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Original Article

Alpha-2-macroglobulin as a Promising Biological Marker of Endothelial Function

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***Aims*: Alpha-2-macroglobulin (α_2MG) is thought to be associated with inflammatory reactions and procoagulant properties that might cause ischemic stroke. Endothelial dysfunction plays an important role in atherosclerosis development and in the occurrence of cardiovascular events. In this study, we investigated whether serum α_2MG levels, endothelial function, and endothelial progenitor cell (EPC) number were associated in patients with chronic stroke or cardiovascular risk factors.**

***Methods*: Patients with a history of stroke or any established cardiovascular risk factors were enrolled in this study ($n=102$; 69 men, 70.1 ± 9.2 years). Endothelial function was assessed by flow-mediated dilation (FMD). EPC numbers (CD34+/CD133+) were measured using flow cytometry ($n=91$). Serum α_2MG levels were measured by nephelometry.**

***Results*: Patients in the highest tertile of serum α_2MG levels were older ($P=0.019$) and more frequently exhibited dyslipidemia ($P=0.021$). Univariate-regression analysis revealed that increased α_2MG levels were negatively associated with FMD values ($r=-0.25$; $P=0.010$), whereas increased EPC numbers were positively associated ($r=0.21$; $P=0.044$). Multivariate-regression analysis adjusted for male gender, hypertension, and severe white-matter lesions showed that serum α_2MG levels were independently associated with FMD values (standardized partial regression coefficient [β] −0.185; $P=0.033$), although not significantly associated with EPC numbers.**

***Conclusion*: Serum α_2MG levels might reflect endothelial dysfunction evaluated by FMD in patients with chronic stroke or cardiovascular risk factors.**

Key words: Endothelial dysfunction, Biological marker, Flow-mediated dilation, Endothelial progenitor cell, Stroke

Introduction

Alpha-2-macroglobulin (α_2MG) is a large plasma glycoprotein that functions as a broad-spectrum proteinase inhibitor[1)]. In mammals, α_2MG is synthesized principally in the liver[2)] and enhances procoagulant properties through the neutralization of plasmin, plasminogen activators, and activated protein C[3-5)]. Additionally, α_2MG binds several cytokines, including interleukin (IL)-6, platelet-derived growth factor (PDGF), nerve-growth factor, tumor-necrosis factor (TNF)-α, and IL-1β[6, 7)]. Therefore, α_2MG might play an important role in the interactions between several cytokines and the process of inflammation.

Procoagulant properties and chronic inflammation are associated with vascular risk factors, atherosclerosis, and thromboembolism; however, there are few studies elucidating how α_2MG is associated with these factors in humans. We previously reported that serum α_2MG levels in patients with acute ischemic stroke were higher than those in control subjects[8)].

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Additionally, elevated serum α_2MG levels are independently associated with the severity of white-matter lesions (WMLs), which are thought to be associated with age, vascular risk factors, inflammation, and endothelial dysfunction[9]. Therefore, we hypothesized that serum α_2MG levels might represent a candidate biological marker for endothelial dysfunction.

Ultrasonographic assessment of brachial artery flow-mediated dilation (FMD) is a useful method to measure endothelium-dependent vasodilation[10]. Endothelial progenitor cells (EPCs), which are considered to play an essential role in maintaining endothelial integrity and repair, are also thought to be associated with endothelial function[11]. To test this hypothesis, we evaluated associations between serum α_2MG levels, FMD, and the number of EPCs in patients with chronic stroke or cardiovascular risk factors.

Methods

Patients

This was a single-center hospital-based prospective study. The study protocol was governed by the guidelines of the national government, based on the Helsinki Declaration revised in 1983, and was approved by the Institutional Research and Ethics Committee of the Hiroshima University Hospital (Hiroshima, Japan). All patients were recruited in an outpatient setting and provided written informed consent for participation in the study. Patients with stroke (at least 6 months after the stroke onset) or other atypical neurological problems with any established cardiovascular risk factors (such as hypertension, diabetes mellitus, dyslipidemia, atrial fibrillation, and renal dysfunction) and who were undergoing a clinically indicated ultrasonographic examination of their carotid artery systems were enrolled between November 2012 and April 2014 at the Hiroshima University Hospital. The patients with acute illness (such as infection, acute heart failure, acute renal dysfunction, and so on), severe renal dysfunction, or severe hepatic dysfunction or having undergone recent surgery were excluded. Each patient underwent FMD studies and blood-sample collection in the morning on the same day. The following baseline clinical characteristics were evaluated: age, sex, body mass index, hypertension, diabetes mellitus, dyslipidemia, atrial fibrillation, renal dysfunction, history of stroke, coronary artery disease, and smoking. In addition to obtaining a medical history, relevant risk factors were identified from a self-reported medical history or inferred from medications prescribed by the primary physician. Criteria for hypertension, diabetes mellitus, and dyslipidemia were as previously described[12]. Renal function was calculated with the estimated glomerular filtration rate (eGFR) using a revised equation for the Japanese population as follows: eGFR (mL/min/1.73 m^2) = $194 \times (\text{serum creatinine})^{-1.094} \times (\text{age})^{-0.287} \times 0.739$ (for women)[13]. Renal dysfunction was defined as an eGFR < 60 mL/min/1.73 m^2.

FMD

Patients were requested to abstain from smoking and consumption of alcohol and caffeine on the examination day of FMD, which was conducted during a fasting state in the morning, with only drinking water given to the patients. Most of the medications taken by the patients were withheld, and only those deemed necessary (such as antithrombotic therapies) were administered at the discretion of the attending physician. The types of medications prescribed to enrolled patients are shown in **Supplemental Table 1**. A high-resolution linear artery transducer that was coupled to computer-assisted analysis software (UNEXEF18G; UNEX Co., Nagoya, Japan) was used to evaluate FMD. The protocol for FMD measurement was previously described in detail[14]. Briefly, the longitudinal image of the brachial artery was assessed prior to and following the generation of a vascular response to reactive hyperemia by a 5-min period of forearm occlusion to evaluate FMD. FMD was automatically calculated as the percent change in peak vessel diameter from the baseline value, with percent FMD (peak diameter − baseline diameter/baseline diameter) used for the analysis. A single investigator (T.N.) who was unaware of the clinical details of the patient performed the FMD evaluations of all patients. The intra-observer coefficients of variation were 1.2% for the baseline brachial artery diameter among all patients and 11.1% for the FMD of 18 randomly selected patients.

Magnetic Resonance Imaging (MRI)

All patients underwent MRI performed with a 1.5 T scanner (SIGNA; GE Medical Systems, Fairfield, CT, USA or Magneton Symphony Advanced or Avanto, Siemens Medical Systems, Erlargen, Germany) or a 3.0 T scanner (SIGNA). The imaging protocol consisted of a T1-weighted spin-echo, a T2-weighted spin-echo, and fluid-attenuated inversion recovery. The severity of WMLs was rated visually from the fluid-attenuated inversion-recovery images using the Fazekas scale as follows: no lesions (grade 0), punctate lesions (grade 1), early confluent lesions (grade 2), and confluent lesions (grade 3)[15]. Patients were classified into one of two groups according to their Fazekas scale: the mild WML group (0−1) or the severe WML group (2−3). Two stroke neurologists (T.N. and M. A.) who were unaware of the clinical details of the patients graded WML severity for all patients.

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Table 1. Patient characteristics classified according to tertiles of Alpha-2-macroglobulin values.

	Alpha-2-macroglobulin values			*P*
	Tertile 1 (*n*=34)	Tertile 2 (*n*=34)	Tertile 3 (*n*=34)	
Age	66.5±9.5	71.5±9.1	72.3±8.2	0.019
Male	26 (76.5)	24 (70.6)	19 (55.9)	0.174
Body mass index (kg/m^2)	22.0±2.7	23.4±2.7	23.0±3.4	0.139
History of smoking	16 (47.1)	17 (50.0)	12 (35.3)	0.434
Hypertension	21 (61.8)	26 (76.5)	25 (73.5)	0.371
Diabetes mellitus	7 (20.6)	8 (23.5)	15 (44.1)	0.068
Dyslipidemia	15 (44.1)	22 (64.7)	26 (76.5)	0.021
Atrial fibrillation	3 (8.8)	8 (23.5)	4 (11.8)	0.194
Renal dysfunction	9 (26.5)	10 (29.4)	15 (44.1)	0.255
History of stroke	29 (85.3)	20 (58.8)	23 (67.7)	0.051
History of coronary artery disease	4 (11.8)	2 (5.9)	4 (11.8)	0.642
Physiological findings				
FMD (%)	5.2±2.5	4.0±2.0	3.6±1.6	0.008
Laboratory findings				
White blood cell (10^3/μL)	5.66±1.26	6.12±2.71	5.55±1.53	0.440
hs-CRP (ng/mL)	0.223±0.598	0.466±1.359	0.133±0.224	0.267
endothelial progenitor cell (/μL) (*n*=91)	0.645±0.376 (*n*=29)	0.555±0.283 (*n*=31)	0.514±0.199 (*n*=31)	0.215
MRI findings				
Severe white matter lesions	8 (23.5)	13 (38.2)	19 (55.9)	0.022

The data are presented as the mean±SD for age, body mass index, FMD value, and laboratory findings or numbers of patients (%) for others.
Tertiles of Alpha-2-macroglobulin values: Tertile 1, 109−176 (mg/dL); Tertile 2, 178−216 (mg/dL); Tertile 3, 218−393 (mg/dL).
FMD, flow-mediated dilation; hs-CRP, high-sensitivity C-reactive protein; MRI, magnetic resonance imaging.

Blood Samples

Peripheral blood samples from each patient were collected after FMD examination. Serum α_2MG levels were measured using laser nephelometry according to the manufacturer's instructions. Serum high-sensitivity C-reactive protein (hs-CRP) was measured using a CRP-Latex kit (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. To determine the number of EPCs, fluorescence-activated cell analysis was performed using a single platform and a lyse-no-wash procedure as previously reported[16-18]. We defined EPCs as CD34+/CD133+ cells. Briefly, peripheral blood was collected into EDTA-containing tubes following venous puncture. Nonspecific antibody binding of whole blood (200 μL) was blocked using 10 μL FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 min on ice. Whole-blood samples were then stained with the conjugated antibodies anti-CD34-APC (Beckman Coulter, Brea, CA, USA) and anti-CD133-PE (Miltenyi Biotec) for 30 min on ice in the dark. After incubation, erythrocytes were lysed with 1 mL of VersaLyse lysing solution (Beckman Coulter) for 10 min in the dark, and the remaining cells were analyzed by a flow cytometer (Cyan-ADP; Beckman Coulter). One investigator (T.N.) measured the number of EPCs in 91 subjects (89.2%); however, the number of EPCs could not be measured in the remaining 11 patients because of mechanical troubles related to the flow cytometer. All samples were stained in duplicate, and the mean number of EPCs was analyzed. The inter-assay coefficient of variation was 13.3% for the number of EPCs. The detailed methods of measuring the number of EPCs were as previously described[19].

Statistical Analysis

Statistical analysis was performed using JMP 12.01 statistical software (SAS Institute Inc., Cary, NC, USA). Data are expressed as the means±standard deviations (SDs) or medians (25th and 75th percentiles) for continuous variables and as frequencies and percentages for discrete variables. The statistical significance of intergroup differences was assessed by χ^2 tests, unpaired *t* tests, and the Mann-Whitney *U* test, as appropriate. Relationships between FMD value and the other variables were examined by Spearman's correlation. Indicators of the severity of endothelial dysfunction (FMD value) were identified using multiple linear regression

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with all factors listed in **Table 1**, using a backward-selection procedure incorporating $P>0.10$ for the likelihood-ratio test as an exclusion criterion. Differences were considered statistically significant at $P<0.05$.

Results

A total of 102 patients (69 men, 70.1 ± 9.2 years) were studied. Of these, 18 had a history of cardioembolism, 10 had a history of large-artery atherosclerosis, 19 had a history of small-artery occlusion, 14 had a history of stroke of other determined etiology, three had a history of transient ischemic attack, and eight had a history of intracranial hemorrhage caused by hypertension. **Table 1** shows the baseline clinical characteristics of the patients, who were divided into three groups according to the tertiles of serum α_2MG levels (109−176, 178−216, and 218−393 mg/dL). Patients with the highest α_2MG levels were significantly older ($P=0.019$) and had more frequent dyslipidemia ($P=0.021$). Additionally, these patients had a tendency to have diabetes mellitus ($P=0.068$), and WML severity was associated with serum α_2MG levels ($P=0.022$). FMD values were significantly lower among the patients with the highest α_2MG levels ($P=0.008$), whereas there was no significant association between the number of EPCs and serum α_2MG levels.

Scatter plots of the relationships between serum α_2MG levels, age, FMD value, and the number of EPCs are shown in **Fig. 1**. Univariate-regression analysis revealed a significant positive relationship between patient age and serum α_2MG level ($r=0.23$; $P=0.021$) and a negative relationship between patient age and FMD value ($r=-0.27$; $P=0.007$) or EPC number ($r=-0.25$; $P=0.015$). **Fig. 1** also shows that increased serum α_2MG levels were negatively correlated with FMD values ($r=-0.25$; $P=0.010$), whereas increased EPC numbers were positively correlated ($r=0.21$; $P=0.044$), although no correlation was observed between serum α_2MG levels and the number of EPCs.

Univariate-regression analysis revealed that FMD values were also associated with male gender, body mass index, hypertension, diabetes mellitus, renal dysfunction, and WML severity (**Table 2**). Multivariate-regression analysis showed that serum α_2MG levels (standardized partial regression coefficient [β] −0.185; $P=0.033$), male gender (β −0.237; $P=0.008$), hypertension (β −0.192; $P=0.032$), and WML severity (β −0.334; $P<0.001$) were independently associated with FMD values (**Table 2**). Conversely, the number of EPCs was not associated with FMD value according to multivariate-regression analysis using the backward-selection procedure.

Discussion

This study revealed that serum α_2MG levels were negatively associated with FMD values among patients with chronic stroke or cardiovascular risk factors. Additionally, serum α_2MG levels were independently associated with endothelial function as measured by FMD after adjustment for male gender, hypertension, and severe WMLs. Our results indicated that serum α_2MG levels might, therefore, represent a biomarker of endothelial function in these patients.

α_2MG binds various cytokines, such as basic fibroblast growth factor (bFGF), PDGF, nerve-growth factor, IL-1β, and IL-6[7]. Proinflammatory cytokines (IL-1, IL-6, and TNF) increase during inflammation, as does IL-6-induced expression of α_2MG[20, 21]. However, the biological activity of some cytokines is inhibited when bound to α_2MG, including that of IL-1β and bFGF. Therefore, serum α_2MG is thought to modulate inflammatory status. Additionally, elevated α_2MG levels might stimulate blood coagulant properties[4]. Consistent with this finding, several studies reported that serum α_2MG levels might constitute a marker of microvascular complications in patients with diabetes mellitus[22, 23]. Furthermore, we previously reported that serum α_2MG levels, which were associated with high-grade WMLs, might reflect the chronic condition of cerebral small-vessel disease among patients with acute ischemic stroke[8]. In the present study, we demonstrated that increased serum α_2MG levels were independently associated with endothelial dysfunction, following adjustment for male gender, hypertension, and severe WMLs among patients with cardiovascular risk factors, most of whom had a history of stroke. Therefore, serum α_2MG levels might indirectly reflect the severity of endothelial dysfunction related to chronic inflammatory or coagulant status in patients with cardiovascular risk factors.

Human α_2MG is a ~720-kDa plasma glycoprotein that forms a tetramer composed of two non-covalently associated dimers of structurally identical disulfide-linked subunits[24]. Recently, serum monomeric α_2MG (182 kDa), termed the cardiac isoform of α_2MG, was considered to represent a promising biomarker for several cardiac diseases[25-27]. Further studies will be needed to clarify whether monomeric α_2MG is also associated with endothelial function.

Previous studies reported that the number of EPCs is associated with vascular risk factors and FMD values[11, 28]. In this study, the number of EPCs was not significantly associated with FMD values according to multivariate-regression analysis, although they were positively related according to Spearman's correlation. However, the definition of EPCs differs between studies

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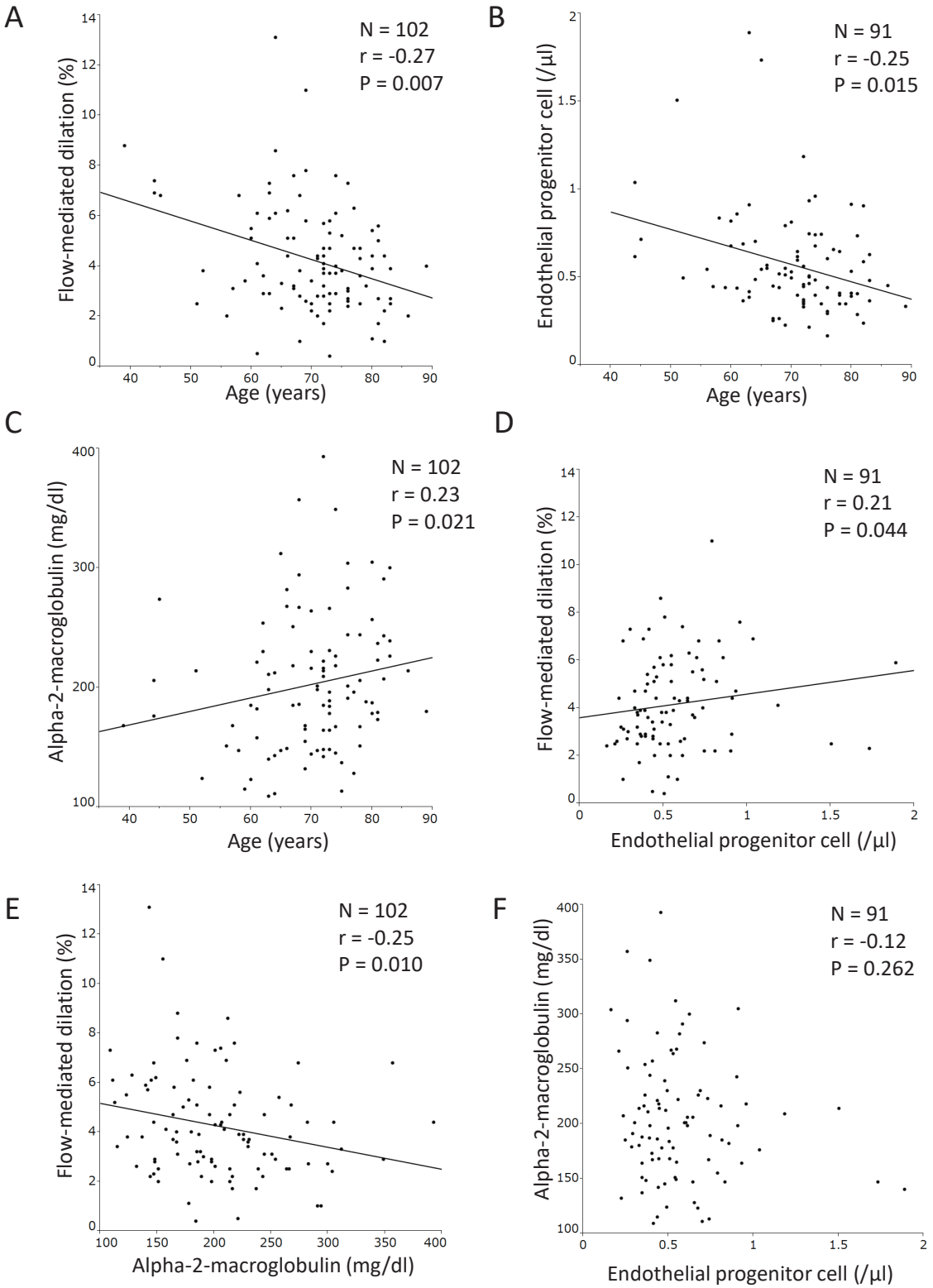

Fig. 1. Scatter plot of the relationships between age, flow-mediated dilation value, number of endothelial progenitor cells, and serum alpha-2-macroglobulin level. Scatter plot of the relationship between A) age and flow-mediated dilation value, B) age and number of endothelial progenitor cells, C) age and serum alpha-2-macroglobulin level, D) number of endothelial progenitor cells and flow-mediated dilation value, E) serum alpha-2-macroglobulin level and flow-mediated dilation value, and F) number of endothelial progenitor cells and serum alpha-2-macroglobulin level.

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Table 2. Associations between cardiovascular risk factors, MRI findings, laboratory findings, and flow mediated dilation (FMD) value.

	Flow mediated dilation			
	Spearman's correlation		Multiple linear regression	
	r	*P*	β coefficient	*P*
Age	−0.266	0.007	−	−
Male	−0.309	0.002	−0.237	0.008
Body mass index (kg/m^2)	−0.255	0.010	−	−
History of smoking	−0.132	0.188	−	−
Hypertension	−0.305	0.002	−0.192	0.032
Diabetes mellitus	−0.277	0.005	−	−
Dyslipidemia	−0.093	0.351	−	−
Atrial fibrillation	0.056	0.576	−	−
Renal dysfunction	−0.208	0.036	−	−
History of stroke	−0.106	0.287	−	−
History of coronary artery disease	−0.095	0.344	−	−
Laboratory findings				
White blood cells (10^3/μL)	−0.012	0.902	−	−
hs-CRP, log (ng/mL)	−0.224	0.024	−	−
Alpha-2-macroglobulin (mg/dL)	−0.253	0.010	−0.185	0.033
endothelial progenitor cell (/μL) (n=91)	0.212	0.044	−	−
MRI findings				
Severe white matter lesions	−0.454	<0.001	−0.334	<0.001

β coefficient indicates the standardized partial regression coefficient.
hs-CRP, high-sensitivity C-reactive protein; MRI, magnetic resonance imaging.

and is based on various subsets of circulating progenitors derived from bone-marrow pluripotent stem cells. For example, it was reported that EPCs displayed immunopositivity for CD34, CD45, CD133, and kinase-inert domain receptor[28]. Moreover, the functional properties of EPCs as assessed by the number of endothelial colony-forming units demonstrated their influences with respect to cardiovascular events and mortality[11, 29]. In particular, the migration of circulating progenitor cells was significantly lower in patients with atherosclerosis obliterans than that in healthy controls[30]. These studies might indicate that the function, as well as the number, of EPCs plays important roles in endothelial function. It should be noted that our findings of associations between EPCs and FMD values might not be conclusive, because we defined EPCs only by their combined CD34- and CD133-positivity and could not evaluate their function. However, our results demonstrated that serum α$_2$MG levels showed a greater degree of association with FMD values than did the number of EPCs (CD34+/CD133+).

This study had several limitations. First, the study was based on a cross-sectional analysis allowing the assessment of only association rather than causal relationship. Second, this was a single-center study with a small sample size, which may have resulted in selection bias. Third, we defined EPCs only as those showing combined CD34- and CD133-positivity. As noted, the published definition of EPCs varies; therefore, this requires investigations, using other means of measurement, of the relationships involving EPC number and function. Fourth, we could not evaluate the number of EPCs in all patients mechanical troubles with the flow cytometer. Although the patients without measured EPC numbers had histories with a higher prevalence of coronary artery disease and had higher white blood cell or CRP levels than for those with EPC measurements (data not shown), there were no differences in other baseline characteristics. Fifth, in this study, the intra-observer coefficient of variation was 11.1% for the FMD of 18 randomly selected patients. The reproducibility of FMD might be relatively low as compared with that of a previous report[31], although the intra-observer coefficient of variation for the baseline brachial artery diameter among all patients was relatively good as compared with that of a previous report (1.2% vs. 2.8%)[30]. Finally, several medications, such as ACE inhibitors, ARB, statins, and cilostazol, reportedly improve endothelial function[32-37], although inconsistent results have also been reported[38]. In this study,

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FMD values were not associated with each medication in univariate-regression analysis (data not shown). Here most of the medications taken by the patients were withheld on the day of the FMD examination; however, certain medications (such as antithrombotic therapies) were taken by the patients without interruption, as withholding of such medications could have resulted in cardiovascular events. Therefore, those medications (withheld or continuously taken) might have influenced the results.

Conclusion

Serum α_2MG levels were independently associated with endothelial function as measured by FMD among patients with cardiovascular risk factors. A recent systematic review and meta-analysis showed that impaired FMD is a predictor of future cardiovascular events[39]. Further studies will be required to determine whether serum α_2MG levels might also serve as a prognostic indicator of cerebral and cardiovascular events.

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Conflict of Interest

Naohisa Hosomi reports an honorarium from Mochida Pharmaceutical Co., Ltd., which is not associated with the submitted work. Hirofumi Maruyama reports grants from Bayer Yakuhin, Ltd, DAIICHI SANKYO COMPANY, LIMITED, Otsuka Pharmaceutical Co., Ltd., Sumitomo Dainippon Pharma Co., Ltd., Nihon Medi-Physics Co.,Ltd., Pfizer Japan Inc., SHIONOGI & CO., LTD., and Eisai Co., Ltd., which are not associated with the submitted work. Masayasu Matsumoto reports honoraria from DAIICHI SANKYO COMPANY, LIMITED, Takeda Pharmaceutical Company Limited, Otsuka Pharmaceutical Co., Ltd., Nippon Boehringer Ingelheim Co., Ltd., Sumitomo Dainippon Pharma Co., Ltd., Novartis Pharma K.K., and Bristol-Myers Squibb, and reports grants from Bayer Yakuhin, Ltd, DAIICHI SANKYO COMPANY, LIMITED, Takeda Pharmaceutical Company Limited, Otsuka Pharmaceutical Co., Ltd., Nippon Boehringer Ingelheim Co., Ltd., Sumitomo Dainippon Pharma Co., Ltd., Bristol-Myers Squibb, MOCHIDA PHARMACEUTICAL CO.,LTD., Kyowa Hakko Kirin Co., Ltd., Nihon Medi-Physics Co.,Ltd., MSD K.K., Pfizer Japan Inc., SHIONOGI & CO., LTD., Mitsubishi Tanabe Pharma Corporation, and Eisai Co., Ltd, which are not associated with the submitted work.

Other authors declare that their having no conflicts of interest.

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Supplemental Table 1. The types of medications.

Medications	Numbers of patients (%)
Antiplatelet agent	55 (53.9)
Aspirin	25 (24.5)
Cilostazol	16 (15.7)
Clopidogrel	18 (17.6)
Anticoagulant agent	24 (23.5)
Warfarin	22 (21.6)
Other anticoagulant agent	2 (2.0)
Antihypertensive agents	59 (57.8)
ACE inhibitor or ARB	43 (42.2)
Calcium channel blocker	35 (34.3)
Other antihypertensive agents	16 (15.7)
Statin	52 (51.0)
Oral hypoglycemic agent	26 (25.5)

ACE, angiotensin-converting enzyme; ARB, angiotensin Ⅱ receptor blocker.