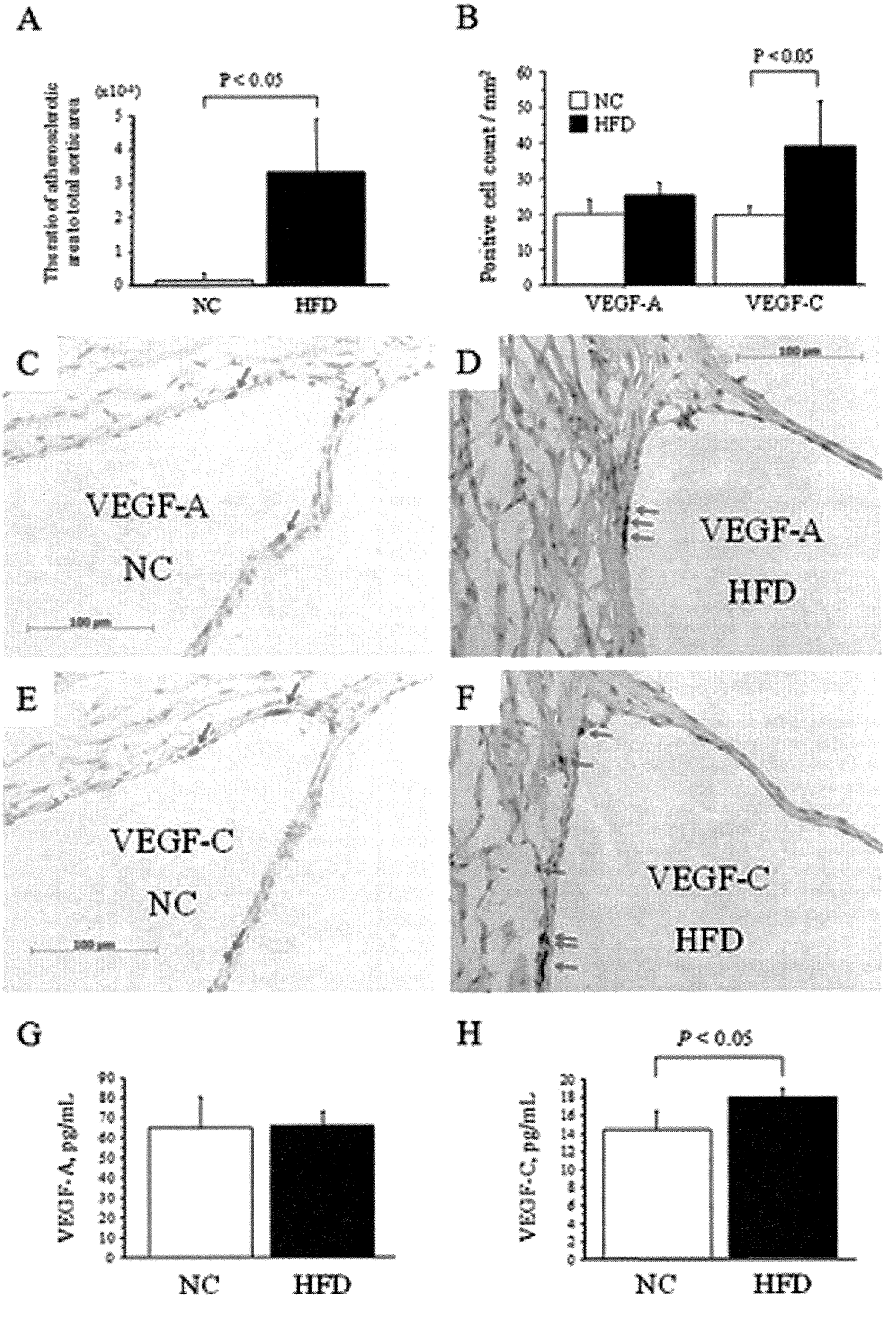
A
P < 0.05
(x10⁴)
The ratio of atherosclerotic area to total aortic area
NC
HFD
B
P < 0.05
NC
HFD
Positive cell count / mm²
VEGF-A
VEGF-C
C
VEGF-A
NC
100 μm
D
100 μm
VEGF-A
HFD
E
VEGF-C
NC
100 μm
F
VEGF-C
HFD
G
VEGF-A, pg/mL
NC
HFD
H
P < 0.05
VEGF-C, pg/mL
NC
HFD

Figure 2. Serum and expression levels in atheromatous plaque of VEGF-A and VEGF-C in apoE-deficient mice. A. Quantification of the lesion size in the proximal aortas of apolipoprotein E (apoE)-deficient mice fed normal chow (NC, n = 3) or a high-fat-diet (HFD, n = 3). The ratio of the atherosclerotic area to the total area was significantly greater in HFD than NC mice. B. Quantification of the expression of vascular endothelial growth factor-A (VEGF-A) and vascular endothelial growth factor-C (VEGF-C) in NC and HFD mice. The expression of VEGF-C, but not VEGF-A, was significantly intensified by feeding HFD compared to NC. C–F. Representative microscopic views (x400) of the expression of VEGF-A in the aortic sinus of apoE-deficient mice fed NC (C) or a HFD (D), and those of VEGF-C in NC (E) or HFD (F) mice. The red arrows indicate VEGF-A- or VEGF-C-positive cells. G and H. Serum levels of VEGF-A (G) and VEGF-C (H) in apoE-deficient mice fed a HFD or NC for 16 weeks. The data are means ± SD.
doi:10.1371/journal.pone.0029351.g002

Therefore, VEGF-C might have more impact on atherosclerosis and future cardiovascular events than VEGF-A in humans.

VEGF-C induces lymphangiogenesis, which is involved in the draining of interstitial fluid and in immune function and inflammation [11]. It has been reported that VEGF-C levels are elevated in patients with refractory hypertension, and that VEGF-C/VEGFR-3 signaling in macrophages is a major determinant of the extracellular volume and blood pressure homeostasis [19]. The trapping of VEGF-C by soluble VEGF receptor-3 blocks VEGF-C signaling, and elevates the blood pressure in response to a high-salt-diet [19]. Thus, VEGF-C seems to be up-regulated to compensate for salt-diet-induced hypertension. Similarly, VEGF-C might be up-regulated to compensate for the development and progression of atheromatous plaque by draining lipid and/or inflammatory cells in response to dyslipidemia. However, further investigation is required regarding this matter.

While lymphatic vessels are rare in the atherosclerotic intima [20], membrane-bound VEGF receptor-2 (VEGFR-2) is up-regulated in atherosclerotic lesion in human coronary arteries [21]. A recent report suggested that a soluble form of VEGFR-2 (sVEGFR-2) inhibits lymphangiogenesis by blocking the VEGF-C function, and that the tissue-specific loss of the sVEGFR-2 gene induces lymphatic invasion of the normally alymphatic cornea and hyperplasia of skin lymphatics without affecting the blood vasculature [22]. These findings suggest that naturally occurring sVEGFR-2 acts as a molecular uncoupler of blood and lymphatic vessels [22]. We recently demonstrated that serum levels of sVEGFR-2 are increased in sera from subjects with metabolic syndrome in association with insulin resistance [17]. Thus, it is of interest to elucidate the interaction between VEGF-C and sVEGFR-2 in the regulation of lymphangiogenesis at vessel walls and in the progression of atherosclerosis.

Several study limitations should be considered. First, the present study was limited due to its moderate sample size. However, all the study participants did not receive any medications including statins or renin-angiotensin-system inhibitors, which could substantially affect serum levels of angiogenesis-related factors. Thus, the relationships between these biomarkers and established risk factors in this study are physiological. Second, this human study was cross-sectional, and, thus, the results cannot help answer the question of whether elevations of VEGF-C are merely consequences of metabolic abnormality or causes of future cardiovascular events in these subjects. Third, to elucidate its prognostic significance, the direct relationship of VEGF-C with cardiovascular events and/or atherosclerosis in patients should be investigated in future studies. Finally, at present, the sources of endogenous VEGF-C in human sera, and the relationships of their levels with cardiovascular lymphangiogenic activity are unclear.

Nevertheless, the present study first demonstrates that circulating levels of VEGF-C are closely associated with dyslipidemia and atherosclerosis. Future investigations are warranted to determine the precise role of lymphangiogenesis in the pathogenesis of atherosclerosis, and the clinical utility of serum VEGF-C levels.

Acknowledgments

We thank Akira Yamada, Yuko Iida, Haruhiko Eda, and Ryosuke Takeoka for technical assistance and Akemi Wada for technical and secretarial assistance.

Author Contributions

Conceived and designed the experiments: HW. Performed the experiments: SU SK NS TH TT RT . Analyzed the data: HW KH KO MA MA T. Morimoto MF AS. Contributed reagents/materials/analysis tools: T. Murayama MY. Wrote the paper: HW.

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Geriatrics Gerontology INTERNATIONAL 1st Impact Factor in June 2010 Medline Indexed

Geriatr Gerontol Int 2012; **12:** *16–22*

COMMISSION REPORT

Toward the realization of a better aged society: Messages from gerontology and geriatrics

Hidenori Arai,[1] Yasuyoshi Ouchi,[2] Masayuki Yokode,[1] Hideki Ito,[3] Hiroshi Uematsu,[4] Fumio Eto,[5] Shinichi Oshima,[6] Kikuko Ota,[7] Yasushi Saito,[8] Hidetada Sasaki,[9] Kazuo Tsubota,[7] Hidenao Fukuyama,[1] Yoshihito Honda,[1] Akihisa Iguchi,[10] Kenji Toba,[6] Takayuki Hosoi[6] and Toru Kita[11] for the Members of the Subcommittee for Aging

[1]Kyoto University Graduate School of Medicine, Kyoto, [2]Graduate School of Medicine and Faculty of Medicine, The University of Tokyo, [3]Tokyo Metropolitan Geriatric Hospital, [4]Tokyo Medical and Dental University Graduate School, Tokyo, [5]National Rehabilitation Center for Persons with Disabilities, Tokorozawa, [6]National Center for Geriatrics and Gerontology, Obu, [7]Keio University, Fujisawa, [8]Chiba University, Chiba, [9]Sendai Tomizawa Hospital, Sendai, [10]Aichi Shukutoku University, Nagakute, and [11]Kobe City Medical Center General Hospital, Kobe, Japan

1. Background: Recent medical advancements, and improvements in hygiene and food supply have led to Japan having the longest life expectancy in the world. Over the past 50 years, the percentage of the elderly population has increased fourfold from 5.7% in 1960 to 23.1% in 2010. This change has occurred at the fastest rate in the world. Compared with France, where the percentage of the elderly population has increased just twofold in the past 100 years, Japanese society is aging at an unprecedented rate. In addition, the percentage of the very elderly (aged 75 years and over), comprising more frail people, exceeded 10% of the nation's population in 2008. In such a situation, many elderly Japanese wish to spend their later years healthy, and wish to achieve great accomplishments in their lives. To achieve that, rather than considering an aging population as a negative social phenomenon, we should create a society where elderly people can enjoy a healthy, prosperous life through social participation and contribution.

Factors that hamper the elderly from leading a healthy life include various psychological and social problems occurring in older age, as well as a high incidence of diseases. Therefore, gerontology, which focuses on health promotion of the elderly by encompassing the study of social welfare, psychology, environment and social systems; and geriatrics, which focuses on health care of elderly people and carried out research, education and practices to promote health in the elderly, are becoming more important. Furthermore, along with a need for multidisciplinary care to support geriatric medicine, the development of a comprehensive education system for aged-care professionals is awaited. Thus, we should now recognize the importance of gerontology and geriatrics, and a reform of medical-care services should be made in order to cope with the coming aged society.

Population aging is a global phenomenon. The actions being taken by Japan, the world's most aged society, have been closely watched by the rest of the world. Japan's aged society has been posing not only medical, nursing and welfare problems, but also complex problems closely associated with economy, industry and culture. Therefore, to solve these

Accepted for publication 3 October 2011.

Correspondence: Professor Hidenori Arai MD PhD, Department of Human Health Sciences, Kyoto University Graduate School of Medicine, 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Email: harai@kuhp.kyoto-u.ac.jp

Proposal from The Subcommittee for Aging, The Science Council of Japan

problems, a macroscopic integration and cooperation among industries, education institutions, administration and community through an interdisciplinary approach including medical science, nursing science, nursing care, study of social welfare, social science, engineering, psychology, economics, religion and ethics should be made. Regarding the promotion of gerontology, the "**Committee for Establishing a Scientific Community for Sustainable Aged Society**" of the Science Council of Japan also prepared a proposal and this was announced on 20 April 2011.

2. Current situation and problems

(1) Promotion of social participation and contribution of elderly people

In Japan, the overall labor force rate is expected to decrease in the near future as a result of the low birth rate and high life expectancy. In contrast, many elderly people, particularly the young-old, have sufficient physical strength to fulfil their job duties and make a social contribution. For these people, a social structure where elderly people can work should be developed through re-educating the elderly and providing various job types. Promotion of social participation and contribution of the elderly is expected to cause a substantial increase in the labor force. Furthermore, it is also expected to contribute to not only the upturn of national economic activity through an increase in total consumption, but also a decrease in the number of elderly people who are likely to be in need of care. Therefore, in order for elderly people to be engaged in various social activities, strategies for developing a social structure for re-education, various employment statuses and employment opportunities should be prepared. However, as the total number of jobs is fixed, consideration should also be given to young workers.

(2) Fostering medical specialists for aging

Older people often suffer from many diseases, together with geriatric syndromes with multiple etiologies. Signs and symptoms vary according to each individual, and are often atypical; therefore, the patients visit different hospitals and receive many screening tests and prescriptions at the same time. To solve this problem, an effective screening system carried out by a primary-care doctor, and privacy-preserving medical data sharing among hospitals and clinics are needed. In a geriatric clinical setting, health-care professionals should be aware of the physical traits of older people who often develop not only dementia, but also geriatric syndromes, such as depression, falls and urinary incontinence, so that a holistic approach with consideration of nursing care is required. However, the existing Japanese medical education system is not prepared for medical professionals enabled to respond to the aforementioned requirements. Thus, the fostering of medical professionals who can provide comprehensive care – especially for the oldest-old – such as geriatric specialists and medical professionals who understand the principles of elderly care, is urgently needed.

(3) Diagnosis of elderly-specific diseases and reform of medical-care services

In Japan, the diagnostic system for elderly-specific diseases, including dementia, and reform of medical care services are markedly delayed. The current status concerning diagnosis, care and nursing should be investigated to collect academic data. In order to accumulate evidence for providing safe elderly care and nursing, the promotion of clinical research and a marked expansion of geriatric medical centers with high-level medical services are eagerly awaited.

(4) Promotion of home-based care and multidisciplinary care

To reduce the length of stay in acute hospitals, to reduce the physical burden of health-care professionals working at acute hospitals and to meet the demand of older people who prefer to remain in their own homes, further promotion of home-based care is needed. In addition, "multidisciplinary care" is increasingly needed to meet various demands in the medical care and welfare of the elderly. It is considered important to share countermeasures against the problems of disease prevention, medicine, care and welfare among health-care professionals in medicine, care and welfare, and cooperate by making the best use of health-care professionals' specialties.

3. Contents of the proposal

The subcommittee for aging, thus, provided the following proposal:

1 Development and promotion of systems that enable elderly people to participate socially and make a contribution using an interdisciplinary approach among the various areas,