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<https://doi.org/10.15017/1931779>

出版情報 : Kyushu University, 2017, 博士 (医学) , 課程博士
バージョン :
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The Relation between Serum Endostatin Level and Carotid Atherosclerosis in Healthy Residents of Japan: Results from the Kyushu and Okinawa Population Study (KOPS)

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Aim: To examine the association between the serum endostatin levels and subclinical atherosclerosis independent of traditional risk factors in a healthy Japanese population.

Methods: Among 1,057 residents who attended free public physical examinations between 2010 and 2011, we evaluated the data of 648 healthy residents for whom the serum endostatin level and common carotid intima-media thickness (IMT) were successfully measured.

Results: The median endostatin level was 63.7 ng/mL (interquartile ranges: 49.7–93.2 ng/mL), and the mean carotid IMT was 0.68 ± 0.12 mm. Residents with above median endostatin had significantly higher carotid IMT than did those with below median endostatin (0.71 ± 0.14 vs. 0.65 ± 0.09 mm, $P < 0.001$). Multiple linear regression analysis demonstrated that increased serum endostatin is significantly associated with carotid IMT (above vs. below median endostatin level; $\beta = 0.11$, $P = 0.03$), independent of the known covariates of age, sex, body mass index, drinking and smoking status, systolic blood pressure, diastolic blood pressure, hemoglobin A1c, low density lipoprotein cholesterol, estimated glomerular filtration rate, and log-transformed high sensitive C-reactive protein.

Conclusions: A higher serum endostatin level reflected subclinical atherosclerosis in this Japanese population.

See editorial vol. 24: 1014-1015

Key words: Atherosclerosis, Carotid intima-media thickness, Endostatin, Epidemiology

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Introduction

Atherosclerosis is a pathologic process that causes intima medial thickening, plaque formation, and sometimes occlusion of the coronary, cerebral, and peripheral arteries¹. Endostatin is a cleavage of the C-terminal domain of collagen XVIII, a component of the extracellular matrix¹, and a potent endogenous angio-

genesis inhibitor *in vivo* and *in vitro*^{2,3}. Recent observational studies have shown that circulating endostatin is associated with cerebrovascular diseases⁴, the severity of kidney dysfunction⁵, hypertensive organ damage⁶, and future cardiovascular mortality⁷. Thus, the circulating level of endostatin is thought to reflect extracellular matrix remodeling and be a useful marker for subclinical cardiovascular damage. However, these reports were limited to elderly participants or patients with cardiovascular or chronic kidney disease (CKD); thus, further research in a relatively healthy population is necessary.

Carotid arterial intima-media thickness (IMT), a marker of subclinical atherosclerosis⁸, is a predictor of future cardiovascular events⁹ and the incidence of

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Received: January 12, 2017

Accepted for publication: March 13, 2017

CKD¹⁰). It is also reported that matrix metalloproteinases (MMPs), which generate endostatin from the extracellular matrix, are associated with carotid IMT^{11, 12}. Based on these findings, we hypothesized that the serum endostatin levels would be positively associated with carotid IMT. To examine this hypothesis, we analyzed this relationship in a healthy Japanese population.

Subjects and Methods

Study Participants

This study is part of the Kyushu and Okinawa Population Study (KOPS) survey of vascular events associated with lifestyle-related diseases^{10, 13-15}. Eligible participants were 1,057 residents who took part in free public physical examinations between 2010 and 2011¹³. The following residents were excluded from analysis: 1) 28 because of insufficient data; 2) 44 who did not agree to undergo carotid ultrasonographic measurement; 3) 77 who had a history of cardiovascular disease, malignancy, or a chronic inflammatory disease (collagen disease or inflammatory bowel disease); 4) 260 receiving treatment for hypertension, diabetes, or dyslipidemia. After exclusions, the data of 648 subjects (200 men and 448 women) were available for analysis. The age of the subjects ranged from 24 to 84 years [mean \pm standard deviation (SD): 56.3 ± 10.6 years]. Written informed consent was obtained from each participant prior to the examination. The study was conducted in accordance with the principles of the Helsinki Declaration of 1975, as revised in 2000. Some of the data from the KOPS were published previously^{10, 13-15}.

Anthropometric Measurement and Questionnaire

Anthropometric measurements were performed with each subject wearing indoor clothing and without shoes. Body mass index (BMI) was calculated as weight [kg] divided by height [m] squared. Systolic and diastolic blood pressure (SBP and DBP) were measured on the right arm, in the sitting position, with an automated sphygmomanometer (HEM-780, Omron Healthcare, Kyoto, Japan) after a five minute rest.

Each subject completed a self-administrated questionnaire to gather information about personal medical history, family medical history, use of drugs, smoking status (current or non-current), and alcohol consumption (habitual or non-habitual). The questionnaire was checked for unfilled or inconsistent answers, first by nurses and again by our staff physicians.

Laboratory Measurements

As part of a free public physical examination,

blood samples were collected after an 8 hour overnight fast to determine the serum levels of creatinine, hemoglobin A1c (HbA1c), and low density lipoprotein (LDL) cholesterol. Aliquots of whole blood and fresh plasma and serum samples after separation were stored at 4°C in refrigerated containers and sent to a commercial laboratory (SRL Inc, Tokyo, Japan). The HbA1c level was measured from a fresh whole blood sample using the immune coherent method (RAPIDIA Auto HbA1c, Fujirebio Diagnostics Inc., Tokyo, Japan), with results expressed as the US National Glycohemoglobin Standardization Program format level (%). The serum level of LDL cholesterol was determined by automated standardized enzymatic analysis (Determiner L LDL-C, Kyowa Medex Co., Ltd, Tokyo, Japan). The serum creatinine level was measured by enzymatic assay. The estimated glomerular filtration rate (eGFR) was calculated using the modification of diet in renal disease study equation modified for Japanese subjects: $eGFR \text{ (mL/min/1.73 m}^2\text{)} = 194 \times \text{age}^{-0.287} \times \text{serum creatinine (mg/dL)}^{-1.094}$ (if woman $\times 0.739$)¹⁶. High sensitive C-reactive protein (hs-CRP) was measured by means of particle-enhanced immunonephelometry (N-latex CRP II, Siemens Healthcare Diagnostics K.K., Tokyo, Japan).

All remaining fasting serum samples were immediately frozen and stored at -80°C until assayed. The serum endostatin level (range: 16–500 ng/mL) was measured from defrosted samples using a commercially available enzyme-linked immunosorbent assay kit (R&D Systems Inc., Minneapolis, USA). Assessment of reproducibility testing showed good results, the recovery rate for spiked samples was 91%–108%, and there was no influence of interfering substances at normal levels. The intra- and inter-assay coefficients of variation were 5.0% and 6.4%, respectively.

Ultrasonographic Measurement

Carotid IMT was assessed by ultrasound. The subjects were supine with a slight hyperextension and rotation of the neck in the direction opposite the probe. Carotid artery lesions were measured using high resolution B-mode ultrasonography with a 7.5 MHz linear array probe (UF-4300R®, Fukuda Denshi Co., Ltd, Tokyo, Japan) by the well trained physicians of our department. Images were obtained 20 mm proximal to the origin of the carotid bulb at the far wall by the IMT measurement software, Intimascope (Media Cross Co., Ltd, Tokyo, Japan)¹⁷. The mean value of the bilateral average mean-IMT level was used as mean carotid IMT level.

Statistical Analysis

Data are presented as the means \pm SD or percent-

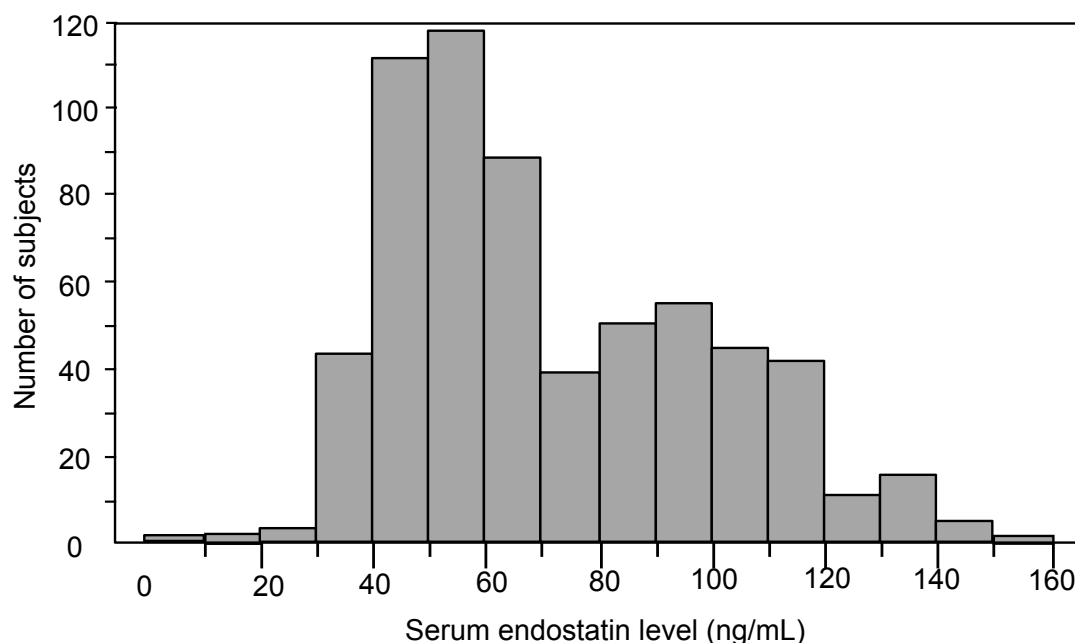


Fig. 1. Distribution of the serum endostatin level of 648 healthy Japanese subjects.

age. Because the distributions of the serum endostatin and hs-CRP levels were highly skewed, they were log-transformed before the statistical analysis and expressed as the median (interquartile ranges). The univariate associations between carotid IMT and clinical variables were assessed using Pearson's correlation coefficient analysis (categorical variables were compared with the difference between groups). For comparisons of participants with an above/below median serum endostatin level, unpaired Student's *t*-test was used to compare mean values, and the chi-square test was used to evaluate differences in prevalence rates. Analysis of covariance was performed to detect differences between participants with an above/below median serum endostatin level after adjustment for confounding factors. The statistical analysis was performed using SPSS ver.22.0 (SPSS Inc., IBM, Somers, NY). A two-tailed *P* value of <0.05 was considered statistically significant.

Results

Clinical Characteristics

The median endostatin level was 63.7 ng/mL (interquartile range: 49.7–93.2 ng/mL) and the mean endostatin level was 72.2 ng/mL (**Fig. 1**). The clinical characteristics of the subjects with above (≥ 63.7 ng/mL) and below (<63.7 ng/mL) median endostatin levels are presented in **Table 1**. Subjects with above median endostatin had significantly higher carotid IMT than those with below median endostatin ($0.71 \pm$

0.14 vs. 0.65 ± 0.09 mm, $P < 0.001$). Age, sex, habitual drinking, SBP, DBP, HbA1c, LDL cholesterol level, and eGFR were also significantly different between the participants with above and below median endostatin levels.

Association between Endostatin and Carotid Atherosclerosis

Univariate analysis determined that age ($r = 0.38$, $P < 0.001$), BMI ($r = 0.10$, $P = 0.008$), HbA1c ($r = 0.18$, $P < 0.001$), and eGFR ($r = -0.11$, $P = 0.003$) were associated with mean carotid IMT. The mean value of carotid IMT was significantly higher for men than women (0.73 vs. 0.65 mm, $P < 0.001$), habitual drinkers than sometime or non-drinkers (0.73 vs. 0.67 mm, $P < 0.001$), and current smokers than past or non-smokers (0.70 vs. 0.67 mm, $P = 0.04$). The log-transformed endostatin level was also significantly associated with carotid IMT ($r = 0.26$, $P < 0.001$). In contrast, SBP, DBP, LDL cholesterol, and log-transformed hs-CRP were not significantly associated with carotid IMT. The log-transformed serum endostatin level was not significantly correlated to carotid IMT in multiple linear regression adjusted for the known covariates of age, sex, BMI, drinking status, smoking status, SBP, DBP, HbA1c, LDL cholesterol, eGFR, and log-transformed hs-CRP (**Table 2: Model 1**). However, in multivariate analysis with categorized serum endostatin [above (≥ 63.7 ng/mL) vs. below (<63.7 ng/mL) median serum endostatin level], above median serum endostatin was independently associated with carotid

Table 1. Clinical characteristics by serum endostatin level

Variable	Below median endostatin (< 63.7 ng/mL, $n = 324$)	Above median endostatin (≥ 63.7 ng/mL, $n = 324$)	<i>P</i> value
Serum endostatin (ng/mL)	49.7 (43.1-56.3)	93.2 (77.1-110.2)	< 0.001
Age (years)	52.4 ± 9.3	60.1 ± 10.4	< 0.001
Man, <i>n</i> (%)	78 (24.1)	122 (37.7)	< 0.001
Body mass index (kg/m ²)	22.5 ± 3.2	22.5 ± 2.9	0.997
Habitual drinker, <i>n</i> (%)	42 (13.0)	80 (24.7)	< 0.001
Current smoker, <i>n</i> (%)	41 (12.8)	33 (10.2)	0.324
Systolic blood pressure (mmHg)	127.4 ± 18.7	122.4 ± 17.0	0.004
Diastolic blood pressure (mmHg)	75.9 ± 12.7	73.4 ± 11.9	0.035
HbA1c (%)	5.4 ± 0.5	5.5 ± 0.4	< 0.001
LDL-cholesterol (mmol/L)	3.2 ± 0.8	3.1 ± 0.8	0.009
eGFR (ml/min/1.73 m ²)	82.3 ± 15.2	74.6 ± 13.3	< 0.001
hs-CRP (mg/L)	0.26 (0.11-0.58)	0.28 (0.13-0.64)	0.089
Carotid IMT (mm)	0.65 ± 0.09	0.71 ± 0.14	< 0.001

Data are presented as the mean \pm standard deviation, median (interquartile ranges), or number of subjects (percent) for categorical variables. Overall *P* values were calculated by unpaired *t*-test or chi-square test.

HbA1c: hemoglobin A1c, LDL: low density lipoprotein, eGFR: estimated glomerular filtration rate, hs-CRP: high sensitive C-reactive protein, IMT: intima-media thickness

Table 2. Multiple linear regression analysis of the association between carotid IMT and serum endostatin

	Variable	Beta	<i>P</i> -value
Model 1 *	Age (years)	0.27	< 0.001
	Sex (woman = 0, man = 1)	0.20	< 0.001
	Body mass index (kg/m ²)	-0.01	0.894
	Log-transformed serum endostatin	0.09	0.107
Model 2 *	Age (years)	0.26	< 0.001
	Sex (woman = 0, man = 1)	0.20	< 0.001
	Body mass index (kg/m ²)	-0.01	0.891
	High vs. low serum endostatin group (below median = 0, above median = 1)	0.11	0.029

Beta coefficient and *P*-value by multiple linear regression.

* Adjusted for drinking status, smoking status, systolic BP, diastolic BP, HbA1c, LDL-cholesterol, eGFR, and log-transformed hs-CRP.

IMT: intima-media thickness, BP: blood pressure, LDL: low density lipoprotein, eGFR: estimated glomerular filtration rate, hs-CRP: high sensitive C-reactive protein

IMT (**Table 2: Model 2**), but SBP, HbA1c, and LDL cholesterol were not.

In addition, we evaluated the carotid IMT levels of participants with above (≥ 63.7 ng/mL) and below (< 63.7 ng/mL) median endostatin levels (**Fig. 2**). Even after adjustment for the known covariates age, sex, BMI, drinking and smoking status, SBP, DBP, HbA1c, LDL cholesterol, eGFR, and log-transformed hs-CRP, the participants with above median endostatin had a higher mean carotid IMT level than did those with below median endostatin (0.67 vs. 0.64 mm, $P = 0.029$).

Discussion

The main findings of the present study are that a high serum endostatin level was significantly associated with carotid IMT in this healthy population, but that traditional risk factors for atherosclerosis, such as blood pressure, blood glucose, and blood lipids¹⁸⁾, were not. To the best of our knowledge, this is the first study to show an association between the circulating endostatin level and subclinical atherosclerosis in healthy individuals with low cardiovascular risk.

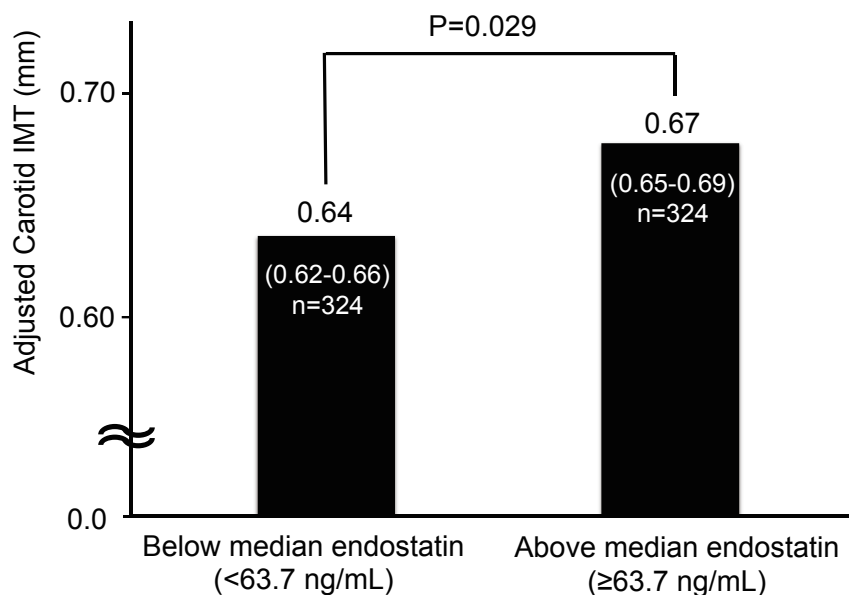


Fig. 2. Comparison of the mean carotid IMT of subjects with an above versus below median serum endostatin level.

Data shown as mean carotid IMT and 95% confidence intervals in comparison of the subjects with an above or below median serum endostatin level.

Carotid IMT was adjusted for age, sex, body mass index, drinking status, smoking status, systolic blood pressure, diastolic blood pressure, HbA1c, LDL cholesterol, eGFR, and log-transformed hs-CRP.

Intergroup differences calculated by the post hoc Tukey's HSD test.

IMT: intima-media thickness, HbA1c: hemoglobin A1c, LDL: low density lipoprotein, eGFR: estimated glomerular filtration rate, hs-CRP: high sensitive C-reactive protein.

In our study, the serum endostatin level was associated with subclinical atherosclerosis. Over the past few years, several studies have reported that elevation of the circulating endostatin level is an independent predictor of cardiovascular mortality⁷⁾, recurrence of cerebrovascular disease⁴⁾, and the incidence of CKD¹⁹⁾. Furthermore, it has been reported that serum endostatin is elevated in patients with acute myocardial infarction²⁰⁾ or CKD²¹⁾ and that it is associated with endothelial function, urinary albumin, and left ventricular mass⁶⁾. The results of our study are in accordance with these results. Moreover, because the average age of our participants was 56.7 years and patients with a past history of atherosclerotic disease or who were taking antihypertensive, lipid-lowering, or glucose lowering drugs were excluded, our results also suggest that the serum endostatin level is associated with atherosclerotic diseases of otherwise healthy individuals.

It is known that hypertension, diabetes, and dyslipidemia are traditional risk factors for atherosclerosis. However, in this study, blood pressure, blood glucose, and blood lipids were not independently associated with carotid atherosclerotic change after casting

serum endostatin into a multivariate analysis. We had hypothesized that serum endostatin would be more strongly related to early atherosclerotic change than these traditional risks. In addition, because blood pressure, glucose metabolism, and lipid metabolism were almost normal in the population studied, the influence of these traditional risk factors on atherosclerosis might not be strong. Although whether or not traditional risk factors related to atherosclerosis are independently associated with serum endostatin in patients with high cardiovascular risk has not been studied, the impact of serum endostatin on atherosclerosis of patients with cardiovascular risk may be relatively small.

The direct mechanisms related to the serum endostatin level and carotid atherosclerotic changes remain unclear. Endostatin is cleaved from collagen XVIII by proteinases such as MMP-3, -7, -9, -13, -14, and -20, elastase, and cathepsin L²²⁻²⁶⁾. It has also been reported that specific serum MMPs are secreted by foam cells in atherosclerosis lesions²⁷⁾ and that they are positively associated with carotid IMT^{11, 12)}. MMP-9 is a key mediator in the development and progression of atherosclerosis²⁸⁾. On the basis of these findings, it is pos-

sible that circulating endostatin is elevated in atherosclerosis by degradation of the extracellular matrix through the above proteinases, which would make it a useful marker of atherosclerosis. Although the serum CRP level is also said to predict future cardiovascular events, the association between subclinical atherosclerosis, including carotid IMT and CRP or other markers of inflammation, were not established in multivariate analysis after adjusting for traditional risk factors or BMI²⁹⁾. Furthermore, we found no independent correlation of log-transformed endostatin to carotid IMT in multiple linear regression analysis; thus, serum endostatin may be related to atherosclerosis beyond a certain cut-off point, but not with a linear correlation. Beta error is also possible because the study design for such a low risk group may require a higher number of residents; thus a larger-scale examination will be necessary. On the other hand, endostatin is known to be a potent endogenous angiogenesis inhibitor, and high-dose endostatin treatment can prevent the progression of atherosclerosis^{27, 30-32)}. Further studies are required to assess the causal associations between circulating endostatin and atherosclerosis.

There are some limitations to this study. First, the cross-sectional observational design makes it difficult to draw concrete inferences regarding causality between the serum endostatin level and atherosclerosis. Second, all of the subjects of our study were Japanese. Third, non-traditional risk factors associated with carotid IMT, such as thyroid function and monokine induced by gamma interferon, were not explored in the present study^{33, 34)}. Finally, we used the results of a single time measurement for our evaluation of serum endostatin. In spite of these limitations, this study is the first to show an association between the serum endostatin level and subclinical atherosclerosis in residents with low cardiovascular risk, based on findings from a large-scale study of a healthy population. We believe that our findings will contribute to the clarification of the usefulness of serum endostatin measurement in the management of cardiovascular diseases in such populations.

We found that the serum endostatin level is independently associated with subclinical atherosclerosis in a Japanese population. Our results indicate that a high circulating endostatin level reflects early atherosclerotic change. Future, longitudinal studies will be necessary to assess the clinical usefulness of the circulating endostatin level as an indicator of future atherosclerotic disease in otherwise healthy populations.

Financial Support

This study was funded by the Japan Multi-insti-

tutional Collaborative Cohort Study (J-MICC Study), Grants-in-Aid for Scientific Research on Priority Areas of Cancer [No. 17015018] and Innovative Areas [No. 221S0001] and by Grants-in-Aid for Scientific Research (A) [JPSP KAKENHI Grant Number JP 16H06277] from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Research design: Y Kato and N Furusyo

Data analysis: Y Kato and N Furusyo

Collection and assembly of data: Y Kato, Y Tanaka, T Ueyama, S Yamasaki, M Masayuki, and J Hayashi

Wrote or contributed to the writing of the manuscript: Y Kato and N Furusyo

Final approval of the article: N Furusyo

Acknowledgement

We are grateful to Drs. Mosaburo Kainuma, Eiichi Ogawa, Kazuhiro Toyoda, Hiroaki Ikezaki, Takeo Hayashi, Takeshi Ihara, Koji Takayama, Fujiko Mitsumoto-Kaseida, Kazuya Ura, Ayaka Komori, Eri Kumade and Masaru Sakiyama from our department for their assistance.

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