Original Article

Serum Levels of Thiobarbituric Acid-Reactive Substances are Associated with Risk of Coronary Heart Disease

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Aim: Evidence of relationships between lipid peroxidation and the incidence of coronary heart disease is limited. We therefore investigated this association in a Japanese general population.

Methods: We prospectively studied 1945 individuals who were apparently healthy at the baseline. Cox proportional-hazards models were used to investigate the relationship between serum levels of thiobarbituric acid-reactive substances (TBARS) as a marker of lipid peroxidation and the incidence of coronary heart disease, adjusted for established risk factors including age, sex, current smoking, body mass index, systolic blood pressure, glycohemoglobin A1c, serum total cholesterol, and serum high-density lipoprotein cholesterol levels.

Results: During 20,226 person-years of follow-up (median follow-up, 10.9 years), 44 coronary events were documented. The risk of coronary heart disease increased in consecutive tertiles of the TBARS level. On age- and sex-adjusted analysis, the level of TBARS was significantly associated with the incidence of coronary heart disease. The hazard ratio in the highest tertile compared with the lowest tertile was 3.22 (95% confidence interval, 1.38 to 7.53; $p=0.007$). On multivariate analysis adjusted for age, sex, and other established risk factors, this association remained significant (hazard ratio, 3.23; 95% confidence interval, 1.28 to 8.16; $p=0.01$).

Conclusions: Serum levels of TBARS are a strong and independent predictor of coronary heart disease. These findings support the hypothesis that lipid peroxidation is an important risk factor for coronary heart disease.

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Key words; Thiobarbituric acid-reactive substances (TBARS), Lipid peroxide, Coronary heart disease (CHD), Cohort study

Introduction

Oxidative stress and subsequent lipid peroxidation is important in the initiation and development of atherosclerosis¹⁻³. The oxidative free radicals released by vascular cells are proposed to be responsible for oxidative modifications of low-density lipoprotein

(LDL) *in vitro*¹, and the presence of oxidized LDL in atherosclerotic lesions and plasma has been identified *in vivo*^{4, 5}. Furthermore, biologically relevant sources of oxidative stress in the arterial wall, including myeloperoxidase⁶, lipoxygenase⁷, and NAD(P)H oxidase⁸, have been extensively studied, suggesting that oxidative stress and subsequent lipid peroxidation is closely related to the development of atherosclerosis.

The oxidation products of unsaturated fatty acids, including malondialdehyde, react with thiobarbituric acid to form red compounds called thiobarbituric acid-reactive substances (TBARS)⁹⁻¹¹. Reactions with thiobarbituric acid (TBA) have been widely used as a sensitive assay for lipid peroxidation of blood

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samples¹⁰⁾ and LDL¹⁾. Although some reports have raised questions about its specificity¹²⁾, we adopted this assay for the following reasons: first, it is simple enough to perform in a hospital laboratory; second, it is applicable to a large number of samples from a cohort study; third, it may be more specific for lipid hydroperoxide than other TBA assays by virtue of its lipid-isolating procedure with the use of phosphotungstic acid before the TBA reaction¹⁰⁾.

Several reports have examined the relationship between oxidative stress markers and the incidence of cardiovascular disease. Walter MF *et al.* have reported that serum levels of TBARS were associated with cardiovascular events in patients with stable coronary artery disease¹³⁾. Plasma oxidized LDL and oxidized phospholipids/apolipoprotein B have also been shown to be associated with the risk of cardiovascular disease by Meisinger C *et al.* and Kiechl S *et al.*^{14, 15)}. Regarding superoxide-generating enzymes, Meuwese MC *et al.* have reported that serum myeloperoxidase levels were associated with the future risk of coronary artery disease in apparently healthy individuals¹⁶⁾. As for antioxidant enzymes, Blankenberg S *et al.* have reported that glutathione peroxidase 1 activity but not erythrocyte SOD activity was inversely associated with the risk of cardiovascular events in patients with coronary artery disease¹⁷⁾; however, these were case-control studies in general populations^{14, 16)} or small cohort studies in patients with coronary artery disease^{13, 17)}, and cohort studies in general populations are limited¹⁵⁾. The present study focused on the impact of an oxidative stress marker, serum TBARS, on the incidence of coronary heart disease (CHD) in a Japanese general population. We estimated the multivariate adjusted hazard ratio for the incidence of CHD in a 10.9-year follow-up study.

Subjects and Methods

Study Population

The surveyed population included residents 20 years of age or older, who participated in a cardiovascular risk survey in 1993 in Hidaka town, a typical rural community in Japan¹⁸⁾. A total of 2155 individuals participated in this survey. These subjects were not specifically selected, but were allowed to freely participate in this survey. Of these, 115 participants had a history of cardiovascular disease. The remaining 2040 participants were eligible for this study, representing 41.6% of the total population of the town. Of these, 95 participants were lost to follow-up. Consequently, 1945 subjects (1151 women, 794 men; mean age, 58.4 years; range, 20 to 93 years) were used for

the analysis.

All study protocols were approved by the Medical Ethics Committee of Hidaka Medical Center. Informed consent was obtained from subjects at both the baseline examination and follow-up survey.

Baseline Examination

The baseline survey was conducted between July 8 and August 6, 1993. Community nurses interviewed the participants at community centers. Information was obtained on current and past health conditions, medication, and lifestyle. Blood pressures were measured with a standard mercury sphygmomanometer on the right arm after the participants had been sitting at rest for at least 3 minutes. Hypertension was defined as systolic blood pressure ≥ 140 mmHg, and/or diastolic blood pressure ≥ 90 mmHg, or current use of antihypertensive agents. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in meters. All participants underwent a physical examination and laboratory testing of blood samples. Participants reported the time since their last meal, which was ≤ 2 hours for 4.3%, 3 to 7 hours for 77.5%, and ≥ 8 hours for 18.2% of total participants. Most blood samples were thus drawn in a non-fasting state. Serum concentrations of total cholesterol, triglycerides and high-density lipoprotein (HDL) cholesterol were measured enzymatically.

Measurement of serum TBARS was conducted as reported by Yagi¹⁰⁾. Concentrations of TBARS were expressed in terms of malondialdehyde ($\mu\text{mol/L}$ serum). The substances reacting with TBA in this system have been reported to be metabolites of unsaturated fatty acid hydroperoxides¹¹⁾. The advantage of the present TBA assay is to isolate lipids by precipitating them with serum protein using phosphotungstic acid, followed by determination by the TBA reaction. By this procedure, TBA-reacting substances in blood serum, other than lipid peroxide, can be easily removed¹⁰⁾.

Follow-Up

Information on the incidence of CHD was collected by means of self-administered, mailed questionnaires or interviews by telephone in June 2004.

Responses were obtained from 1945 subjects, with a response rate of 95.3%. Information about deaths and incidences of diseases was also obtained from reviews of vital statistics through the municipal register, hospital records, death certificates and autopsy reports with permission from the participants or their relatives. Although almost all events were reviewed using hospital records, only three cases of coronary

artery bypass graft surgery were self-reported by the participants. Follow-up was conducted until May 2007.

Confirmation of Events

The endpoint of the study was the development of CHD, including fatal or non-fatal myocardial infarction, angina pectoris, performance of coronary bypass surgery or angioplasty, and sudden cardiac death. Myocardial infarction was confirmed if the subject met the World Health Organization criteria for the Monitoring Trends and Determinants of Cardiovascular Disease project (MONICA Project)¹⁹. Angina pectoris was diagnosed according to the criteria of the American Heart Association²⁰. In addition to these criteria that comprise the clinical symptoms, angina pectoris was considered present if at least one of the following criteria was met: 1) local abnormality of cardiac wall motion on echocardiography during an attack or 2) at least 75% stenosis on coronary angiography. Ischemic cardiac arrest in the criteria for MONICA Project was also considered as angina pectoris. Sudden cardiac death was defined as death within 1 hour after onset of acute illness, occurring in a patient without any restriction of daily activities and who was not hospitalized prior to the onset of illness. All coronary events were reviewed and confirmed by three cardiologists who were blinded to baseline examination data.

Statistical Analysis

Participants were categorized into tertiles according to baseline serum TBARS levels and other variable levels. Data were analyzed continuously, or as tertiles with the lowest tertile as the reference category. Hazard ratios (HR) for future CHD according to tertiles of TBARS level with 95% confidence intervals (CI) were calculated and adjusted for established risk factors including age, sex, current smoking, BMI, systolic blood pressure, glycohemoglobin A1c, serum total cholesterol, and serum HDL cholesterol levels by Cox proportional-hazards models. Cumulative event-free curves according to tertiles of the TBARS level were estimated by the Kaplan-Meier method and compared using the log-rank test. Among the 1945 subjects with complete follow-up, 71 subjects, including 59 subjects for glycohemoglobin A1c, 21 subjects for waist circumference, five subjects for BMI, five subjects for TBARS, and one subject each for several variables, were excluded from the analysis due to missing data at baseline. $P < 0.05$ was considered to be significant, and all tests were two-tailed. SPSS for Windows version 11.01J software (SPSS Japan, Tokyo) was used to perform all statistical analyses.

Results

Baseline characteristics of the study cohort are presented in **Table 1**. During the 20,226 person-years of follow-up (median follow-up, 10.9 years), we documented 44 coronary events, including 2 deaths due to fatal myocardial infarction, 17 nonfatal myocardial infarctions, 5 cases of angina pectoris, 7 coronary artery bypass or angioplasty procedures, and 13 sudden cardiac deaths.

Table 2 shows age- and sex-adjusted analysis of risk factors in tertiles or in dichotomous variables for the incidence of CHD. The HR for the highest tertile of the serum TBARS level as compared with the lowest tertile was 3.22 (95% CI, 1.38 to 7.53; $p = 0.007$). Other potential confounders in this analysis were waist circumference (HR, 3.23; 95% CI, 1.21 to 8.63; $p = 0.02$) and serum levels of triglycerides (HR, 2.81; 95% CI, 1.26 to 6.27; $p = 0.01$). These results suggest that lipid peroxidation, hypertriglyceridemia and abdominal obesity may be associated with the risk of CHD in our population, even though non-HDL cholesterol did not show a significant association with coronary events. A history of hypertension and diabetes were also associated with the events (HR, 2.97; 95% CI, 1.55 to 5.69; $p = 0.001$ and HR, 3.44; 95% CI, 1.59 to 7.45; $p = 0.002$, respectively); however, we could not find any associations of current smoking, systolic blood pressure, total cholesterol, or HDL cholesterol levels with the risk of CHD in this analysis.

Fig. 1 shows the Kaplan-Meier estimate for event-free survival according to tertiles of the serum TBARS level. The rate of coronary events increased in a stepwise fashion according to increasing tertiles of the baseline serum TBARS level. Differences among all curves were significant according to the log-rank test ($p = 0.03$).

Furthermore, a Cox proportional-hazards model with stepwise variable selection was used to identify variables of potential risk for CHD. We adopted covariates including age, sex, current smoking, BMI, systolic blood pressure, glycohemoglobin A1c, serum total cholesterol level, HDL cholesterol level, history of hypertension, history of diabetes, and serum TBARS level for the analysis. Of these, we identified age, sex, history of hypertension, history of diabetes, and the serum TBARS level as variables associated with the risk of coronary events (data not shown).

Table 3 shows the HR of future coronary events according to tertiles of the serum TBARS level. In multivariate analysis adjusted for established risk factors including age, sex, current smoking, BMI, systolic blood pressure, glycohemoglobin A1c, serum

Table 1. Baseline characteristics of the study cohort

Characteristics*	Entire cohort <i>n</i> = 1945	Females <i>n</i> = 1151	Males <i>n</i> = 794
Age (years)	58.4 ± 14.7	58.4 ± 14.9	58.4 ± 14.4
BMI (kg/m ²) [†]	22.5 ± 3.0	22.5 ± 3.0	22.5 ± 2.9
Waist circumference (cm)	72.8 ± 8.3	70.0 ± 7.3	76.9 ± 7.9
Current smoker (%)	22.3	2.1	51.5
History of hypertension (%)	33.1	34.6	30.9
Antihypertensive medication (%)	15.9	17.3	14.0
History of diabetes (%)	5.3	4.4	6.7
Blood pressure (mmHg)			
Systolic	133 ± 21	133 ± 21	135 ± 21
Diastolic	77 ± 12	76 ± 12	79 ± 12
Hypertension (%) [‡]	45.7	44.6	46.7
Glycohemoglobin A1c (%)			
Median	4.8	4.8	4.9
Interquartile range	0.5	0.6	0.6
Cholesterols (mg/dL) [§]			
Total	199 ± 36	206 ± 36	189 ± 34
HDL	59 ± 15	61 ± 14	57 ± 15
Non-HDL cholesterol (mg/dL) [†]	78 ± 42	84 ± 41	70 ± 41
Triglycerides (mg/dL)			
Median	89	85	97
Interquartile range	65	59	78
TBARS (mol/L)	5.0 ± 1.9	4.9 ± 1.6	5.2 ± 2.1

* Plus-minus values are the means ± standard deviation.

As glycohemoglobin A1c and triglycerides were skewed distributions, values are expressed as medians and interquartile ranges.

[†] BMI is weight in kilograms divided by the square of height in meters.

[‡] Hypertension was defined as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg, or current use of antihypertensive agents.

[§] To convert cholesterol values to millimoles per liter, multiply by 0.02586; HDL, high-density lipoprotein

^{||} To convert triglycerides values to millimoles per liter, multiply by 0.01129.

total cholesterol level, and HDL cholesterol level, the HR remained nearly unchanged compared with age- and sex-adjusted analysis (HR, 3.23; 95% CI, 1.28 to 8.16; $p=0.01$; model 1, **Table 3**). To exclude confounders caused by associations between increased lipid peroxidation and hyperlipidemia^{21, 22}, we included the serum level of triglycerides as a covariate in the model. Even after further adjustment for the serum level of triglycerides, the HR remained nearly unchanged (HR, 3.33; 95% CI, 1.30 to 8.53; $p=0.01$; Model 2, **Table 3**). Furthermore, on multivariate analysis adjusted for history of hypertension, history of diabetes, waist circumference, and serum triglycerides level, all were significantly associated with the risk of CHD in age- and sex-adjusted analysis (**Table 2**), this association remained significant (HR, 2.76; 95% CI, 1.15 to 6.63; $p=0.02$) with a slight decrease in the HR (Model 3, **Table 3**).

Discussion

This population-based cohort study demonstrated that elevated serum lipid hydroperoxide measured as the TBARS level was associated with the incidence of CHD. This association was independent of established risk factors even after adjustment in multivariate analysis. Our findings provide evidence that increased lipid peroxidation is a risk factor for CHD in a general population.

Mortality and the incidence rates of CHD in Japan are the lowest in the world²³⁻²⁵, which is generally attributed to lifestyle rather than genetic differences²⁶. The ratio of polyunsaturated fatty acids to saturated fatty acids in the typical Japanese diet has traditionally been higher than in the Western diet²⁴, and a diet rich in polyunsaturated fatty acids is favorable for preventing CHD; however, these fatty acids

Table 2. Age- and sex-adjusted hazard ratios for CHD according to risk factors

Variables	Hazard ratio* (95%CI)	<i>p</i>
Current smoking	1.56 (0.77-3.16)	0.22
History of hypertension	2.97 (1.55-5.69)	0.001
History of diabetes	3.44 (1.59-7.45)	0.002
BMI	2.15 (0.99-4.66)	0.05
Waist circumference	3.23 (1.21-8.63)	0.02
Systolic blood pressure	1.82 (0.72-4.59)	0.21
Diastolic blood pressure	1.28 (0.63-2.61)	0.49
Glycohemoglobin A1c	2.07 (0.83-5.17)	0.12
Total cholesterol	2.14 (0.99-4.63)	0.05
HDL cholesterol	0.55 (0.25-1.20)	0.13
Non-HDL cholesterol [†]	2.03 (0.97-4.25)	0.06
Triglycerides	2.81 (1.26-6.27)	0.01
TBARS	3.22 (1.38-7.53)	0.007

*Hazard ratios as determined from Cox proportional-hazards models.

[†]Non-HDL cholesterol denotes the differences between total cholesterol and HDL cholesterol.

Subjects with continuous variables were divided into tertiles. Subjects in the lowest tertile served as the reference group. Current smoking status, history of hypertension, and history of diabetes were dichotomous variables. Subjects with the absent dichotomous variable served as the reference group.

CHD, coronary heart disease; CI, confidence interval; HDL, high-density lipoprotein; TBARS, serum levels of thiobarbituric acid-reactive substances

are susceptible to lipid peroxidation. The origin of TBARS in the present assay has been reported to be the decomposition of lipid hydroperoxide^{11, 27)} or prostaglandin endoperoxide²⁸⁾, both of which are derived from n-6 polyunsaturated fatty acids. Production of TBA pigment in this assay and the extent of lipid peroxidation were closely correlated *in vitro*¹¹⁾. Since atherosclerosis is well recognized as an inflammatory disease^{29, 30)}, oxidative metabolites of n-6 polyunsaturated fatty acids such as arachidonic acid-derived prostanoids may cause vascular inflammation, leading to atherosclerosis. A recent clinical trial has demonstrated favorable effects of long-chain n-3 polyunsaturated fatty acid on preventing CHD³¹⁾. This result may be derived, at least in part, from anti-inflammatory effects of n-3 polyunsaturated fatty acid on vascular walls, as opposed to n-6 polyunsaturated fatty acids³²⁾.

Recent clinical trials have failed to prove any preventive effects of antioxidant vitamins on cardiovascular diseases³³⁾. Moreover, recent studies have demonstrated that antioxidant vitamins may inhibit reverse cholesterol transport by reduction-oxidation-sensitive gene expression³⁴⁾, or prevent health-promoting effects

Fig. 1. Cumulative survival with a risk of CHD according to tertiles of serum level of TBARS.

Differences among all curves were statistically significant according to the log-rank test ($p=0.03$). Five subjects with missing data were excluded from the analysis.

of physical exercise such as induction of insulin sensitivity and endogenous antioxidant defense³⁵⁾. Conversely, studies in patients with end-stage renal disease and in diabetic patients with the haptoglobin 2-2 genotype, both known to be under oxidative stress, have demonstrated beneficial effects of vitamin E on cardiovascular disease^{36, 37)}. Taken together, antioxidant vitamins should be considered only for high-risk patients with increased oxidative stress; however, most clinical trials with antioxidant vitamins for cardiovascular disease prevention have not evaluated oxidative stress with certain oxidative stress markers, leading to inappropriate interventions. Pharmacological interventions for profound reduction-oxidation signals should thus be performed with caution, and evaluation of oxidative stress by oxidative stress markers is warranted in such clinical trials. The clinical significance of measuring the TBARS level has not yet been well-established; however, it is of particular interest to investigate whether aspirin, antioxidant vitamins, and other agents, which have potential effects on the cellular reduction-oxidation state, would reduce the serum TBARS level and subsequently decrease the risk of CHD.

The advantage of the present assay method is that lipids are isolated by precipitation with serum protein by phosphotungstic acid, followed by the TBA reaction. In this procedure, TBARS other than lipid hydroperoxide can be easily removed¹⁰⁾. The present assay method may thus be more useful than other

Table 3. Hazard ratio of future CHD according to tertiles of serum levels of TBARS at baseline

Variable	Tertiles of TBARS (mol/L)			<i>p</i>
	Tertile 1 < 4.2	Tertile 2 4.2-5.3	Tertile 3 > 5.3	
Crude analysis*				
n	647	656	637	
HR (95%CI)	1	1.82 (0.73-4.54)	3.52 (1.50-8.22)	0.008
P		0.20	0.004	
Age-, sex-adjusted analysis*				
n	647	656	637	
HR (95%CI)	1	1.49 (0.59-3.72)	3.22 (1.38-7.53)	0.009
P		0.40	0.007	
Multivariate analysis model 1 *†‡				
n	611	638	625	
HR (95%CI)	1	1.68 (0.63-4.51)	3.23 (1.28-8.16)	0.03
P		0.30	0.01	
Multivariate analysis model 2 *‡§				
n	611	638	625	
HR (95%CI)	1	1.71 (0.64-4.58)	3.33 (1.30-8.53)	0.02
P		0.29	0.01	
Multivariate analysis model 3 *§				
n	638	647	626	
HR (95%CI)	1	1.48 (0.58-3.74)	2.76 (1.15-6.63)	0.04
P		0.41	0.02	

HR, hazard ratio; CI, confidence interval

* Due to missing data on TBARS, 5 subjects were lost to analyze.

† Multivariate model 1 includes covariates of age, sex, BMI, smoking status (current smoker vs. non-smoker), systolic blood pressure, serum total cholesterol, HDL cholesterol, and glycohemoglobin A1c.

‡ Multivariate model 2 includes covariates of model 1 plus serum triglycerides.

§ Multivariate model 3 includes covariates including age, sex, history of hypertension, history of diabetes, waist circumference, serum triglycerides level.

|| Due to missing data on glycohemoglobin A1c and smoking status, 2 cases were lost from models 1 and 2, leaving 42 coronary events. Model 3 lost 1 case due to missing data on a history of hypertension, leaving 43 cases.

TBA assays based on crude samples; however, we should not rule out the possibility of potential effects of residual compounds in the sample even after this procedure.

We failed to demonstrate an association between established risk factors (including systolic blood pressure, serum total cholesterol, and HDL cholesterol) and coronary events in age- and sex-adjusted analyses. Three possible explanations can be suggested for these results.

First, lipid-modifying and/or antihypertensive treatments during the follow-up period may have affected the incidence of CHD in this study. In addition to coronary events, we also sent mailed questionnaires to the participants whose total cholesterol levels were ≥ 220 mg/dL at baseline and asked them whether they had been prescribed lipid-modifying agents during the follow-up period. Indeed, most participants

with hypercholesterolemia received statin therapy after the baseline study, which may have attenuated the deleterious effects of hypercholesterolemia on the development of CHD in this population (data not shown). The relatively small number of events in this study might be, at least in part, due to statin therapy; therefore, this study is important as a cohort study in which statins have been widely used and is distinct from previous studies in which effective drugs were not available.

Second, a recent study has reported that the non-fasting triglycerides level is useful to predict the incidence of future cardiovascular disease, whereas non-fasting total and LDL-cholesterol levels are less useful³⁸. Our baseline blood samples were mostly drawn under non-fasting states, which may have attenuated any association between cholesterol levels and the incidence of coronary events.

Third, a lack of statistical power should be considered because of the relatively small number of events ($n=44$).

In conclusion, we have demonstrated an association between the serum TBARS level and future CHD in a general population. These results strongly suggest that lipid peroxidation is involved in the development of CHD. The present TBA assay is easy to perform in clinical procedures and large clinical trials. This assay may be useful in evaluating the systemic status of oxidative stress and subsequent lipid peroxidation in general populations, and may contribute to the further prevention of CHD in the era of statin therapy.

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