Original Article

Association of Serum Lipocalin-Type Prostaglandin D Synthase Levels with Subclinical Atherosclerosis in Untreated Asymptomatic Subjects

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Recent studies suggest that lipocalin-type prostaglandin (PG) D synthase (L-PGDS), which converts PGH_2 to PGD_2, is implicated in the pathogenesis of atherosclerosis. However, clinical evidence for the association between serum L-PGDS levels and atherosclerosis has not been reported. In this study, we measured the serum L-PGDS concentration using sandwich enzyme-linked immunosorbent assay (ELISA) and investigated the association with traditional cardiovascular risk factors and surrogate atherosclerotic indices, such as the maximum score of the intima-media complex thickness of the carotid artery (C-IMT_{max}) and the brachial-ankle pulse wave velocity (ba-PWV), in 500 non-treated asymptomatic subjects. The serum concentration of L-PGDS was 0.56±0.01 (mean±SEM, range 0.25–1.27, median 0.54) mg/L. Serum L-PGDS levels increased with age and were higher in men than in women. Serum L-PGDS was higher in subjects with hypertension and increased with increasing numbers of the traditional atherosclerotic risk factors. When the subjects were divided into four groups according to the levels of serum L-PGDS, the age-adjusted values of C-IMT_{max} and ba-PWV were significantly increased in subjects with higher serum L-PGDS levels (quartile 3 and quartile 4) compared to those in the lowest quartile (quartile 1), for both genders. Multiple regression analysis including risk factors revealed that serum L-PGDS was an independent determinant for ba-PWV (β=0.130, p<0.001). Serum L-PGDS tended to associate with C-IMT_{max} but was not statistically significant (β=0.084, p=0.075). In conclusion, our results suggest that an increase in serum L-PGDS concentration is associated with the progression of atherosclerosis. (*Hypertens Res* 2008; 31: 1931–1939)

***Key Words*: lipocalin-type prostaglandin D synthase, intima-media complex thickness, pulse wave velocity, atherosclerosis, arterial stiffness**

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Introduction

Lipocalin-type prostaglandin (PG) D synthase (L-PGDS) is the enzyme responsible for the biosynthesis of the PGD_2 produced in the arachidonic acid cascade from a common precursor of various prostanoids, PGH_2. This enzyme has recently been reported to have an association with cardiovascular diseases. L-PGDS is expressed in the vascular wall (*1*, *2*) and is overexpressed in the atherosclerotic intima (*1*). Exogenous L-PGDS inhibits cell proliferation and migration in vascular smooth muscle cells (*3*). The decrease in L-PGDS production in plaque macrophages increases the risk of plaque rupture through matrix metalloproteinase-9 activation (*4*). L-PGDS knockout mice fed a high-fat diabetic diet showed accelerated atherosclerosis (*5*).

The downstream products of L-PGDS, PGD_2 and its naturally occurring metabolites in the PGJ_2 family, such as PGJ_2, Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), have also been reported to show atheroprotective effects by a variety of mechanisms. PGD_2 inhibits platelet aggregation (*6*), induces vasodilation (*7*) and suppresses the mRNA expression of pro-inflammatory cytokines such as inducible nitric oxide and plasminogen activator inhibitor-1 in vascular wall cells (*8*, *9*). Among the PGJ_2 family members, 15d-PGJ_2, a potent endogenous ligand for peroxisome proliferator–activated receptor-γ (PPAR-γ), inhibits inducible nitric oxide synthase expression (*10*), inflammatory cytokine production (*11*), and matrix metalloproteinase activity in macrophages (*12*). The PGJ_2 family also strongly inhibits proliferation and promotes differentiation in vascular smooth muscle cells (*13*–*15*).

In addition to these findings, several clinical studies have suggested the usefulness of serum L-PGDS measurement in predicting cardiovascular dysfunction. Serum L-PGDS levels were increased in the coronary circulation in patients with angina pectoris (*1*) and essential hypertension (*16*), and were suggested to predict the occurrence of restenosis after coronary angioplasty (*17*). However, these studies analyzed a relatively small population, and the association between serum L-PGDS and atherosclerosis in asymptomatic subjects has not been well examined yet. Therefore, in the present study, we measured the serum L-PGDS concentration using enzyme-linked immunosorbent assay (ELISA) in untreated asymptomatic subjects and investigated the association with the traditional risk factors of atherosclerosis. We also quantitatively assessed the atherosclerotic changes of the vascular wall using two common methods—the maximum value of the intima-media complex thickness of the carotid artery ($C\text{-}IMT_{max}$) and the brachial-ankle pulse wave velocity (ba-PWV)—and examined the associations with the serum L-PGDS level.

Methods

Subjects

This study involved 512 untreated asymptomatic subjects who participated in an annual health check program from 2001 to 2003 at Kisei-Town (Ise-City, Mie Prefecture, Japan), conducted by Keio University Ise Hospital. Six subjects with severe diabetes (HbA1c≥8.0%), 4 subjects with severe hyperlipidemia (total cholesterol ≥7.8 mmol/L [300 mg/dL] or triglyceride ≥4.5 mmol/L [400 mg/dL]), and 2 subjects with renal impairment (serum creatinine ≥115 μmol/L [1.3 mg/dL]) were excluded and, consequently, 500 subjects were analyzed. Hypertension was defined as systolic blood pressure (SBP) ≥140 mmHg and/or diastolic BP (DBP) ≥90 mmHg. Dyslipidemia was defined as low-density lipoprotein (LDL) cholesterol ≥3.6 mmol/L (140 mg/dL). Diabetes mellitus was defined as fasting plasma glucose (FPG) ≥7.0 mmol/L (126 mg/dL). This study was conducted in accordance with the Declaration of Helsinki, and the study protocol was approved by the ethics review committee of the Keio University Ise Hospital. Written informed consent was obtained from all subjects.

Clinical Parameters

At the physical examination, blood pressure, body mass index (BMI), and hematological and biochemical profiles were determined in the morning after an overnight fast. Serum levels of triglyceride, total cholesterol, high-density lipoprotein (HDL) cholesterol, LDL cholesterol, fasting plasma glucose (FPG), and creatinine were measured using routine laboratory methods.

Measurement of Serum L-PGDS Concentration

Venous blood was collected in the absence of anticoagulant in the morning after an overnight fast. After centrifuge, the serum was aliquoted and stored at −80°C prior to use. Serum L-PGDS levels were determined by sandwich ELISA using two monoclonal antibodies, Mab-7F5 and Mab-1B7, as described previously (*18*). In brief, the samples were incubated at 30°C for 90 min in Maxi Sorp F96 microtiter plates (Nalge Nunc, Roskilde, Denmark) precoated with unlabelled Mab-7F5 (4.4 mg/L) at 4°C overnight. After a wash, the plates were incubated at 30°C with peroxidase-conjugated Mab-1B7 (2.0 μg/mL) for 90 min. Thereafter, 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid solution (Boehringer-Mannheim, Mannheim, Germany) was added to each well, and the reaction was stopped by adding 1.5% oxalic acid. The plates were read on a Spectra-Max 250 microplate reader (Molecular Devices, Sunnyvale, USA) at 405–492 nm. All samples were measured in duplicate and the results were averaged. The minimal detectable concentration of this assay system

Table 1. Clinical Characteristics of the Participants

	Men (n=158)	Women (n=342)	p
Age, years	61.8	59.6	0.041
Body mass index, kg/m^2	24.0	23.6	n.s.
Systolic BP, mmHg	133.6	130.8	n.s.
Diastolic BP, mmHg	79.8	76.2	0.003
Total cholesterol, mmol/L	5.48	5.67	0.024
HDL cholesterol, mmol/L	1.49	1.64	<0.001
LDL cholesterol, mmol/L	3.36	3.50	n.s.
Triglyceride, mmol/L	1.37	1.15	<0.001
FPG, mmol/L	5.70	5.16	<0.001
Serum creatinine, μmol/L	63.9	84.4	<0.001
Habitual smoking, %	29.3	8.2	0.046
Obesity, %	32.3	29.8	n.s.
Hypertension, %	37.3	33.9	n.s.
Dyslipidemia, %	39.2	43.9	n.s.
Diabetes, %	7.6	3.2	0.030

Values are the mean or frequency. p values refer to unpaired t-test for continuous variables and χ^2 analysis for categorical variables. n.s., not significant; BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FPG, fasting plasma glucose.

was 0.03 mg/L, and the intra- and interassay coefficients of variation were 3.2 and 5.7%, respectively.

Carotid Artery Ultrasonography

Ultrasonography of the common carotid arteries was performed with a high-resolution Duplex scanner (SSA-390A; Toshiba Medical Co., Ltd., Tokyo, Japan) using a probe at a frequency of 7.5 MHz for the B-mode scan. The subjects were investigated in the supine position. The carotid arteries were carefully examined with regard to wall changes from different longitudinal (anterior oblique, lateral, and posterior oblique) and transverse views. Each ultrasound image was recorded on a computer with an on-line digital filing system, and the intima-media complex thickness (IMT) was measured off-line and analyzed. This measurement was performed by two independent sonographers blinded from the clinical data. C-IMT$_{max}$ was defined as the maximum value of the IMT in the whole area examined, including plaques in bilateral common carotid arteries. The intra-observer and inter-observer coefficients of variation using 50 subjects were 4.6% and 4.3%, respectively.

Measurement of the ba-PWV

The oscillometric ba-PWV was automatically measured by AT form (Nihon-Colin AT Co., Komaki, Japan) as described previously (*19*). In brief, this device records the phonocardiogram, electrocardiogram, volume pulse form and arterial blood pressure at the bilateral brachia and ankles. The level of the ba-PWV was calculated by time-phase analysis between the right brachium and ankle. The intra-observer and inter-observer coefficients of variation using 50 subjects were 2.1 and 2.3%, respectively.

Statistical Analysis

Data are presented as means, means±SEM or frequency. The values for the serum L-PGDS level were used for regression analysis after logarithm-transformation, since these raw values were not normally distributed when we performed the Shapiro-Wilk test and instead showed a logarithm distribution. To compare the mean values between two groups, the unpaired t-test was used for continuous variables and the χ^2 analysis was used for categorical variables. In a simple regression analysis, Pearson's correlation coefficients were used for continuous variables and Spearman's correlation coefficients for categorical variables. To compare the age-adjusted mean values among groups, analysis of covariance was used. Multivariate-adjusted correlations were analyzed by multiple regression models. In a multivariate-adjusted analysis for the C-IMT$_{max}$ and ba-PWV, sex (men: 1 or women: 0), age, habitual smoking (yes: 1 or no: 0), obesity (yes: 1 or no: 0), hypertension (yes: 1 or no: 0), dyslipidemia (yes: 1 or no: 0), diabetes mellitus (yes: 1 or no: 0) and $\log_{10}$ L-PGDS were entered into the model. The above statistical analyses were performed using the JMP IN, version 5.1.1. J (SAS Institute Inc., Cary, USA). The appropriate cut-off values of serum L-PGDS levels for the atherosclerotic indices in the logistic regression model were calculated by plotting the receiver-operating characteristic (ROC) curve using SAS statistical software (SAS Institute Inc.). We defined significant atherosclerosis as a ba-PWV≥14.0 m/s (yes: 1 or no: 0) or a C-IMT$_{max}$≥1.0 mm (yes: 1 or no: 0), and the points closest to the upper left-hand corner of the graph were chosen as the cut-off points. p<0.05 was

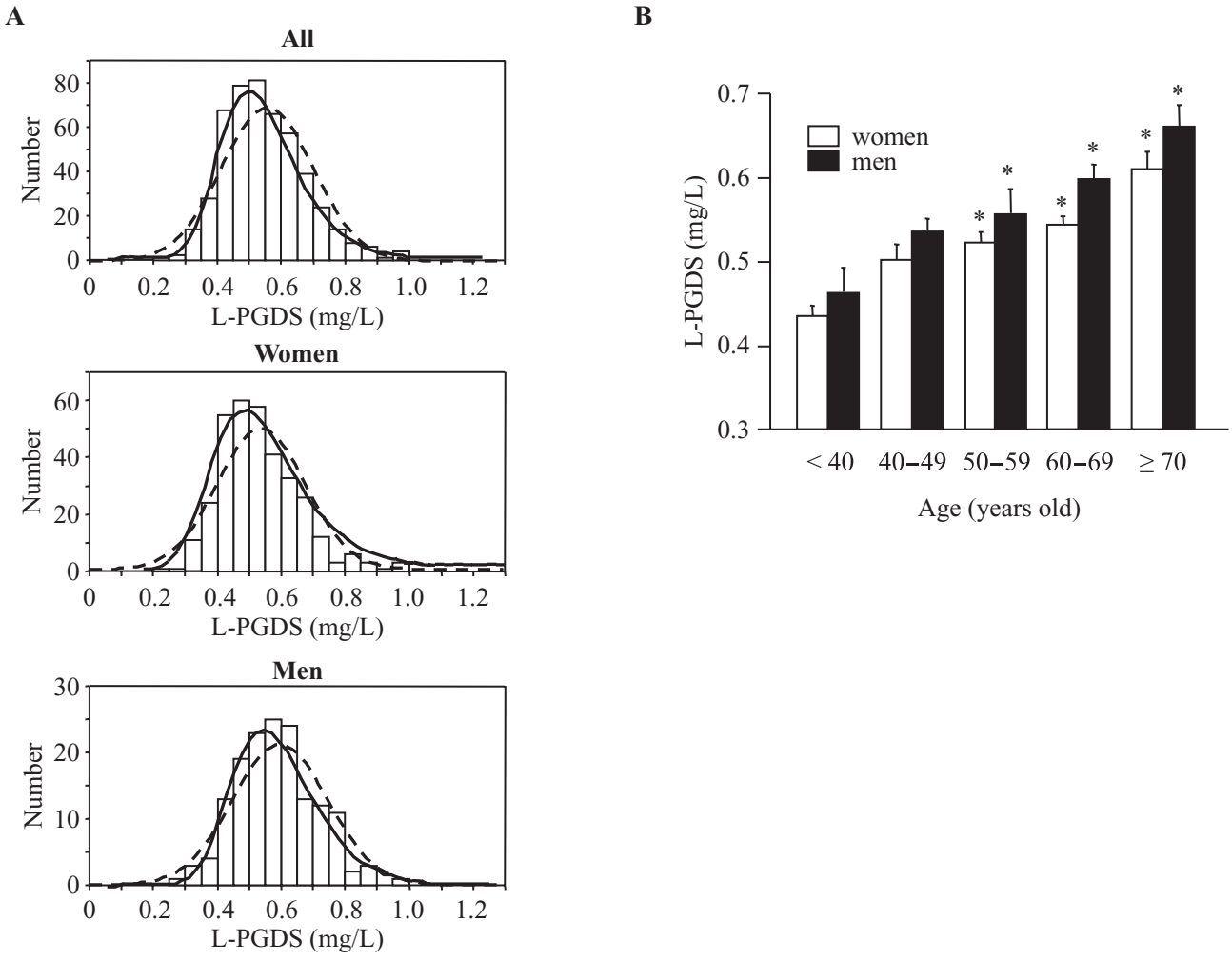

Fig. 1. *A: Distribution of the serum L-PGDS concentration. The solid and dashed lines represent logarithmic and normal distributions, respectively. B: Serum L-PGDS levels in the indicated age groups. *p < 0.05 vs. < 40 years old. L-PGDS, lipocalin-type prostaglandin D synthase.*

considered statistically significant.

Results

Clinical Characteristics

The characteristics of the participants categorized by sex are presented in Table 1. Ages ranged from 34 to 88 (60.3±0.5) years. Men had higher levels than women of diastolic BP, triglyceride, FPG, and serum creatinine, and lower levels of total cholesterol and HDL cholesterol. The prevalence of atherosclerotic risk factors was 15% for habitual smoking, 31% for obesity, 35% for hypertension, 42% for dyslipidemia and 5% for diabetes. The ratios of habitual smoking and diabetes mellitus were higher in men.

Serum L-PGDS Level and the Association with Traditional Cardiovascular Risk Factors

The serum level of L-PGDS was 0.56±0.01 mg/L (range 0.25–1.27, median 0.54) and showed a logarithm distribution (Fig. 1A). Serum L-PGDS levels increased with aging; the subjects over 50 years old had significantly higher serum L-PGDS levels than those under 40 years old (Fig. 1B). Furthermore, men (0.60 mg/L) had a significantly greater level of serum L-PGDS than women (0.54 mg/L) ($p<0.001$). This trend did not change when the subjects were categorized by age (Fig. 1B). In a linear regression analysis, the serum L-PGDS (log scale) was positively correlated with age ($r=0.382$, $p<0.001$), SBP ($r=0.231$, $p<0.001$), DBP ($r=0.142$, $p=0.001$), and serum creatinine ($r=0.428$, $p<0.001$) and inversely correlated with HDL cholesterol ($r=-0.181$, $p<0.001$). These associations did not change

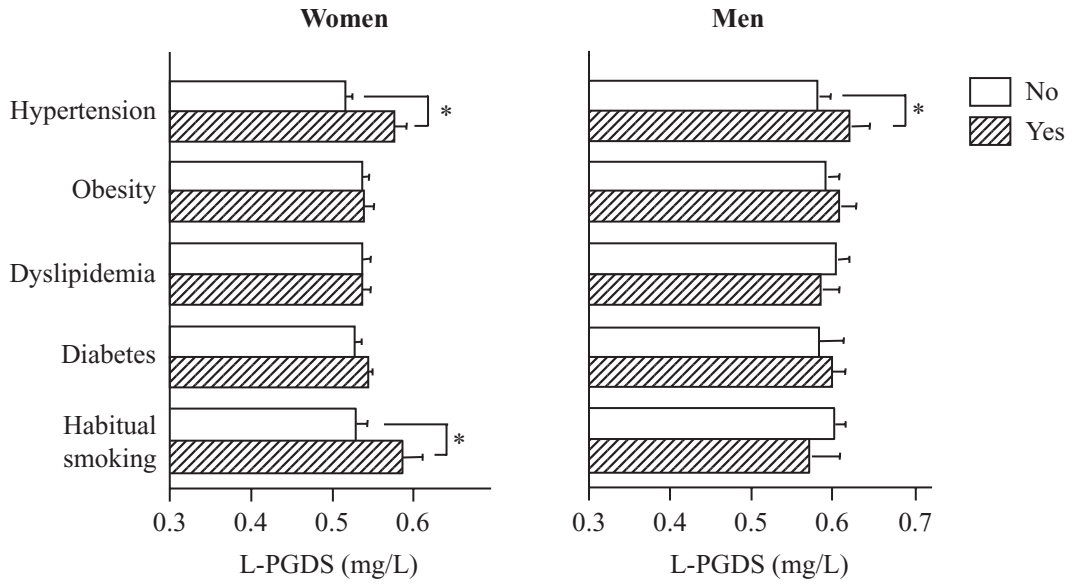

Fig. 2. *Comparison of L-PGDS levels between subjects with traditional cardiovascular risk factors and those without. *p < 0.01. L-PGDS, lipocalin-type prostaglandin D synthase.*

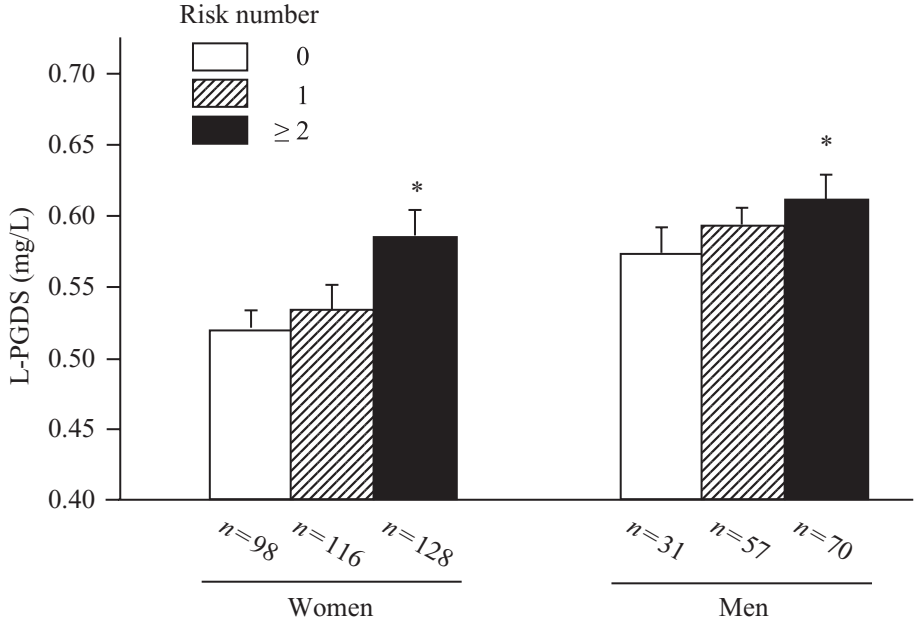

Fig. 3. *The serum levels of L-PGDS among groups categorized by number of the traditional atherosclerotic risk factors (obesity, habitual smoking, hypertension, dyslipidemia and diabetes). *p < 0.05 vs. the subjects without risk factors (risk number 0). L-PGDS, lipocalin-type prostaglandin D synthase.*

when subjects were categorized by gender (data not shown). When the subjects were categorized by the traditional cardiovascular risk factors (habitual smoking, obesity, hypertension, dyslipidemia and diabetes), age-adjusted serum L-PGDS levels were significantly higher in patients with hypertension for both men and women (Fig. 2). In women but not in men, smokers also had higher serum L-PGDS levels than non-smokers. Obesity, dyslipidemia, and diabetes did not show significant differences. However, when the subjects were divided into three groups according to the number of

Table 2. Clinical Characteristics of the Groups Divided by Serum L-PGDS Level

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	*p*
Women					
Number	86	86	86	84	
Serum L-PGDS, mg/L (range)	0.39 (0.25–0.44)	0.48 (0.44–0.52)	0.56 (0.52–0.61)	0.72 (0.61–1.21)	<0.001
Age, years	54.3	57.8	61.1	65.3	<0.001
Habitual smoking, %	2.3	8.1	16.3	14.5	0.010
Body mass index, kg/m^2	23.0	23.9	23.9	23.9	n.s.
Systolic BP, mmHg	122.8	130.3	131.6	138.5	<0.001
Diastolic BP, mmHg	73.6	76.9	77.2	77.2	n.s.
HDL-cholesterol, mmol/L	1.7	1.7	1.7	1.6	n.s.
LDL-cholesterol, mmol/L	3.4	3.5	3.6	3.5	n.s.
FPG, mmol/L	5.3	5.2	5.0	5.1	n.s.
Serum creatinine, μmol/L	60.8	61.3	62.1	71.7	<0.001
Men					
Number	39	40	39	40	
Serum L-PGDS, mg/L (range)	0.43 (0.25–0.49)	0.54 (0.50–0.58)	0.62 (0.58–0.67)	0.79 (0.67–1.27)	<0.001
Age, years	56.4	58.2	63.4	69.1	<0.001
Habitual smoking, %	28.2	30.0	23.1	20.0	n.s.
Body mass index, kg/m^2	24.1	23.3	24.6	23.9	n.s.
Systolic BP, mmHg	132.3	130.5	129.4	142.2	0.039
Diastolic BP, mmHg	78.2	77.4	78.6	84.8	0.047
HDL-cholesterol, mmol/L	1.6	1.6	1.4	1.4	0.032
LDL-cholesterol, mmol/L	3.4	3.5	3.4	3.2	n.s.
FPG, mmol/L	5.9	5.5	5.5	5.9	n.s.
Serum creatinine, μmol/L	77.8	80.5	85.4	93.6	<0.001

Values are the means or frequency. *p* values refer to ANOVA. n.s., not significant; L-PGDS, lipocalin-type prostaglandin D synthase; other abbreviations as shown in Table 1.

risk factors, age-adjusted L-PGDS levels were significantly elevated in subjects with two or more risk factors, for both genders, compared to those without risk factors (Fig. 3).

Association of Serum L-PGDS with Surrogate Atherosclerotic Indices

Next, we examined the association between serum L-PGDS and subclinical atherosclerotic indices. The values of C-IMT$_{max}$ and ba-PWV were 1.20±0.03 mm (men: 1.40±0.07; women: 1.11±0.03) and 15.7±0.2 m/s (men: 16.4±0.3; women: 15.3±0.2), respectively. In a simple regression analysis, C-IMT$_{max}$ showed a significant positive correlation with age, serum creatinine and LDL cholesterol. The values of ba-PWV were also associated with age, BP, FPG, serum creatinine and serum L-PGDS. The serum L-PGDS level (log scale) was significantly associated with both C-IMT$_{max}$ (women: r=0.125, p=0.021; men: r=0.108, p=0.175; all: r=0.150, p<0.001) and ba-PWV (women: r=0.328, p<0.001; men: r=0.360, p<0.001; all: r=0.354, p<0.001) in a linear regression model. When the subjects were divided into four groups according to the L-PGDS levels (Table 2), age-adjusted values of C-IMT$_{max}$ and ba-PWV were significantly higher in subjects in quartile 3 and quartile 4 compared to those in quartile 1, in both genders (Fig. 4). In a multiple regression analysis including traditional atherosclerotic risk factors, age and hypertension were independently associated with ba-PWV (Table 3). The serum L-PGDS level was also detected as an independent determinant for ba-PWV (β=0.130, p<0.001). For C-IMT$_{max}$, age, obesity, dyslipidemia and serum creatinine were detected as independent factors. Serum L-PGDS tended to associate with C-IMT$_{max}$, but this was not statistically significant (β=0.084, p=0.075). Finally, we determined the appropriate cut-off values of serum L-PGDS in relation to each atherosclerotic index using ROC curve analysis. The optimal cut-off points of the regression model for increased ba-PWV (≥14.0 m/s) and C-IMT$_{max}$ (≥1.0 mm) were 0.57 and 0.58 mg/L in men, and 0.53 and 0.53 mg/L in women, respectively.

Discussion

In the present study, the serum L-PGDS concentration measured by ELISA was comparable to those obtained in healthy subjects in previous studies (*16*, *20*, *21*). Serum L-PGDS levels increased with aging and showed a significant difference between genders. Furthermore, serum L-PGDS levels increased with increasing numbers of the traditional athero-

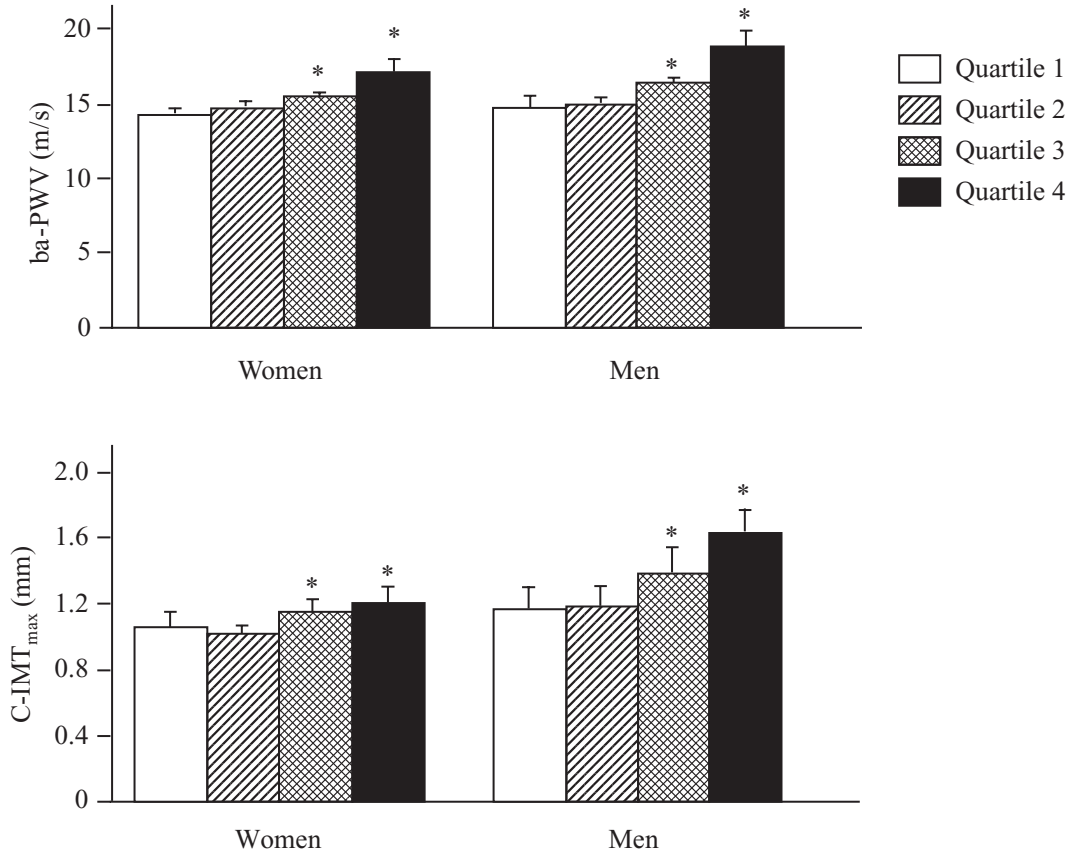

Fig. 4. *Comparison of the age-adjusted values of the atherosclerotic indices (top, ba-PWV; bottom, C-IMT$_{max}$) among groups divided according to the serum L-PGDS levels. *p < 0.05 vs. quartile 1. ba-PWV, brachial-ankle pulse wave velocity; C-IMT$_{max}$, maximum intima-media complex thickness of the carotid artery.*

Table 3. Multiple Regression Analyses for Vascular Properties

Risk factors	C-IMT$_{max}$		ba-PWV	
	β	p	β	p
Men	0.093	0.064	0.058	0.180
Age	0.458	<0.001	0.463	<0.001
Habitual smoking	0.074	0.069	−0.008	0.815
Obesity	0.093	0.021	−0.029	0.408
Hypertension	0.017	0.687	0.251	<0.001
Dyslipidemia	0.083	0.036	0.011	0.741
Diabetes	0.003	0.942	0.028	0.417
Serum creatinine	0.105	0.049	0.013	0.771
$\log_{10}$ L-PGDS	0.084	0.075	0.130	<0.001

C-IMT$_{max}$, maximum intima-media complex thickness of the carotid artery; ba-PWV, brachial-ankle pulse wave velocity; L-PGDS, lipocalin-type prostaglandin D synthase.

sclerotic risk factors and were associated with the atherosclerotic changes of the vascular wall (*i.e.*, C-IMT$_{max}$ and ba-PWV). When we determined the cut-off values of L-PGDS for the atherosclerotic indices calculated by ROC curve analysis, these were approximately equal to the median values (men=0.58 mg/L, women=0.52 mg/L). As shown in Fig. 4, age-adjusted values of the atherosclerotic markers increased from quartile 3 (=median values) in men and women, suggesting that these cut-off values are reasonable and useful to predict the atherosclerotic changes of the vascular wall. How-

ever, because this study was cross-sectional in design, further investigation is required to confirm whether these are actually appropriate values.

Although it remains largely unknown how serum L-PGDS levels are regulated, a possible source of L-PGDS is the vascular endothelium in normal vessels. We previously reported that fluid shear stress induced L-PGDS expression in vascular endothelial cells and subsequently released downstream PGs, PGD_2 and 15d-PGJ_2, into the culture medium (*22*). L-PGDS expression in endothelial cells depends on the strength of the shear stress, and therefore the hemodynamic changes accompanying elevated BP and the morphological and functional changes of the vascular wall could alter the serum L-PGDS levels. The increase in serum L-PGDS in hypertensives, in our results as well as in the previous report (*16*), supports this hypothesis. Another possible source is the intimal de-differentiated VSMCs in the atherosclerotic region because the expression of L-PGDS was also found in the atherosclerotic intima (*1*). In our results, the serum L-PGDS level showed a strong correlation with aging and hypertension, which are the most powerful inducers of the atherosclerotic change of the vascular wall, and increased as the number of risk factors increased. Moreover, serum L-PGDS levels were associated with C-IMT_{max}, which reflects the degree of intimal thickening. Therefore, it seemed probable that L-PGDS is induced by the neointimal VSMCs in the atherosclerotic lesion of the vascular wall.

We found no significant association between serum L-PGDS and diabetes mellitus. Other researchers have reported that plasma concentrations of L-PGDS were slightly higher in patients with diabetes mellitus than in control subjects, although the differences did not reach significance (*20*). However, a recent report suggested that a high concentration of glucose induces L-PGDS expression and that exogenous L-PGDS inhibits cell proliferation and migration in vascular smooth muscle cells explanted from Goto-Kakizaki rats, a model of type II diabetes (*2*). Furthermore, one of the downstream PGs of L-PGDS, 15d-PGJ_2, is well known as a strong endogenous ligand of PPAR-γ, which is closely associated with insulin sensitivity. Since we excluded the severe diabetic subjects, further detailed study in a large population is required to clarify this issue.

Another interesting finding is the inverse correlation between serum L-PGDS and HDL-cholesterol. Considering the significant association between atherosclerotic risk factors and serum L-PGDS, a direct correlation might seem natural because decreased HDL cholesterol is one of the risk factors of atherosclerosis. However, we recently found an association between the human L-PGDS gene polymorphism and HDL-cholesterol levels (*23*). In subjects with the A/A genotype of 4111A>C, serum levels of HDL cholesterol were significantly higher than in those with the A/C and C/C genotypes. Although further studies are required, L-PGDS may play a role in lipid transport because L-PGDS belongs to the lipocalin superfamily, a group of proteins that bind and transport small lipophilic molecules.

There are several limitations of this study. Because its design is cross-sectional, we could not confirm whether the increase in serum L-PGDS in subjects with mild atherosclerosis is a cause or a consequence at this stage. Additionally, the L-PGDS concentration might not represent its enzymatic activity. To determine the L-PGDS activity, measurement of the serum levels of downstream PGs may be useful; however, PGD_2 and PGJ_2 series are unstable substances and measuring their serum levels is quite difficult. To determine the precise role of L-PGDS in the pathogenesis of atherosclerosis, further clinical investigations using prospective designs and basic research using animal models such as L-PGDS knockout mice are required.

In conclusion, our results showed that serum L-PGDS levels increase with the increasing number of traditional atherosclerotic risk factors in a relatively large asymptomatic population. For the first time, we provided evidence that the increase in serum L-PGDS is associated with the atherosclerotic changes of the vascular wall. L-PGDS may be involved in the development of atherosclerosis, although further study is necessary to clarify its pathophysiologic role.

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