Serum Phospholipid Transfer Protein Mass as a Possible Protective Factor for Coronary Heart Diseases

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Background Phospholipid transfer protein (PLTP) can generate pre- β -high-density lipoprotein (HDL), an efficient acceptor of peripheral cholesterol, by mediating a process called HDL conversion. The transfer of phospholipids to immature HDL is also essential in maintaining reverse cholesterol transport. The phospholipid transfer activity of PLTP has been associated with various patho-physiological conditions; however, little information is available concerning the relationship between PLTP mass and disease.

Methods and Results Using a sandwich enzyme-linked immunosorbent assay, PLTP concentration was measured and related to the risk of developing cardiovascular disease in a worksite-based cohort of Japanese men ($n=2,567$). Multiple linear regression analysis showed significant associations between PLTP and HDL cholesterol, triglycerides, low-density lipoprotein cholesterol, and body mass index (standardized $\beta=0.395$, -0.191 , -0.064 , and -0.064 , respectively; $R^2=0.31$). During the follow-up period, there were 10 cases of coronary heart disease (CHD) and 7 of stroke. The multivariate adjusted relative risk of CHD was 0.46 (95% confidence interval, 0.20–1.07) for an increase of 1 standard deviation in the PLTP value ($p=0.071$). PLTP concentration was not related to the risk of stroke.

Conclusions The results of this prospective study indicate that the serum PLTP concentration would serve as a predictor of CHD, independent of HDL cholesterol, triglycerides and other established risk factors. (Circ J 2004; 68: 11–16)

Key Words: Cohort study; Coronary heart disease; High-density lipoprotein; Japan; Phospholipid transfer protein

An inverse relationship between high-density lipoprotein (HDL) cholesterol and cardiovascular diseases has been demonstrated in epidemiologic studies^{1,2} and is often explained by the capacity of HDL to transport cholesterol from peripheral tissues to the liver, known as reverse cholesterol transport.³ In this process, pre- β -HDL, a quantitatively minor subfraction of HDL, is thought to play a critical role because it acts as an efficient acceptor in the cholesterol efflux process from the plasma membrane of peripheral cells.^{3,4} Phospholipid transfer protein (PLTP) is capable of generating pre- β -HDL by inducing HDL conversion, a process that remodels a homogeneous HDL fraction into populations of large and small HDL particles.^{5–8} The transfer of phospholipids to immature HDL in the process of HDL maturation is also essential in maintaining reverse cholesterol transport.^{5,9,10} In addition, PLTP has other roles, such as the transfer of α -tocopherol to cell membrane for the maintenance of endothelial function, or the transfer of lipopolysaccharide to

lipoproteins, which leads to neutralization of its potent inflammatory function.⁵ Altogether, PLTP can be considered to play an important role in the prevention of atherosclerosis, and thus it has recently attracted much attention.

Previous studies regarding PLTP examined its phospholipid transfer activity in patho-physiologic states in human or experimental models.^{11–14} Sandwich enzyme-linked immunosorbent assay with 2 monoclonal antibodies to human PLTP has been recently introduced to assay PLTP concentration,^{15,16} but the significance of the PLTP concentration in human diseases is incompletely understood.^{5,17} It is, therefore, important to evaluate whether an elevated concentration of serum PLTP is associated with an increased or decreased risk of cardiovascular diseases. We examined serum PLTP in relation to newly diagnosed cardiovascular diseases in a middle-aged worksite-based cohort of men in Japan. We also examined the association of serum PLTP concentration with other cardiovascular risk factors.

Methods

Subjects

The present analysis is based on a cohort of male workers in a worksite in Aichi prefecture, central Japan.^{18,19} Its aim was to determine factors associated with the development of cardiovascular diseases. The study began in 1997, when 2,896 male employees of a manufacturing company, aged 34–59 years, were recruited as potential participants. A self-administered questionnaire was used to assess the

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Table 1 Baseline Characteristics of the Study Subjects

	Total (n=2,567)	Cases		
		Any cardiovascular diseases (n=17)	CHD (n=10)	Stroke (n=7)
Age (years)	50±7	52±5	52±6	52±3
PLTP (μg/ml)	14.9±4.3	13.1±3.8	12.2±2.5	14.4±5.1
Body mass index (kg/m ²)	22.8±2.7	23.4±3.7	23.5±4.3	23.4±3.0
Systolic blood pressure (mmHg)	129±19	143±15	141±13	145±19
Glycosylated hemoglobin (%)	5.7±0.6	6.5±1.3	6.6±1.5	6.3±1.1
HDL cholesterol (mg/dl)	54±14	46±11	46±12	46±11
LDL cholesterol (mg/dl)	128±31	136±30	150±20	117±31
Triglycerides (mg/dl)	132±94	114±41	130±60	194±131
Smoking status (%)				
Never	33.6	31.3	30.0	33.3
Past smoker	13.2	6.3	10.0	0.0
Current smoker				
1–24 cigarettes/day	36.0	31.3	20.0	50.0
≥25 cigarettes/day	17.2	31.3	40.0	16.7
Alcohol intake (%)				
None	26.4	23.5	20.0	28.6
Light	21.8	23.5	30.0	14.3
Moderate	26.0	35.3	40.0	28.6
Heavy	25.8	17.6	10.0	28.6
Leisure-time physical activity (%)				
Not very active	82.8	76.5	80.0	71.4
Somewhat active	12.6	5.9	0.0	14.3
Regularly active	4.5	17.6	20.0	14.3

PLTP, phospholipid transfer protein; CHD, coronary heart diseases; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Data are expressed as means±standard deviations.

Fig 1. Distribution of phospholipid transfer protein (PLTP).

baseline characteristics of the participants. Of the 2,896 workers, 2,567 (88.6%) participated in the study by completing a questionnaire and donating a residual serum sample, which was taken during an annual health check-up after fasting at least 12 h overnight. Samples were kept frozen in a -80°C deep freezer immediately after centrifuging until determination of serum PLTP concentration in the year 2000. The study protocol and informed consent procedure was approved by the ethics committee of Nagoya University Graduate School of Medicine.

Follow up and Identification of Cases of Cardiovascular Diseases

All participants were followed from the date of enrollment until the date of the last health check-up unless they had developed one of the prospectively defined endpoints, which were reported by the healthcare professionals of the

worksite using a standardized form. The median follow-up period was 37 months. Cardiovascular diseases here refer to coronary heart diseases (CHD), identified as myocardial infarction or cardiac sudden death, and stroke. Medical and surgical interventions, such as percutaneous transcatheter angioplasty and coronary artery bypass graft, were also regarded as CHD events. Myocardial infarction included definite fatal and non-fatal myocardial infarction according to the MONICA criteria.²⁰ Stroke included cerebral infarction, cerebral hemorrhage, and subarachnoid hemorrhage. Sudden cardiac death was defined as sudden unexpected death within 1 h of the onset of acute symptoms and signs. The diagnosis was confirmed by a medical or death certificate.

Serum PLTP Concentration and Phospholipid Transfer Activity of PLTP

Serum PLTP concentration was measured by a sandwich enzyme-linked immunosorbent assay method using 2 monoclonal antibodies specific to PLTP.¹⁵ Circulating PLTP has been demonstrated to be present in 2 forms, one catalytically active and the other inactive.^{21,22} The assay measures the total concentration of PLTP. The assay range was 1.2–30.0 mg/L, and the intra- and inter-assay coefficients of variations were <3.0% and <4.2%, respectively.

Phospholipid transfer activity of PLTP was measured by a liposome-HDL₃ system as previously described.¹⁵ The activity was expressed as μmol of phosphatidylcholine transferred to HDL₃ per milliliter of plasma per hour. The activity was measured only in a sample of 41 subjects, which consisted of 32 normolipidemics and 9 dyslipidemics (triglyceride concentration ≥ 150 mg/dl and HDL cholesterol <40 mg/dl) for the purpose of describing the correlation with circulating PLTP concentration.

Table 2 Correlation Coefficients Between the Serum PLTP Concentration and Age, Body Mass Index, and Other Cardiovascular Risk Factors

Age	BMI	SBP	HbA1c	LDLC	HDLC	TG
-0.03	-0.27*	-0.09*	-0.11*	-0.18*	0.51*	-0.41*

* $p < 0.001$.

PLTP, phospholipid transfer protein; BMI, body mass index; SBP, systolic blood pressure; HbA1c, glycosylated hemoglobin; LDLC, low-density lipoprotein cholesterol; HDLC, high-density lipoprotein cholesterol; TG, triglycerides.

Table 3 Parameter Estimates of Stepwise Multiple Regression With Serum PLTP as the Dependent Variable

Variable	Standardized	p value
HDLC	0.395	<0.0001
TG	-0.191	<0.0001
LDLC	-0.064	<0.001
BMI	-0.064	<0.001

$R^2 = 0.31$.

Independent variables excluded from the model were systolic blood pressure, glycosylated hemoglobin, and age.

PLTP, phospholipid transfer protein; HDLC, high-density lipoprotein cholesterol; TG, triglycerides; LDLC, low-density lipoprotein cholesterol; BMI, body mass index.

Definition of Covariates

The study participants provided information on their smoking history, drinking habits, and physical activity. Smoking status was classified into 4 levels (never, past, and current smokers of 1–24 cigarettes or ≥ 25 per day). Drinking habit was first assessed by the number of drinking days per week (none, 1–3, 4–6, and daily).²³ If present (not none), it was further categorized into 3 levels by weekly consumption (light, moderate, heavy); that is, daily alcohol consumption multiplied by days of drinking per week. Leisure-time physical activity was assessed by 2 questions: frequency (seldom, 1–3 times per month, 1–2 times per week, ≥ 3 times per week) and intensity (vigorous, moderate, light). Those who engaged in vigorous activity 1–2 times or more per week, or moderate activity 3 times or more per week, were classified as ‘regularly active’. Those who engaged in vigorous activity 1–3 times per month, moderate activity 1–2 times per week, or light activity 3 times or more per week were classified as ‘somewhat active’. All others with no missing data on these questions were classified as ‘not very active’. Vigorous activity was defined in the questionnaire as the level that leaves participants out of breath. Similarly, moderate activity was defined as the level that leaves participants breathing rather hard.

Body-mass index (BMI) was calculated as weight in kilograms divided by the square of the height in meters. Serum level of low-density lipoprotein cholesterol (LDL-C), HDL cholesterol (HDL-C) and triglycerides (TG) were measured enzymatically. Glycosylated hemoglobin concentration was analyzed by high-performance liquid chromatography. Serum TG concentration was natural logarithmically transformed to approximately normalize the distribution.

Statistical Analysis

Forward stepwise multiple regression with serum PLTP concentration as the dependent variable was used in order to evaluate associations of PLTP with established cardiovascular risk factors. Independent variables entered into the model included age, systolic blood pressure, BMI, LDL-C, HDL-C, TG and glycosylated hemoglobin. A selection threshold of a p-value of less than 0.05 was employed.

Cox proportional-hazards regression models were used to calculate the relative risks (RR) for CHD, stroke and cardiovascular diseases in relation to serum PLTP concentration. Because the distribution of serum PLTP was normally distributed, we treated it as a continuous variable, and presented the RR that represent change in risk per increment of 1 SD (4.3), which was calculated from the distribution in all subjects. Adjusted estimates of risk were obtained with multivariate models that also controlled for age, smoking status, alcohol intake, and leisure-time physical activity (multivariate model 1), variables entered in the multivariate model 1, BMI, systolic blood pressure, LDL-C, and glycosylated hemoglobin (multivariate model 2), and variables entered in the multivariate model 2, HDL-C and TG (multivariate model 3).

All reported p-values were 2-sided and a p-value of less than 0.05 was considered statistically significant. However, a p-value of less than 0.1 was treated as of marginal significance. The 95 percent confidence intervals (95% CI) are presented for all RR. All statistical analyses were performed with the SPSS statistical package for Windows, version 11.5 (Chicago, IL, USA).

Results

The baseline characteristics of the participants are presented in Table 1. The mean serum PLTP concentration was 14.9 $\mu\text{g/ml}$. The PLTP was normally distributed (Kolmogorov-Smirnov test; range 1.2–29.8) (Fig 1). There was no correlation between the serum PLTP concentration and age (Pearson's $r = -0.03$, NS) (Table 2). There was a strong positive correlation between the PLTP and serum HDL-C concentrations (Pearson's $r = 0.51$, $p < 0.001$). There was a significant negative correlation between the PLTP concentration and TG (Pearson's $r = -0.41$, $p < 0.001$). Significant correlation was not found between PLTP concentration and PLTP activity in normolipidemics nor in dyslipidemics (Spearman's $r = 0.230$, $p = 0.21$ ($n = 32$) and $r = -0.092$, $p = 0.81$ ($n = 9$), respectively). Stepwise multiple linear regression analysis performed with serum PLTP concentration as the dependent variable and with age, BMI, systolic blood pressure, LDL-C, HDL-C, TG and glycosylated hemoglobin as independent variables, showed signifi-

Table 4 Cox Proportional Hazard Models Examining the Relation Between the Serum PLTP and the Risk of Cardiovascular Diseases*

Variables adjusted for	Any cardiovascular diseases			Coronary heart diseases			Stroke		
	No. of Cases/ No. of Subjects	RR (95%CI)	p value	No. of Cases/ No. of Subjects	RR (95%CI)	p value	No. of Cases/ No. of Subjects	RR (95%CI)	p value
Univariate	17/2,567	0.66 (0.41–1.07)	0.093	10/2,567	0.52 (0.28–0.97)	0.039	7/2,567	0.92 (0.45–1.91)	0.830
Multivariate model 1	16/2,494	0.59 (0.36–0.98)	0.039	10/2,494	0.49 (0.25–0.93)	0.029	6/2,494	0.76 (0.34–1.70)	0.498
Multivariate model 2	15/2,493	0.60 (0.34–1.04)	0.069	9/2,493	0.45 (0.21–0.97)	0.041	6/2,493	0.80 (0.35–1.84)	0.593
Multivariate model 3	15/2,493	0.73 (0.37–1.44)	0.371	9/2,493	0.46 (0.20–1.07)	0.071	6/2,493	1.37 (0.50–3.76)	0.545

*The concentration of phospholipid transfer protein was analyzed as a continuous variable.

The relative risks (RR) are per increment of 1 SD (4.3) in PLTP value. CI, confidence interval; PLTP, phospholipid transfer protein.

Multivariate model 1 adjusted for age, smoking status, alcohol intake, and leisure-time physical activity.

Multivariate model 2 adjusted for variables entered in the multivariate model 1, body mass index, systolic blood pressure, low-density lipoprotein cholesterol, and glycosylated hemoglobin.

Multivariate model 3 adjusted for variables entered in the multivariate model 2, HDL cholesterol and triglycerides.

cant and independent associations between serum PLTP concentration and HDL-C, TG, LDL-C, and BMI (standardized β = 0.395, -0.191 , -0.064 , and -0.064 , respectively) (Table 3). Thirty-one percent of the variance in serum PLTP concentration was explained by this model; however, 26% of variance was explained only by HDL-C, and an additional 4% was explained by TG.

During follow-up of 7,037 person-years, CHD developed in 10 subjects (8 cases of non-fatal acute myocardial infarction, 1 of unstable angina, which ended up as coronary artery bypass graft operation, and 1 sudden cardiac death), and stroke developed in 7 subjects (5 cases of non-fatal cerebral infarction and 2 of fatal subarachnoid hemorrhage). Crude incidence rates for CHD and stroke were 142.1 (per 100,000 person-years) and 99.5, respectively and the age-adjusted incidence rates to the world standard population²⁴ were 112.9 (per 100,000 person-years) and 62.1, respectively.

The results relating the serum PLTP to the development of any cardiovascular diseases, CHD or stroke are shown in Table 4. The concentration of serum PLTP was significantly and inversely related to the risk of cardiovascular diseases in the multivariate model 1 ($p=0.039$). In the multivariate model 2 adjusting for BMI, systolic blood pressure, LDL-C, and glycosylated hemoglobin, a marginally significant association was observed ($p=0.069$). Further adjustment with TG and HDL-C (multivariate model 3) substantially attenuated the association. The concentration of serum PLTP was, however, inversely related to the risk of CHD with marginal significance even after adjustment for HDL-C and TG. The relative risk of CHD in the multivariate model 3 was 0.46 (95%CI 0.20–1.07) for an increase of 1 SD in the PLTP value ($p=0.071$). The concentration of serum PLTP was not related to the risk of stroke ($p=0.545$ in multivariate model 3).

Discussion

The results of this prospective, observational study indicate that there is a graded inverse association between serum PLTP concentration and the risk of cardiovascular disease, especially CHD. The observed association appeared to be independent of age and other covariates included in the multivariate models. In the analysis adjusted for the concentration of HDL-C and TG, which explained 30% of the variance in PLTP concentration, there was still a marginally significant association between serum PLTP concentration and the risk of CHD. The reason for the lack of association between stroke and the

PLTP concentration is unclear. Though the limited number of stroke cases makes it difficult to speculate specifically, classic risk factors such as systolic blood pressure or glycosylated hemoglobin had a significant, or marginally significant, direct association with the risk of stroke (relative risk of 1.04 ($p=0.06$) for the increment of 1 mmHg in blood pressure, and 2.13 ($p=0.02$) for the increment of 1% in glycosylated hemoglobin, in the multivariate model 3). Exclusion of 2 cases of subarachnoid hemorrhage, which may have a different etiology from cerebral infarction, did not alter the association (data not shown). To elucidate whether the association of serum PLTP concentration exists only with the development of CHD, further investigations with more cases are needed.

This is the first report showing a significant inverse relationship between the serum concentration of PLTP mass and the development of CHD. Previous studies regarding PLTP and CHD or other patho-physiologic conditions have mainly focused on its phospholipid transfer activity.^{25,26} The assay used in the present study measures the total amount of serum PLTP mass, and it is reported that it does not differentiate the catalytically active form from the inactive form of PLTP.²² It is also suggested that majority of the PLTP mass exists in the inactive form in the circulation in healthy subjects.^{16,17} We obtained the data regarding PLTP activity in a sample of subjects, and the results indicated that the concentration of serum PLTP mass did not correlate with its activity. Thus, the inverse association between PLTP mass and CHD found in this study may not be solely attributed to the phospholipid transfer activity of PLTP.

The reason for the presence of PLTP with low phospholipid transfer activity is not yet clear. Oka et al found that PLTP activity in hypoalphalipoproteinemia was comparable to that in normolipidemic controls, although PLTP concentration was significantly lower in hypoalphalipoproteinemia subjects than in controls.¹⁷ They found that the low PLTP concentration in hypoalphalipoproteinemia was because of a lack of inactive PLTP. Dyslipidemic states, such as low HDL-C or elevated TG,^{16,22} or intravenous infusion of apo A-I/phosphatidylcholine discs, which increases the plasma pre- and apo A-I and HDL-C concentrations,²⁷ were demonstrated to modify the distribution of active and inactive pools of PLTP. It is, therefore, necessary to conduct further research to elucidate the mechanisms involved in the existence of inactive PLTP, with regard to its function in lipoprotein metabolism. Clinical studies that measure both the activity and the concentration of PLTP are also needed to understand the reason for the disparity between them in various pathophysiological

settings. Nevertheless, the present results show that the serum PLTP concentration, which probably reflects the amount of the inactive form of PLTP, was significantly and inversely associated with the development of CHD, even after adjustment of HDL-C and TG.

Our finding that an elevated concentration of PLTP may be a protective marker of CHD is consistent with several previous reports that indicated that the function of PLTP was anti-atherogenic^{7,28} although all these studies did not obtain the concentration of the PLTP mass, except for a few;²⁷ which may limit direct comparisons. Haperen et al showed that accumulation of cholesterol in peritoneal macrophages was significantly reduced in transgenic mice that overexpressed human PLTP⁷ and that result was attributed to the increased production of pre- β -HDL in the transgenic mice. In contrast, overexpression of PLTP in mice heterozygous for the LDL receptor, which is a model for atherosclerosis, significantly increased the area of the atherosclerotic lesion in the mice.²⁶ However, these apparently inconsistent results were from experiments with specific mouse models, and may be attributable to differences in the experimental models.^{9,29} Because studies regarding PLTP and atherosclerosis in humans are scarce, further research must evaluate the present result.

One mechanism that mediates the possible protective effect of PLTP on the risk of CHD is probably the capability of PLTP to generate pre- β -HDL, an efficient acceptor of peripheral cell cholesterol in reverse cholesterol transport. The pre- β -HDL concentration has been found to be significantly lower in CHD cases than in healthy controls.³⁰ Plasma from the transgenic mouse that overexpressed human PLTP had increased potential for pre- β -HDL formation.^{7,31} Furthermore, generation of pre- β -HDL, measured as the change in pre- β -HDL concentration during incubation of plasma from cases and controls, which would reflect a function of PLTP, was significantly higher in controls than in cases.³⁰

The prospective nature of the present study between newly developed CHD and serum PLTP concentration suggests that the decrease in the PLTP concentration preceded the onset of CHD. However, we measured PLTP concentrations only at the time of enrollment and there is still uncertainty about how long this inter-individual variation persisted. Further studies to examine the degree of the intra-individual variability of the PLTP concentration over varying time intervals are needed.

Other limitations of the study are the relatively short follow-up period and that the study population included only Japanese males. Although the study is limited in the number of cases included, the incidence rates of CHD and stroke were both comparable to those of previous reports on Japanese males of approximately the same age range.^{32,33} However, the relation between elevated serum PLTP concentrations and cardiovascular diseases must be evaluated in other cohort studies. If such studies confirm our findings, proof of a causal association between serum PLTP and the development of cardiovascular diseases will require further elucidation of the patho-physiologic mechanisms. Furthermore, studies to investigate the association between lifestyle factors and the serum PLTP concentrations are needed for the development of practical prevention programs.

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