have demonstrated that cholesterol is extracted by HDL particles in the culture medium from cultured cells, including macrophages overloaded with cholesterol.

From these two lines of evidence, HDL is believed to be a "preventive factor" against atherosclerosis. This view is strongly associated with the hypothesis that HDL plays a central role in the recovery of cholesterol molecules from tissues and organs, which cannot be catabolized in peripheral cells, and in their transport to the liver for conversion to bile acids. From the viewpoint of public health, many research results suggest that a decrease in HDL-C contributes more than an increase in LDL-C to the development of ischemic heart disease in Japan. In studies conducted at Nagoya City University, for example, narrowing of the coronary artery was more closely related to triglycerides (TG) and HDL-C than to total cholesterol (TC) or LDL-C[1, 2], and this tendency is commonly observed in many other reports. HDL-C is thus suggested to be a strong determinant of atherosclerosis in Japan and perhaps a more important risk factor than LDL-C from a public health point of view.

HDL is smaller (12 nm or less in diameter) than other lipoproteins, abundant in protein and does not contain much TG, so it has a greater hydrated density than other lipoproteins (d=1.063-1.21). Similarly to other plasma lipoproteins, however, HDL functions to transport cholesterol among cells or organs using the flow of blood or extracellular fluid. Cholesterol, an essential molecule for the life of animals, requires a number of steps and plenty of energy for synthesis, and its dietary intake is not always guaranteed; therefore, the animal body has developed systems to use cholesterol sparingly as a precious material. As a result, little cholesterol is converted to energy in its catabolism, and, with the exception of a very small amount used for the production of steroid hormones, most cholesterol is transported to the liver for conversion to bile acids and is recycled an reused in the intestine before excretion. Its steroid backbone is not degraded in the metabolism in the animal body and finally broken down by microorganisms in the environment. Therefore, cholesterol molecules must be released from most somatic cells for metabolic homeostasis, and HDL receives these cholesterol molecules for their transport. Cholesterol is converted to cholesteryl acylester (CE) as a fatty acyl chain and transferred from phosphatidylcholine to its hydroxyl group to form an ester bond, for packing cholesterol molecules into the core of HDL. CE is recovered by the liver directly from HDL by a selective uptake reaction, or as LDL particles after being transferred to apolipoprotein B-containing lipoproteins by CE transfer protein (CETP). As a result of these activities, HDL is considered to exert a preventive effect against atherosclerosis as it interferes with the excessive accumulation of cholesterol in cells from LDL, etc., by extracting it.

No drug has been marketed yet to independently increases HDL-C; therefore, the question of whether increasing HDL-C is effective for preventing and treating atherosclerotic disorders has not been answered. However, researchers have recently directed more attention to HDL and, accordingly, more research results on HDL metabolism have recently accumulated. Much effort to develop drugs targeting HDL has been initiated. On the other hand, some existing drugs are known to increase plasma HDL-C. Drugs that reduce TG generally increase HDL-C, primarily because these drugs reverse low HDL-C induced by high TG through CETP[3]. In addition, fibrates have been suggested to directly increase HDL production[4]. Many clinical studies have also shown that statins elevate HDL-C as well as decreasing LDL-C. Concerning their mechanism, statins have recently been reported to increase HDL synthesis in the liver, unlike their effects in peripheral tissues[5]. The mechanism of the increase in HDL through exercise and alcohol intake has not been sufficiently elucidated. As mentioned below, the question of whether HDL-C increase by inhibiting CETP prevents atherogenesis has been shelved because of the failure to develop a CETP-inhibiting drug, perhaps due to a business-oriented strategy[6].

Position of HDL in Risk-Reducing Strategies

Large-scale clinical studies targeted to high LDL-C and high TG, major risk factors of atherosclerotic diseases, such as ischemic heart disease, have indicated that ischemic heart disease can be prevented by reducing LDL-C and TG and, particularly, that mortality due to the disease can be lowered by controlling the LDL-C level, with a consequent reduction in the total number of deaths in the high-risk group. In addition, based on stratified analysis of the results of many clinical trials, the conclusion has been reached that an increase in HDL-C contributes to the prevention of diseases as a "statistically independent factor". In consideration of the above-stated marked epidemiological contribution of HDL-C as a "negative risk factor" and the significant "indirect evidence" of an increase in HDL-C in the prevention of atherogenesis, the argument that a standard should be set for the control of HDL-C appears to be well grounded. However, it is also true that a consensus concerning

HDL-C management, similar to that in evidence-based quantitative guidelines for the control of LDL-C and the management and treatment of high TG, is difficult to reach at present, when no therapeutic technique specifically targeted to increase HDL-C has reached a practical level and there is no direct evidence concerning the prevention and treatment of atherosclerotic disorders using such a technique. Thus, any therapeutic guideline regarding HDL-C is merely a "proposal" based on indirect circumstantial evidence until the results of a large-scale clinical trial of a technique to specifically increase HDL-C become available.

Recently, some negative implications have been spread regarding the anti-atherosclerotic effect of an increase in HDL-C, inviting some confusion in the discussion. One is the discontinuation of a large-scale clinical study on the prevention of ischemic heart disease by increasing HDL-C, carried out to develop the CETP inhibitor torcetrapib, due to an increase in the mortality rate in the treated group[6]. Another is a large-scale epidemiological study reporting that a mutation to cause dysfunction of ABCA1, a rate-regulating protein of HDL biogenesis, is not likely to be a risk factor of ischemic heart disease[7]. The first report appears to support the contention of researchers arguing that "an increase in HDL-C by CETP inhibition has no anti-atherosclerotic effect," and allowed the generalized assertion that "the HDL-C increasing strategy is a mistake" to emerge; however, these reports do not necessarily mean the failure of CETP inhibitors themselves, and the pressor effect of a particular drug, torcetrapib, is likely to have led to such results. This incidence postponed an answer to the question of whether increasing HDL-C with a CETP inhibitor is a good idea, the most important medical issue, and markedly complicated the strategy for developing HDL-C elevating agents in general. Also, studies on ABCA1 mutation have shown that the maximum decrease in HDL is about 20%, suggesting that this does not necessarily reject the benefit of high HDL-C.

Under these circumstances, the position has not changed that an elevation of HDL-C is an important part of the anti-atherosclerotic strategy, including CETP inhibition. The above discussion may be summarized as follows: 1) a decrease in HDL-C is a strong risk factor for atherosclerotic disorders, 2) there are rational grounds for the supposition that this risk can be reduced by correcting low HDL-C (increasing HDL-C), but 3) no direct evidence has been obtained that increasing HDL-C is effective for the prevention and treatment of atherosclerotic disorders, 4) changes in HDL-C may include changes in the number and size of HDL particles, and the difference in their clinical significance may become a problem in the future.

Simulation of Atherosclerosis Prevention by Increasing HDL-C

There are qualitative scientific grounds for lowering the LDL-C level to reduce the risk of atherosclerotic disorders or, more specifically from an evidence-based viewpoint, to reduce the probability of the occurrence of ischemic heart disease; however, to prepare specific guidelines for diagnosis and treatment, quantitative criteria are considered indispensable. This is a problem with the concept in setting therapeutic goals for target groups. A quantitative profile of increases in the risk associated with elevations of the LDL-C level is necessary, and, if possible, results directly showing that the treatment reverses this curve of increasing risk must be presented. It is not impossible to set medical goals according to this parameter alone, but how criteria are set markedly affects the cost-effectiveness of treatment depending on the distribution of the HDL-C level and demographic composition of the target population; therefore, simulation involving these factors is one of the tasks that must be implemented to devise guidelines.

Fig. 1B shows the relationship between the LDL-C level and incidence (per 1,000 people) of myocardial infarction (lethal/non-lethal) in the JLIT, a cohort study that followed up a simvastatin-treated group for 5 years[8]. From this graph, the distribution of the HDL-C level in Japanese of corresponding ages (**Fig. 1A**)[9], and the population composition of the Japanese by age, the number of people needed to treat (NNT) and number of patients in whom the disease is prevented can be calculated when the control target is fulfilled 100% by reducing LDL-C (**Fig. 1C**). According to this calculation, the primary prevention efficacy, expressed as the inverse of NNT, is high at a target LDL-C level of 140 mg/dL but begins to fall rapidly as it is reduced to 120 mg/dL. Reflecting this, the incidence of myocardial infarction shows no further decrease when the target control level is set lower than 140 mg/dL. According to this analysis, roughly 140 mg/dL is considered to be medically and medico-economically appropriate as the target control level of LDL-C for primary prevention, at least on the basis of the results of the JLIT. In this case, the maximum preventive effect is 30-35% for myocardial infarction, which is in close agreement with the results of the MEGA study, the only large-scale interventional study of ischemic heart disease conducted in Japan using a statin[10].

Fig. 2B shows the decreases in the risk of ischemic heart disease associated with elevations of the HDL-C level in 3 epidemiological studies with prospective risk evaluation carried out in Japan including the JLIT[8, 11, 12]. While it is difficult to directly compare the incidences because the clinical definition of the endpoint varied among the studies, the peak decrease of the risk associated with increased HDL-C is less notable than that associated with the change of LDL-C in all studies. In other words, HDL-C-dependent decreases in the risk were observed even at HDL-C exceeding 60 mg/dL in all 3 studies. **Fig. 2C** shows the results of simulation similar to that of LDL-C performed using the results of the JLIT, which analyzed the therapeutic outcomes, on the basis of the HDL-C distribution curve in Japanese (**Fig. 2A**)[9] and the population composition. Since decreases in the risk associated with increases in HDL-C have not been directly demonstrated, the simulation was based on the hypothesis that increases in the risk associated with decreases in HDL can be reversed by increasing HDL-C. In contrast with the results concerning LDL, little decrease or peaking of the preventive efficacy associated with increased HDL-C was observed with an HDL-C level over 60 mg/dL. Reflecting this, the preventive effect against myocardial infarction could still be increased by raising the HDL-C level beyond 60 mg/dL. These results suggest that, under the hypothesis that the risk of myocardial infarction is reversibly reduced by elevating HDL-C, myocardial infarction can be prevented in 60-70% of the Japanese population at risk.

As far as these results are concerned, it can be concluded that the criterion of a "low HDL-C level" is unnecessary in guidelines for the control of HDL-C, and that the higher the HDL-C the better; however, according to the results in **Fig. 2A**, some studies have shown relatively large increases in the risk associated with decreases in HDL-C at about 50 mg/dL or below and, particularly, below 40 mg/dL; therefore, it may be reasonable to set a "caution level" around here. On the other hand, views on high HDL-C are divided. First, there is no epidemiological evidence indicating that higher HDL-C is better, even when it exceeds 60 mg/dL. This is probably because the population falling in this category is small (even though high HDL-C is relatively frequent in Japan) and cardiovascular incidence is low, making it difficult to obtain significant results.

In addition, the controversy is further complicated by the inclusion in this category of cases of homozygous CETP-deficient patients, in which elevations of HDL may not be considered to decrease the

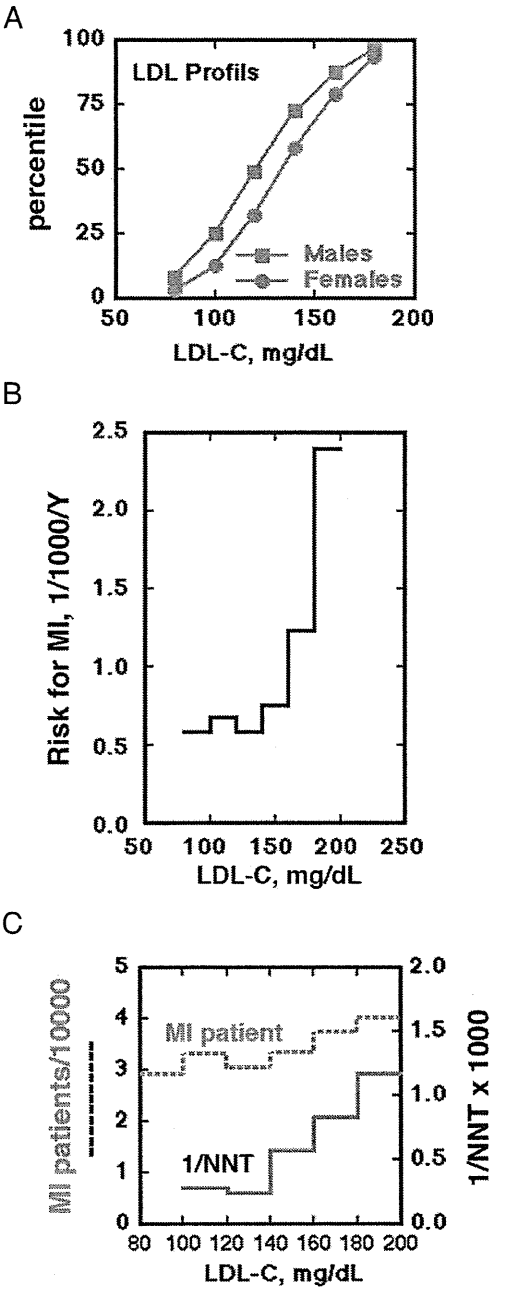

Fig. 1. Prevention of ischemic heart disease in Japanese by reducing LDL.

A: Distribution curve of the plasma LDL-C level in Japanese[9]. B: Relationship between the plasma LDL-C level and risk of "myocardial infarction" observed in the JLIT[8]. C: Simulation of the prevention of "myocardial infarction" based on Graphs A and B and demographic data for Japanese. Solid lines represent the inverse of NNT (·1,000) as an indicator of the treatment efficacy for managing lipoproteins to a target. The value of each horizontal segment is the efficacy when reaching a target LDL-C value at the left end of the segment in all Japanese at ages covered by the JLIT. Each horizontal segment of broken lines represents the number of MI patients when LDL is reduced to or lower than the level of the right end of the segment.

risk. The argument that increased HDL does not necessarily contribute to decreased risk is supported by the absence of a further decrease in the risk when the HDL-C increases above 70 mg/dL and the increased risk in patients with a homozygous CETP defect[13]; however, HDL-C is usually 80 mg/dL or higher and often reaches 100-200 mg/dL or even higher in patients with a homozygous CETP defect[13-16], and such high HDL-C should be considered separately from regular high HDL-C. Still, researchers are not in agreement concerning the increase in risk. In this sense, the differentiation of homozygous CETP deficiency is necessary in patients showing HDL-C exceeding 80 mg/dL, and there is no clinical or experimental evidence pointing to any conclusion about whether HDL-C should be maintained above this level. Nevertheless, the high prevalence of CETP deficiency among Japanese (1/20 for D442G and 1/100 for I14A) may have a limited but significant impact on the association between high HDL and atherogenesis in Japanese.

Proposal of Standards for Management of the HDL-C Level

On the basis of the above discussion, this article summarizes a proposal for the management of the HDL-C level as follows:

1) The evidence status is summarized as (1) A decrease in HDL-C is a strong risk factor for atherosclerotic disorders, particularly in Japan and, from the viewpoint of public health, it may be a more important risk factor than an increase in LDL-C; (2) While there are rational grounds for the argument that elevated HDL-C leads to a decreased risk, (3) there is as yet no direct evidence that elevating HDL-C is effective for the prevention and treatment of atherosclerotic disorders.

2) If elevations of HDL-C through interventional measures cause reversible decreases in the risk, this effect is expected, at least, up to 60 mg/dL or higher, and a simulation indicated that it eventually reduce the incidence of ischemic heart disease in Japan by 60-70%.

3) In risk management, high HDL-C is presently defined as 40 mg/dL or below. While there is no evidence that strongly urges a change in this definition, the results of epidemiological studies support "the higher the HDL-C level, the lower the risk," even in the "normal range" so that elevation of HDL-C may reduce the risk probably at least up to 70 mg/dL; however, there are no supportive data for this effect still being obtained over 80 mg/dL. Patients with a

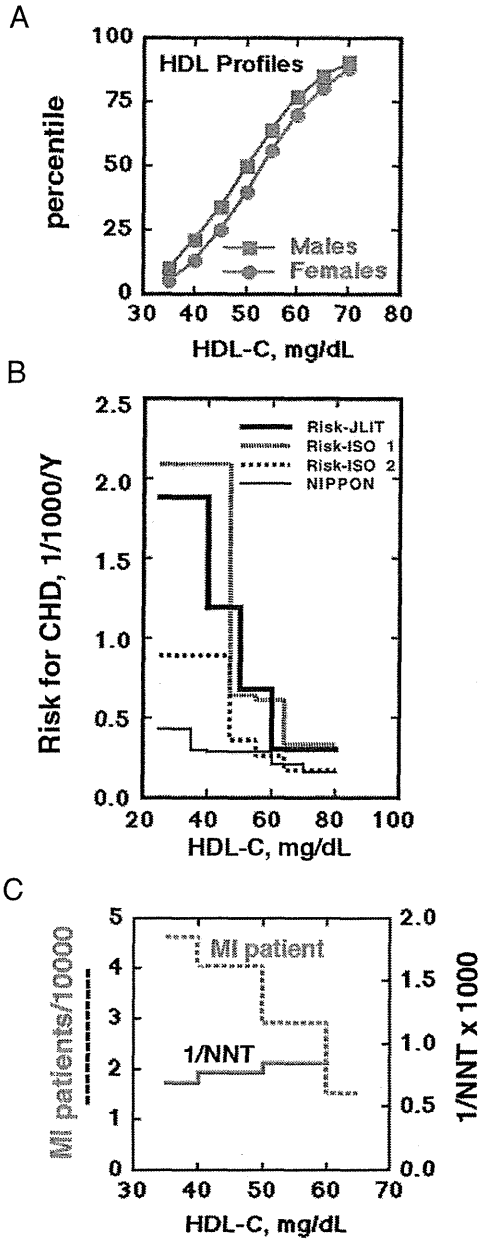

Fig. 2. Prevention of ischemic heart disease in Japanese by increasing HDL-C.

A: Distribution curve of the plasma HDL-C level in Japanese[9]. B: Relationship between the plasma HDL-CH level and risk of ischemic heart disease in Japanese. "Myocardial infarction" in the JLIT[8], "coronary artery disease" and "definitive diagnosis of myocardial infarction" by Kitamura, Iso, *et al.*[11], and "deaths due to cardiovascular diseases" according to NIPPON DATA[12]. C: Simulation for prevention of "myocardial infarction" based on Graphs A and B and demographic data of Japanese. Solid lines represent the inverse of NNT (x 1000) as an indicator of the treatment efficacy for managing lipoproteins to a target. The value of each horizontal segment is the efficacy when reaching a target HDL level at the right end of the horizontal segment in all Japanese at ages covered by the JLIT. Each horizontal segment of broken lines represents the number of MI patients when HDL is raised to the left end of the segment.

homozygous CETP deficiency should be followed-up while controlling other risk factors, not to dismiss the possibility of the risk increase with an extremely elevated HDL-C level. A gender-dependent strategy for HDL-C management should be discussed when further epidemiological and clinical evidence becomes available.

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ORIGINAL ARTICLE: EPIDEMIOLOGY, CLINICAL PRACTICE AND HEALTH

Importance of high-density lipoprotein cholesterol levels in elderly diabetic individuals with type IIb dyslipidemia: A 2-year survey of cardiovascular events

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Aim: The risk factors for ischemic heart disease (IHD) or cerebrovascular accident (CVA) in elderly diabetic individuals with type IIb dyslipidemia are not fully known. Therefore, we investigated the relationship between lipid levels and IHD and CVA in diabetic individuals with type IIb dyslipidemia.

Method: The Japan Cholesterol and Diabetes Mellitus Study is a prospective cohort study of 4014 type 2 diabetic patients (1936 women; age 67.4 ± 9.5 years). The primary end-points were the onset of IHD or CVA. Lipid and glucose levels, and other factors were investigated in relation to the occurrence of IHD or CVA. A total of 462 participants were included in the group of patients with type IIb dyslipidemia.

Results: The 462 diabetic participants with type IIb dyslipidemia were divided into those who were aged <65 years, 65–74 years and >75 years (n = 168, 190 and 104, respectively). High-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol/HDL-C were significantly associated with the risk of cardiovascular events in diabetic individuals with type IIb dyslipidemia who were aged <65 years, and HDL-C and diastolic blood pressure was significantly associated with cardiovascular events in patients aged 65–74 years. Non-HDL-C was not significantly associated with the risk of cardiovascular events. Multiple regression analysis showed that lower HDL-C was significantly associated with the risk of cardiovascular events in diabetic individuals with type IIb dyslipidemia who were aged <65 years and 65–74 years.

Conclusions: Lower HDL-C was an important risk factor for cardiovascular events in diabetic individuals with type IIb dyslipidemia who were aged <75 years. **Geriatr Gerontol Int 2013; ••: ••–••.**

Keywords: cerebrovascular accident, elderly type 2 diabetes, high-density lipoprotein cholesterol, ischemic heart disease, type IIb dyslipidemia.

Introduction

Investigators in Western countries have reported that patients with both hypercholesterolemia and type 2 diabetes have a higher risk of coronary events than patients with hypercholesterolemia alone.[1] The incidence of ischemic heart diseases (IHD) and cerebrovascular

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accident (CVA) in patients with type 2 diabetes is reported to be high in Japan.[2] However, risk factors for IHD or CVA in elderly diabetic individuals with hypercholesterolemia are not fully known.

Many lines of evidence show that low-density lipoprotein cholesterol (LDL-C) is an important risk factor for cardiovascular disease (CVD; CVD = IHD + CVA),[3,4] but it is still debatable whether plasma triglyceride (TG) levels are associated with the occurrence of CVD. However, recent reports have shown that plasma TG levels are an independent risk factor for coronary artery disease (CAD).[3,5–8] In addition, non-fasting TG levels have been shown to be associated with CAD and stroke.[3,9,10] Despite the accumulating evidence against LDL-C and TG, few reports have addressed the effect of type IIb dyslipidemia on cardiovascular disease. We considered the fact that elevated LDL-C and TG along with an increase in atherogenic lipoproteins, such as small and dense LDL, are found in type IIb dyslipidemia, and that this type of dyslipidemia is often associated with type 2 diabetes. It is important to note that when investigating diabetic individuals with type IIb dyslipidemia, there is a synergistic effect of type 2 diabetes and dyslipidemia. This effect might pose a larger risk factor for CVD, but few reports have addressed this association.

Few data were available for the elderly diabetic individuals with type IIb dyslipidemia.[3] Therefore, it is worthwhile to analyze the data from the Japan Cholesterol and Diabetes Mellitus investigation (Japan-CDM), which is a nationwide observational cohort study of a large number of diabetic individuals who were treated in clinical practice. It was designed to assess the relationship between lipid levels and the incidence of CVD in Japanese diabetic individuals.[11,12] We investigated the relationship between lipid levels, IHD and CVA in diabetic individuals with type IIb dyslipidemia in the present study.

Methods

Data source

The Japan Cholesterol and Diabetes Mellitus Study is a single-center prospective cohort study comprising 4014 Japanese diabetic individuals on a consecutive outpatient basis who were recruited between September 2004 and March 2005 (1936 women; age 67.4 ± 9.5 years [range 35–83 years]) from 40 Japanese hospitals. Patients with coronary artery disease, which was defined as previous myocardial infarction, coronary intervention or confirmed angina pectoris and recent stroke, who had been admitted within the past 24 months were excluded. Follow-up information was available for 98.2% and 92.3% of patients enrolled in the first and second years, respectively. Patients were divided into those who were aged <65 years, 65–74 years and <75 years (n = 1267, 1731 and 1016, respectively). The primary end-points were onset of IHD or CVA. Plasma lipid, glucose, glycated hemoglobin (National Glycohemoglobin Standardization Program) and other relevant levels were measured annually. Lipid and glucose levels, and other factors were investigated in relation to occurrence of IHD or CVA.[11,12]

From this study, we investigated patients with type IIb dyslipidemia. Patients with type IIb dyslipidemia were defined by having both TG ≥150 and LDL-C ≥120. A total of 462 participants were included in the patient group showing type IIb dyslipidemia. The study was approved by institutional review boards and by the safety monitoring board. All events were confirmed annually by the organizing committee. The guidelines of the Japan Atherosclerosis Society (2002), stating that LDL-C should be <120 mg/dL and high-density lipoprotein cholesterol (HDL-C) >40 mg/dL in diabetic individuals, and the American Diabetes Association criteria for diagnosis of type 2 diabetes were used.

Statistical analysis

Results are presented as means ± SD. All statistical analyses were carried out using JMP software (SAS Institute, Cary, NC, USA). Incidences were analyzed in relation to risk factors. Univariate and multiple logistic regression analysis were used. We included both SBP and DBP in the same multivariable model, because systolic hypertension is very often observed in the elderly, and those variables did not show a strong correlation in the present study (r = 0.48). Values of $P < 0.05$ were considered significant.

Results

The characteristics of the 462 participants are shown in Table 1. The mean age was 67.4 ± 9.5 years, and 52.2% of participants used antihyperlipidemic agents. The 462 participants with type IIb dyslipidemia were divided into those who were aged <65 years, 65–74 years and <75 years (n = 168, 190 and 104, respectively). IHD and CVA occurred in 1.6 and 1.4% of participants, respectively, over a 2-year study period. The occurrence of IHD and CVA in participants with type IIb dyslipidemia was 2.4 and 1.7%, respectively. Participants with type IIb dyslipidemia made up a higher proportion of occurrence of cardiovascular events (Fig. 1). The relationship between IHD or CVA and background factors, such as LDL-C levels, in each age-group was analyzed by univariate logistic regression. Lower HDL-C was significantly associated with a risk of cardiovascular events in diabetic individuals with type IIb dyslipidemia aged <65 years and 65–74 years (Fig. 2a). Higher diastolic blood

Table 1 Clinical background of diabetic patients with type IIb dyslipidemia

	Total	<65 years (n = 168)	65–74 years (n = 190)	≥75 years (n = 104)
Age	66.4 ± 10.6	54.8 ± 7.2	70.0 ± 2.77	78.6 ± 3.18
Sex ratio (m/f)	0.99	1.46	0.87	0.66
SBP (mmHg)	136.9 ± 18.3	133.8 ± 17.6	138.0 ± 18.4	139.9 ± 18.8
DBP (mmHg)	75.7 ± 11.3	78.5 ± 10.5	74.3 ± 11.8	73.5 ± 10.8
LDL (mg/dl)	145.6 ± 24.1	150.1 ± 26.1	145.1 ± 24.8	139.0 ± 16.7
HDL (mg/dl)	46.8 ± 11.6	47.6 ± 11.2	46.8 ± 12.8	45.7 ± 10.2
LDL-C/HDL-C	3.4 ± 2.4	3.4 ± 1.8	3.6 ± 3.3	3.2 ± 0.9
Non-HDL-C (mg/dL)	187.1 ± 29.8	194.0 ± 31.1	185.6 ± 31.6	178.7 ± 20.5
TG (mg/dL)	211.8 ± 65.1	227.5 ± 79.5	204.4 ± 55.1	199.6 ± 49.9
HbA1c (%)	7.86 ± 1.38	7.96 ± 1.54	7.74 ± 1.29	7.91 ± 1.25
Antihyperlipidemic agents (%)	(52.2)	(60.1)	(51.6)	(40.4)

DBP, diastolic blood pressure; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TG, triglycerides.

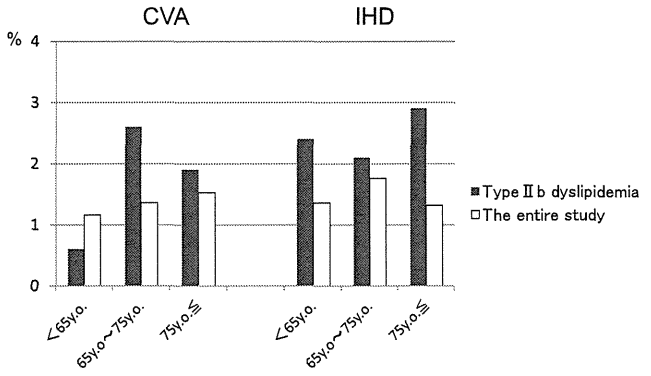

Figure 1 A 2-year survey of cardiovascular events in each generation's type IIb dyslipidemia and of the entire study. CVA, cerebrovascular accident; IHD, ischemic heart disease.

pressure (DBP) was significantly associated with the risk of cardiovascular events in diabetic individuals with type IIb dyslipidemia who were aged 65–74 years, and LDL-C/HDL-C was significantly associated with individuals who were aged <65 years (Fig. 2a,b). Non-HDL-C was not significantly associated with the risk of cardiovascular events. We carried out multiple regression analysis. The data shown were after adjustment for age, sex, systolic blood pressure (SBP), DBP, glycated hemoglobin, plasma lipid levels and antihyperlipidemic agents. With regard to LDL-C/HDL-C, the data obtained were after adjustments for the same factors except for lipid levels. With regard to non-HDL-C, the data obtained were after adjustment for the same factors, but lipid factor is only TG. We investigated three age groups. Lower HDL-C was associated with the risk of cardiovascular events in patients who were aged <65 years and 65–74 years (Table 2).

Discussion

Type IIb dyslipidemia is important, because it sometimes accompanies atherogenic lipid profiles, such as small dense LDL, remnant lipoprotein and low HDL cholesterolemia. It is also associated with type 2 diabetes mellitus, metabolic syndrome and chronic kidney disease (CKD), and most patients with familial combined hyperlipidemia (FCHL) show this phenotype.[3,13–16] Therefore, it is necessary to understand that patients with type IIb dyslipidemia have a high risk for CVD. The management of type IIb dyslipidemia is key to the prevention of CVD.[3] Therefore, we assessed the relationship between lipid levels and IHD, and CVA in diabetic individuals with type IIb dyslipidemia.

The present study showed that lower HDL-C was an important risk factor for cardiovascular events in diabetic individuals with type IIb dyslipidemia who were aged <75 years. Multiple regression analysis showed lower HDL-C levels were associated with the risk of cardiovascular events in patients who were aged <75 years. We could not show the significant association between HDL-C levels and each event. One of the reasons for this inability was the small number of type IIb patients, and the relatively short duration of observation for participants with type IIb dyslipidemia. We showed there was a significant association between HDL-C levels and total events (IHD + CVA). We could not show the significant association between non-HDL-C levels or LDL-C/HDL-C and each event by multiple regression analysis. We speculate that HDL-C was the most important risk factor for cardiovascular events in diabetic individuals with type IIb dyslipidemia. Other studies including Japanese patients with type 2 diabetes (mean age 58.2 years) showed serum TG levels were a leading predictor of coronary

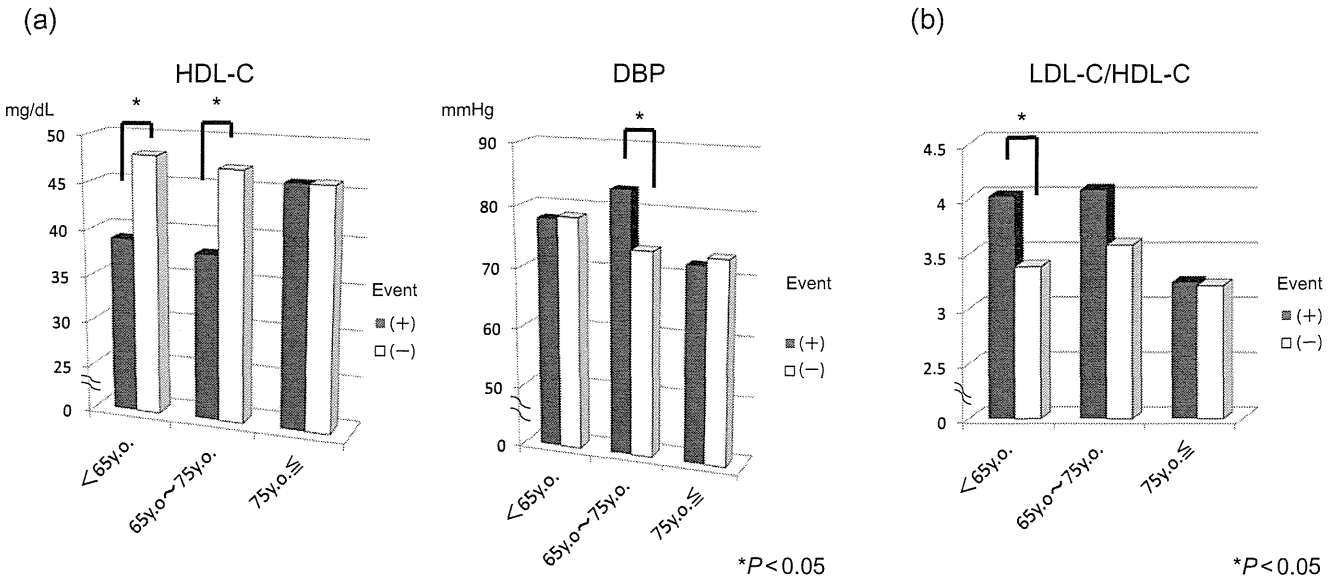

Figure 2 (a) The relationship between high-density lipoprotein (HDL-C) and diastolic blood pressure (DBP) levels, and the occurrence of events. (b) The relationship between low-density lipoprotein cholesterol (LDL-C)/HDL-C and the occurrence of events.

Table 2 Adjusted multiple regression analyses of factors found to be significant by univariate regression analysis for cardiovascular disease, as well as major atherogenic risk factors

	<65 years (n = 168) Adjusted OR (95%CI)	P	65–74 years (n = 190) Adjusted OR (95%CI)	P	≥75 years (n = 104) Adjusted OR (95%CI)	P
Age	1.117 (0.98–1.33)	0.14	0.818 (0.57–1.13)	0.24	1.068 (0.81–1.39)	0.62
LDL-C	0.980 (0.93–1.02)	0.38	1.000 (0.95–1.03)	0.97	1.036 (0.98–1.09)	0.17
HDL-C	0.910 (0.82–0.99)	0.04*	0.900 (0.81–0.98)	0.03*	1.000 (0.92–1.09)	0.99
TG	0.996 (0.98–1.01)	0.62	1.000 (0.98–1.01)	0.94	0.996 (0.97–1.01)	0.68
HbA1c	1.030 (0.56–1.80)	0.92	0.921 (0.38–1.96)	0.84	0.803 (0.37–1.50)	0.52
SBP	1.027 (0.96–1.10)	0.45	1.008 (0.95–1.07)	0.77	0.980 (0.94–1.02)	0.33
DBP	0.992 (0.89–1.10)	0.87	1.065 (0.98–1.17)	0.14	0.975 (0.90–1.06)	0.55
LDL-C/HDL-C	1.120 (0.57–1.57)	0.57	1.161 (0.85–1.39)	0.12	1.173 (0.41–2.68)	0.72
Non-HDL-C	0.988 (0.96–1.01)	0.43	1.002 (0.96–1.03)	0.89	1.016 (0.97–1.06)	0.45

*P < 0.05. DBP, diastolic blood pressure; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; TG, triglycerides.

heart disease (CHD), comparable with LDL-C or HDL-C.[17] However, our former study including all participants showed the importance of HDL-C in CVA in elderly diabetic individuals, and in IHD in middle-aged diabetic individuals.[12] In our previous study, lower HDL-C was significantly related to CVA in participants aged ≥65 years, and especially in those aged >75 years.[12] The Prospective Study of Pravastatin in the Elderly at Risk study showed that simple LDL-C control might not prevent IHD or CVA in elderly individuals.[18] Our former study showed the importance of HDL cholesterol in CVA in elderly diabetic individuals.[12]

In addition to LDL-C, HDL-C is also a key risk factor in elderly diabetic individuals.[11,12] Because diabetic individuals with type IIb dyslipidemia have a higher risk for CVD, the importance of HDL-C might be different than that of usual diabetic individuals. Therapeutic lifestyle changes, including those to diet and exercise, constitute the cornerstone of management in patients with type IIb dyslipidemia. Restriction of dietary cholesterol (less than 300 mg/day) and saturated fat in addition to increasing dietary fiber and plant sterols can lower LDL-C, and restriction of alcohol, sugar, saturated fat and high intake of omega-3 fatty acids can reduce serum TG.[3,19] Because weight reduction can further lower LDL-C and TG, and raise HDL-C levels, maximal improvement in dyslipidemia should be attempted with lifestyle intervention before prescribing lipid-lowering medications.

Risk factors for cardiovascular events appear to change with advancing age.[12] The importance of HDL-C is different for each age-group. The present study on diabetic individuals with type IIb dyslipidemia was small in size, so a larger study will be required. However, HDL-C might help prevent cardiovascular events diabetic patients with type IIb dyslipidemia who are aged <75 years.

With regard to antihypertensive agents, approximately half of the participants used antihypertensive agents. There were no significant relationships between CVD and antihypertensive agents. Although we did not focus on antihypertensive agents in the present study, investigation of antihypertensive agents is important, and further study will be required in the future.

In conclusion, the present study showed that lower HDL-C was an important risk factor for cardiovascular events in diabetic individuals with type IIb dyslipidemia who are aged <75 years. If HDL-C is well controlled in elderly diabetic individuals who are aged <75 years with type IIb dyslipidemia, then IHD and CVA might be decreased to the levels found in diabetic patients of middle-aged cohorts.

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Disclosure statement

All authors have no conflict of interests.

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Original Article

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Plasma Activity of Endothelial Lipase Impacts High-Density Lipoprotein Metabolism and Coronary Risk Factors in Humans

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***Aim*:** Endothelial lipase (EL) is a determinant of plasma levels of high-density lipoprotein cholesterol (HDL-C). However, little is known about the impact of EL activity on plasma lipid profile. We aimed to establish a new method to evaluate EL-specific phospholipase activity in humans.
***Methods*:** Plasma samples were obtained from 115 patients with coronary artery disease (CAD) and 154 patients without CAD. Plasma EL protein was immunoprecipitated using an anti-EL monoclonal antibody after plasma non-specific immunoglobulins were removed by incubation with ProteinA. The phospholipase activity of the immunoprecipitated samples was measured using a fluorogenic phospholipase substrate, Bis-BODIPY FL C_{11}-PC.
***Results*:** The EL-specific phospholipase assay revealed that plasma EL activity was inversely correlated with HDL-C levels ($R = -0.3088$, $p < 0.0001$). In addition, the EL activity was associated with cigarette smoking. Furthermore, EL activity in CAD patients was significantly higher than that in non-CAD patients. Concomitantly, the HDL-C level in CAD patients were significantly lower than that in non-CAD patients.
***Conclusion*:** We have established a method for human plasma EL-specific phospholipase activity by combination of EL immunoprecipitation and a fluorogenic phospholipid substrate. Plasma EL activity was associated with not only plasma HDL-C levels but also the risks for CAD.

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Key words: Endothelial lipase, High-density lipoprotein, Cholesterol, Coronary artery disease, Phospholipase

Introduction

Low plasma levels of high-density lipoprotein cholesterol (HDL-C) are associated with the risk of coronary artery disease (CAD)[1]. This relationship is independent of the effects of therapy in lowering the low-density lipoprotein cholesterol (LDL-C) level[1-3]. Therefore, raising the HDL-C level has emerged as a key strategy for reducing the residual CAD risk in individuals optimally treated for elevated LDL-C[3].

Endothelial lipase (EL) is a member of the triglyceride lipase family that exhibits a substantial phospholipase A1 activity[4-6]. EL shows high substrate specificity to HDL and hydrolyzes phospholipids on HDL particles[7]. As a result, EL promotes the catabolism and remodeling of HDL particles and has a major influence on HDL metabolism, both in humans and mice[5-10]. Moreover, the EL concentrations are increased in patients with metabolic syndrome and inflammation and are associated with the development of coronary atherosclerosis[10-12]. On the other hand,

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statins increase the HDL-C levels partly by reducing the EL mass via the inhibition of RhoA[9]. Because the inhibition of EL results in an increase in HDL particles with anti-inflammatory properties[13], EL is considered to be an attractive molecular target in HDL-C-raising pharmacological therapy.

Cell culture and animal studies suggest that the alteration of the EL expression is proportionally correlated with that of the EL activity[14]. However, previous studies have also reported that the EL activity is regulated by a variety of factors[15-19]. Moreover, it has been postulated that endogenous EL inhibitor(s) exist in human plasma[20, 21]. Based on this line of evidence, more detailed investigations are required regarding the measurement of EL-specific enzymatic activities in human plasma. There are multiple enzymes with lipase activity in the plasma, and it is difficult to discriminate the enzymatic activity of EL from that of other lipases. In the present study, we therefore aimed to establish a method to measure the EL-specific phospholipase activity in the plasma and investigate the correlations between the plasma EL activity and lipid profiles in human subjects. We herein documented associations between the EL activity and the plasma HDL-C level as well as various cardiovascular risk factors.

Materials and Methods

Preparation of the Plasma Samples

A total of 269 Japanese patients with cardiovascular diseases (191 men, age range: 22-88 (mean 64 ± 13) years) admitted to Kobe University Hospital between April 2008 and August 2009 were eligible for this study. Among them, 115 patients with coronary lesions exhibiting ≥ 75% angiographically narrowing of the coronary luminal diameter who underwent coronary intervention within the past six months were categorized as CAD patients. In addition, 154 patients with arrhythmias, valvular disease, non-ischemic cardiomyopathy, pulmonary hypertension or non-ischemic heart failure were categorized as non-CAD patients. The patient characteristics are shown in **Table 1**. Hypertension was diagnosed in patients with a systolic blood pressure of >140 mmHg or a diastolic blood pressure of >90 mmHg and in those treated with antihypertensive drugs. Diabetes mellitus was diagnosed in patients with a fasting serum glucose level of >126 mg/dL or a hemoglobin A1c value of >6.5% (NGSP), according to the clinical guidelines of the Japan Diabetes Society. A diagnosis of diabetes was also recorded in patients treated with antidiabetic drugs. Dyslipidemia was diagnosed in patients with a high serum LDL-C concentration, according to the Japan Atherosclerosis Society Guidelines for the Prevention of Atherosclerotic Cardiovascular Diseases. A diagnosis of dyslipidemia was also recorded in patients treated with antihyperlipidemic drugs. Patients with renal failure (i.e. a serum creatinine level of >2.0 mg/dL), cancer, active inflammatory disease (a C-reactive protein level of >1.0 mg/dL) or emergent admission were excluded. All patients provided their written informed consent, and the clinical study was approved by the Institutional Review Board of Kobe University Graduate School of Medicine. The investigation conformed to the principles outlined in the Declaration of Helsinki.

Table 1. Characteristics of the CAD and non-CAD patients

Variable	Non-CAD (n=154)	CAD (n=115)
Male, n (%)	99 (64.3)	91 (79.1)
Age (years)	60.8 ± 14.3	67.6 ± 9.8*
Body mass index (kg/m^2)	23.5 ± 3.6	24.6 ± 2.9
Hypertension, n (%)	61 (39.6)	89 (77.0)*
Diabetes mellitus, n (%)	25(16.2)	53 (46.0)*
Dyslipidemia, n (%)	56 (36.3)	88 (76.5)*
Current alcohol consumption, n (%)	55 (35.7)	51 (44.3)
Smoking status		
Never smoked, n (%)	64 (41.5)	39 (33.9)*
Former smoker, n (%)	31 (20.1)	54 (47.0)*
Current smoker, n (%)	48 (31.1)	22 (19.1)*
Statin	24 (15.6)	71 (61.7)*
Fibrate	1 (0.6)	2 (1.7)

The values are expressed as the mean ± SD or frequencies (%). *p <0.05 vs. non-CAD. Former smokers had not smoked for ≥ 1 year. CAD, coronary artery disease.

Blood was obtained after an overnight fast without the administration of heparin. The plasma levels of total cholesterol (Tcho), triglycerides (TG), HDL-C, glucose and hemoglobin A1c were measured using a standard assay at the Clinical Laboratory of Kobe University Hospital. The LDL-C level was calculated using the Friedewald formula. The homeostasis model assessment insulin resistance index (HOMA-IR) was calculated as fasting plasma glucose × immunoreactive insulin/405, after excluding patients with a fasting plasma glucose level of >126 mg/dL and/or those treated for diabetes. The plasma levels of interleukin-6 and adiponectin were measured using a latex particle-enhanced turbidimetric immunoassay and a chemiluminescent enzyme immunoassay, respectively, at SRL, Inc. (Hachioji, Tokyo, Japan).

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Preparation of Recombinant Human EL Protein

Recombinant human EL protein (rhEL) was purified from COS7 cells that stably overexpress c-myc epitope tagged-hEL (hEL-COS7)[4]. The hEL-COS7 of 90% confluence was incubated with Production Medium (DMEM without phenol red, 1x glutamine, 1x pyruvate and 2 units/ml of heparin) for 24 hours, after which the culture medium was collected and centrifuged using a Vivaspan 20 (Sartorius Stedim Biotech, Aubagne, France) to concentrate the solution by ~30 times. Glycerol was added to make the final concentration 15%, and the solution was kept at −80℃ until use. The rhEL concentration was determined by comparing the sample with bovine serum albumin (BSA) as an indicator after electrophoresis. rhEL was used as a working standard for the EL activity assay.

Plasma IgG Removal and Immunoprecipitation

To measure the EL-specific phospholipase activity, we immunoprecipitated EL proteins in the plasma with an anti-EL antibody and Protein A. However, because Protein A binds nonspecifically to the heavy chain domain of IgG[22], it was expected that Protein A may bind not only to the sample of EL-immunoprecipitation (EL-IP), but also native IgG in the plasma. Therefore, plasma native IgG was pulled-down using Protein A, prior to EL-IP. In the pilot experiment, we confirmed with SDS-PAGE that plasma IgG was completely removed by pretreatment with 80 μL of nProtein A, when less than 20 μL of plasma was used (*data not shown*). Therefore, 20 μL of plasma was found to be optimal and chosen for the EL activity assay. Briefly, the 20-μL plasma samples were mixed with 2 μL of 10 mmol/L phenylmethanesulfonylfluoride and 80 μL of nProtein A Sepharose 4 Fast Flow (GE Healthcare, Sweden) that was washed three times with 1×IP buffer (20 mmol/L of Tris-HCl, pH 7.5, 50 mmol/L of NaCl). After one hour rotating at room temperature, 400 μL of 1×IP buffer was added, and the sample was incubated for four hours at 4℃ rotating on a rotator. The samples were then centrifuged, and 300 μL of the supernatant was obtained as a conditioned plasma sample.

EL-IP was performed using a monoclonal IgG antibody against human full-length EL proteins raised in mice (clone 26A1, Immuno-Biological Laboratories, Fujioka, Gunma, Japan) that reacts with the amino terminus of EL proteins[12]. 26A1 mouse IgG (10 μL, 1 μg) was crosslinked onto 40 μL of nProtein A and incubated overnight with the 300-μL conditioned plasma samples to immunoprecipitate EL. After several washes, the EL-IP samples were subjected to measurement of the EL activity.

EL Activity Measurement

The EL-IP samples (90 μL/well) were transferred to a 96-well plate (Nunc A/S, Roskilde, Denmark). The reaction was started by adding 10 μl/well of a fluorogenic crude phospholipase A substrate, Bis-BODIPY FL C$_{11}$-PC (B7701, Invitrogen, Carlsbad, CA) to a final working concentration of 0.02 mmol/L[23]. After 10 minutes of pre-incubation at 37℃, the fluorescence intensity (FLI) was continuously monitored using the Fluoroskan Ascent FL (Thermo LabSystems, Cambridge, UK) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm for 21 minutes at 37℃. The data were analyzed and expressed as FLI (arbitrary unit)/min/mL. In each assay, a standard curve was constructed, and the EL activity was calculated and expressed as fluorescence units (see details in the Results section). The intra-assay CV and inter-assay CV for the phospholipase assay were 4.0% and 19.3%, respectively.

Statistical Analysis

The one-way ANOVA was used to compare continuous variables between groups, and the Chi-square test was used to compare categorical values between groups. Relationships between the EL activity and the levels of serum lipids, lipoproteins and other variables were examined using Pearson correlation coefficients. All statistical analyses were performed using the Stata 11.2 software package (Stata, Texas, USA). A value of $P<0.05$ was considered to be statistically significant.

Results

Validation of the EL Activity Assay

Fig. 1A shows the relationship between the level and activity of rhEL standard protein according to the FLI. The EL phospholipase activity was time- and dose-dependent; therefore, the EL activity was calculated as (FLI at 21 min - FLI at 1 min)/20 min and expressed as FLI/min. We created a standard curve using the protein dose (**Fig. 1B**) in each assay and defined the activity of 1 μg of rhEL standard protein as one Unit. The EL activity in the samples was calculated and expressed in Units according to the standard curve. When the activity of different volumes of the rhEL standard protein was assessed before and after IP, the EL activity exhibited a linear relationship with fluorescence consistently, both before and after IP (**Fig. 1C**), which indicates that all EL proteins were successfully pulled down by IP and the EL activity was not affected during the procedure.

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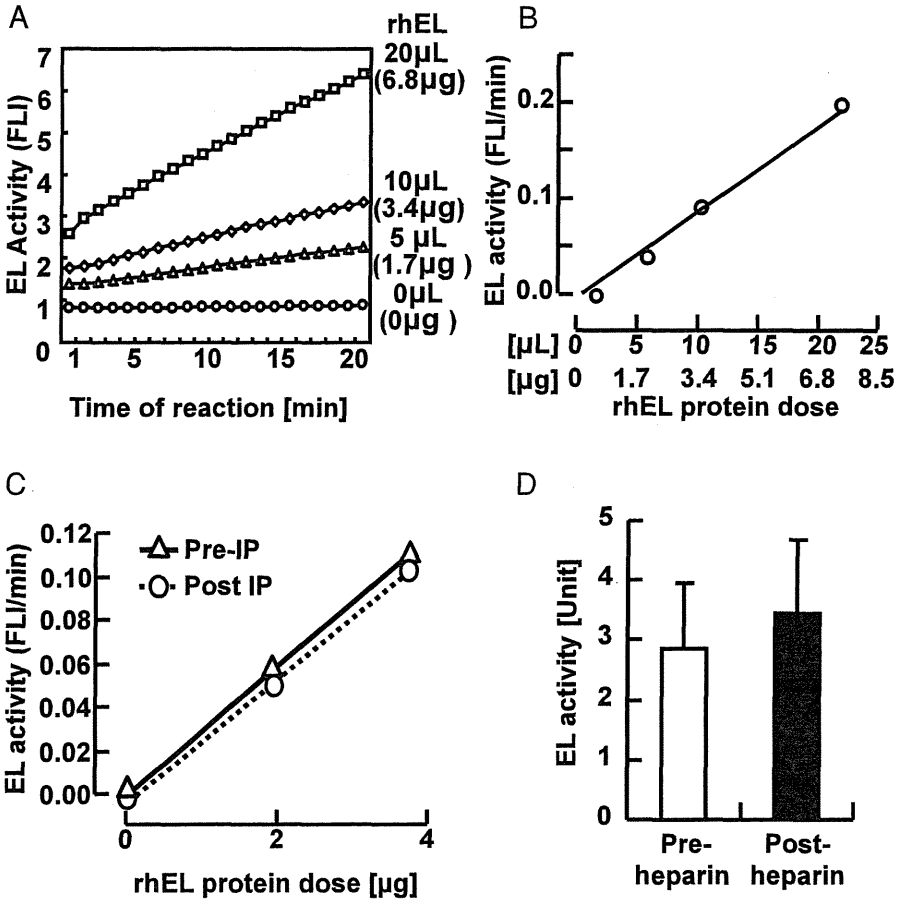

Fig. 1. Validation of the EL activity assay following EL immunoprecipitation

(A) The time- and dose-dependent EL activity of recombinant human EL protein (rhEL) is shown as FLI (fluorescence intensity). Therefore, the EL activity was calculated as (FLI at 21 min - FLI at 1 min)/20 min and expressed as FLI/min in the subsequent analyses.
(B) A representative standard curve of the EL activity (FLI/min) based on the rhEL dose.
(C) The EL activity levels (FLI/min) of indicated rhEL doses showed a linear relationship both before and after the immunoprecipitation (IP) procedure.
(D) There were no significant differences in the EL activity between the pre- and post-heparin treatment plasma samples. One "Unit" represents the phospholipase activity equivalent to 1 μg of standard rhEL, which was determined using the standard curve. The data are presented as the mean ± SE.

Although a previous study reported that the plasma EL mass increases in response to heparin[10], no significant differences in the EL activity were observed between the pre- and post-heparin treatment plasma values (**Fig. 1D**). Therefore, in this study, the human plasma was collected without heparin treatment.

Associations between the EL Activity and the Plasma Lipid Profiles and Coronary Risk Factors

We measured the plasma EL activity in 269 patients with cardiovascular disease. As shown in **Table 2**, there were no significant associations between the EL activity and non-lipid parameters, such as age,

Table 2. Associations between the EL activity and non-lipid cardiovascular risk factors

Variable	R	*p*-value
Age	−0.0252	0.6802
Body mass index	−0.0261	0.6966
Waist circumference	−0.0190	0.7840
Systolic blood pressure	0.0066	0.9172
Diastolic blood pressure	0.0382	0.5485
Fasting glucose	0.0463	0.4510
HOMA-IR	−0.0426	0.4884
Interleukin-6	0.0293	0.6332
Adioponectin	0.0589	0.3374

HOMA-IR, homeostasis model assessment index-insulin resistance.

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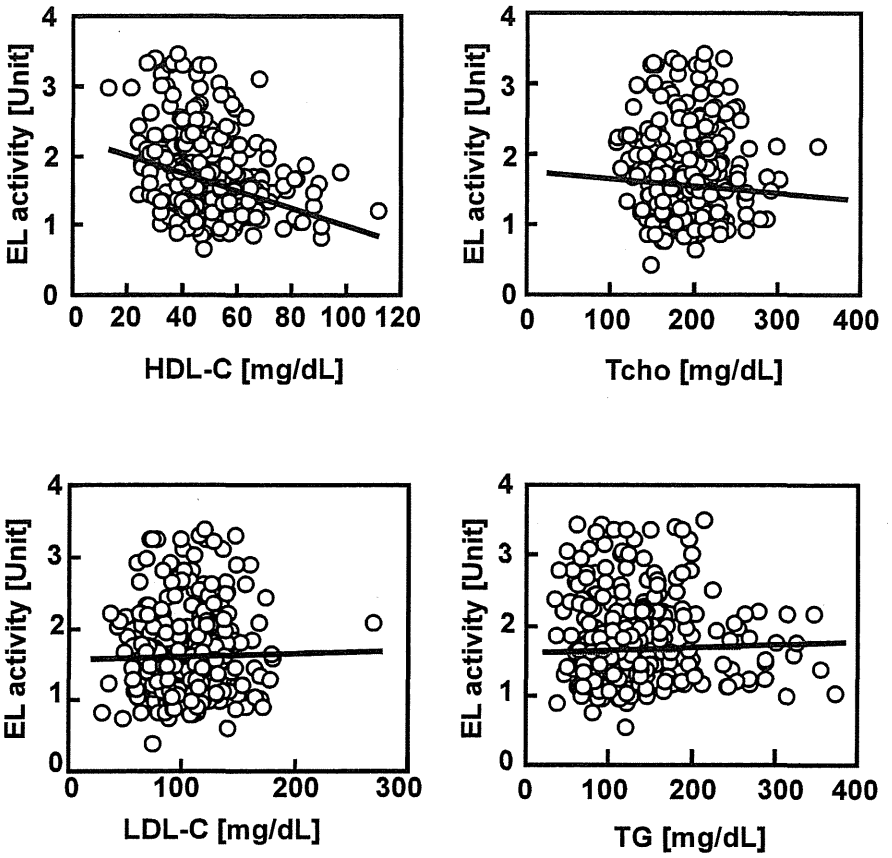

Fig. 2. Plasma EL activity and lipid profiles

The pre-heparin plasma EL-specific activity levels were determined in 269 patients with cardiovascular disease. The relationships between the EL activity and the plasma levels of HDL-cholesterol (HDL-C), total-cholesterol (Tcho), LDL-cholesterol (LDL-C) and triglycerides (TG) are shown. There was an inverse relationship between the EL activity and the HDL-C level ($R=-0.3088$, $P<0.00001$).

body mass index, waist circumference, blood pressure and the glucose, interleukin-6 and adiponectin levels. The associations between the plasma EL activity and lipid profiles are shown in **Fig. 2**. The plasma EL activity was inversely correlated with the HDL-C level ($R=-0.3088$, $p<0.0001$); however, no relationships were observed with the Tcho ($R=-0.0613$, $p=0.3195$), LDL-C ($R=0.0207$, $p=0.7371$) or TG levels ($R=0.0480$, $p=0.4523$).

Associations between the EL Activity and Coronary Risk Factors

Because habitual cigarette smoking is known to be a cause of a low HDL-C level, we analyzed the relationship between the plasma EL activity and cigarette smoking. As shown in **Fig. 3A**, the EL activity levels in the patients who did not smoke were significantly lower than those observed in the former or current smokers, and the EL activity levels in the former smokers were higher than those observed in the current smokers. Interestingly, the plasma HDL-C levels in the non-smokers were significantly higher than those observed in the former or current smokers (**Fig. 3A**). Next, we compared the EL activity levels in the CAD- and non-CAD patients. As shown in **Table 1**, the CAD patients were older and had a higher prevalence of hypertension, diabetes, dyslipidemia and statin use than the non-CAD patients. Although the CAD patients included subjects with habitual cigarette smoking habits (i.e., former and current smokers), many were not current smokers (**Table 1**). Reflecting the associations between the EL activity and the risk factors, the plasma EL activity levels were modestly but significantly higher in the CAD patients than in the non-CAD patients (**Fig. 3B**). Moreover, the HDL-C levels in the CAD patients were 15%

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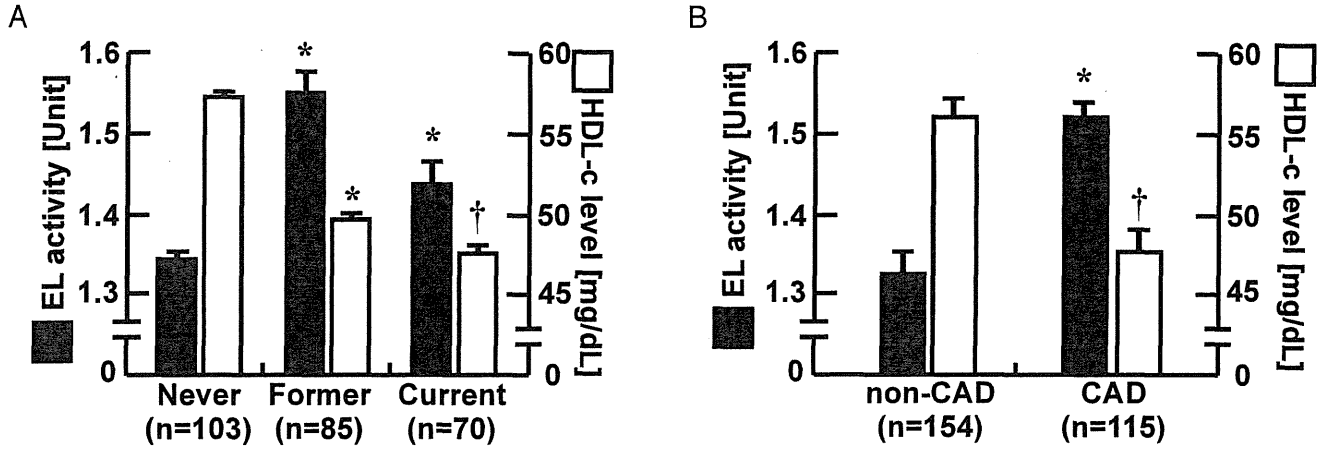

Fig. 3. *Plasma EL activity and cardiovascular risks*

(A) The plasma EL activity levels were higher and the HDL-C levels were lower in the former and current smokers than in the never-smokers. The data are presented as the mean ± SE. $*p<0.05$, $\dagger p<0.01$ vs. the corresponding never-smokers.
(B) The EL activity levels were higher and the HDL-C levels were lower in the coronary artery disease (CAD) patients than in the non-CAD patients. The data are presented as the mean ± SE. $*p<0.05$, $\dagger p<0.01$ vs. the corresponding non-CAD value.

lower than those observed in the non-CAD patients (**Fig. 3B**). These findings indicate that the EL activity is correlated with the risk of CAD.

Discussion

Since the identification of EL in 1999, the role of the plasma EL activity in lipoprotein metabolism has remained incompletely understood. Recently, Miksztowicz and colleagues measured the HL phospholipase activity in the presence of 1 mol/L of NaCl to inhibit the EL activity and calculated the EL activity as the difference between the total- and HL-specific phospholipase activities[24]. In addition, they reported that the EL activity regulates the plasma HDL-C concentration in patients undergoing hemodialysis. The present study successfully established a direct method for assessing the plasma human EL-specific phospholipase activity using a combination of IP and a fluorogenic phospholipid substrate. The principle of this assay has recently been reported and validated for the measurement of the murine plasma EL activity[25]. In the present study, furthermore, we depleted native IgG using pretreatment of plasma samples with Protein A prior to EL-IP, as plasma is abundant in native IgG and may interfere with the IP process. The catalytic triad (Ser-His-Asp), which determines the enzyme activity of lipase, exists in the amino terminal portion of EL, as is the case with LPL and HL[4, 26]. Given that the phospholipase activity was preserved after EL-IP, however, the IP step did not interfere with the EL enzymatic activity.

Interestingly, the EL phospholipase activity was inversely correlated with the plasma HDL-C level (**Fig. 2**). In addition, the EL activity was positively associated with cigarette smoking (**Fig. 3A**). A high EL activity and low HDL-C level were observed not only in current smokers, but also in former smokers (**Fig. 3A**), for unknown reasons. We speculate that this may be because the former smoker group included more CAD patients than the current smoker group (**Table 1**). Furthermore, the EL activity was elevated in the patients with CAD (**Fig. 3B**). Because the majority of CAD patients were treated with statins, which inhibit the EL expression and/or phospholipase activity[9, 27] (**Table 1**), the high EL activity levels observed in the CAD patients were considered to be rather significant. These findings indicate that the EL activity not only regulates the plasma HDL-C level, but is also associated with the risk of CAD. Although previous animal studies suggest that the EL expression *in vivo* affects the plasma concentrations of apoB-containing lipoproteins[8], the present study clearly demonstrated that the EL activity is not associated with the plasma concentrations of LDL-C or triglycerides.

Several studies have documented a significant inverse correlation between the plasma EL mass and the HDL-C level in humans[9, 10, 12]. However, the plasma EL mass in these studies showed a large amount (>100 times) of variation, in contrast to the small amount of variation observed in the HDL-C level (25-100 mg/dL). This discrepancy can be explained, at least in part, by the variation in the EL catalytic activity in the plasma, as a variety of factors, including

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gene polymorphisms or protein modification, affect the enzymatic activity of EL[15-19]. For instance, a naturally occurring variant in the EL gene (LIPG), G26S, has been reported to be associated with an elevated HDL level and exhibits impaired synthesis[15]. Moreover, Singaraja and colleagues demonstrated that several complete or partial loss-of-function mutations in LIPG are associated with a high plasma HDL-C level and an enhanced cholesterol efflux capacity[28]. In addition, the authors indicated that carriers of LIPG mutations exhibit a trend toward a reduced incidence of coronary artery disease[28], while another study focusing on a common and partial mutation (N396S) did not identify a cardioprotective effect, despite the presence of an elevated HDL-C level[29].

The EL activity is partly regulated via posttranscriptional mechanisms. It has been reported that EL forms a head-to-tail dimer in human plasma and that homodimer formation is critical for maintenance of the EL activity[16], as is the case with LPL and HL. In addition, EL is proteolytically processed into 40- and 28-kD fragments and inactivated by proprotein convertases[18, 19]. Furthermore, human heat-inactivated serum inhibits the EL phospholipase activity[20], indicating the existence of endogenous EL inhibitors in human serum. For example, angiopoietin-like 3 is known to act as an endogenous EL inhibitor[21]. EL has five potential N-glycosylation sites, four of which are glycosylated, and the EL activity is regulated by N-glycosylation[17, 30]. This line of evidence supports our speculation of the existence of inactive or less active forms of EL in the plasma, which may account for the inconsistency between the EL mass and the EL activity. Further studies are needed to clarify the association between the EL mass and activity in humans.

EL has several heparin-binding domains and binds to heparan sulfate proteoglycans on the vascular endothelium[31]. It has been postulated that EL is released into the plasma by heparin treatment[10]. In the present study, however, the injection of heparin (30 units/kg) did not result in a change in the plasma EL activity level (**Fig. 1D**). Moreover, we recently reported that the plasma EL mass is similar between pre- and post-heparin plasma samples[12]. Therefore, the effects and dose-dependency of heparin treatment on the plasma EL mass and activity must be determined in detail in further studies. In addition, the interaction between EL proteins and heparan sulfate proteoglycans should be reevaluated.

Conclusion

We herein established a method for assessing the human plasma EL-specific phospholipase activity using a combination of IP and a fluorogenic phospholipid substrate. The plasma EL activity was found to be inversely associated with the plasma HDL-C level. In addition, the plasma EL activity levels were elevated in the CAD patients and smokers. These findings indicate that the plasma EL activity impacts the plasma HDL-C level and risk of cardiovascular disease.

Note

In a recent article by Singaraja R.R. *et al.*, the EL phospholipase activity assay described in the present paper was cited in an abstract[27].

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Conflicts of Interest

No authors have any financial relationships with a biotechnology manufacturer, pharmaceutical company or other commercial entity with an interest in the subject matter or materials discussed in this manuscript.

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ELISA System for Human Endothelial Lipase

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BACKGROUND: Endothelial lipase (EL) regulates the metabolism of HDL cholesterol (HDL-C). However, the role of EL in regulating plasma HDL-C concentrations and EL's potential involvement in atherosclerosis in humans has not been fully investigated due to the lack of reliable assays for EL mass. We developed an ELISA system for serum EL mass.

METHODS: Human recombinant EL proteins, purified from cultured media of human EL–transfected Chinese hamster ovary cells, were used as antigen and calibrator. Two specific monoclonal antibodies were generated in mice against recombinant EL protein for a sandwich ELISA. We measured EL mass in human serum using EL recombinant protein as a calibration standard.

RESULTS: The EL antibodies did not cross-react with lipoprotein lipase and hepatic triglyceride lipase. The detection limit of the ELISA was 20 pg/mL, which is approximately 10 times lower than that of previous ELISA systems. Recovery of spiked EL in serum was 90%–105%. Assay linearity was intact with a >4-fold dilution of serum. Intra- and interassay CVs were <5%. The serum EL mass in 645 human subjects was [mean (SE)] 344.4 (7.7) pg/mL (range 55.2–1387.7 pg/mL). Interestingly, serum EL mass was increased in patients with diagnosed cardiovascular disease and inversely correlated with serum HDL-C concentrations. There was no difference in EL mass between pre- and postheparin plasma samples.

CONCLUSIONS: This ELISA should be useful for clarifying the impact of EL on HDL metabolism and EL's potential role in atherosclerosis.

A large number of studies have established an inverse relationship between HDL cholesterol (HDL-C)[6] and risk for cardiovascular disease (CVD) in humans *(1–5)*, and low HDL-C is considered one of the most important negative risk factors for atherosclerotic CVD *(6)*. Even after LDL cholesterol (LDL-C) is intensively controlled to low concentrations with statin therapy, low HDL-C remains a clinically significant cardiovascular risk factor *(7, 8)*. Furthermore, low HDL-C is frequently accompanied by hypertriglyceridemia, and these lipid disorders synergistically contribute to an increased risk for CVD *(9)*. Increased plasma triglyceride (TG) concentrations and low plasma concentrations of HDL-C have emerged as diagnostic criteria for the metabolic syndrome. Despite the therapeutic potential of HDL in combating CVD, there is a limited therapeutic strategy available for selectively raising HDL-C concentrations. Moreover, because of the multiplicity of HDL metabolism in humans, it is difficult to make an etiological diagnosis for the cause of high or low HDL-C concentrations in clinical settings.

Endothelial lipase (EL), a member of the triacylglyceride lipase family, is synthesized by vascular endothelial cells *(10–13)*. Experiments in engineered mice with a disrupted native EL locus, as well as in mice overexpressing human EL (hEL), have revealed an inverse relationship between plasma HDL-C concentration and EL expression *(11, 14)*. Previous studies have shown that plasma EL mass measured by ELISA is inversely correlated with HDL-C concentrations in humans *(15, 16)*. Association-based human genetic studies have provided evidence that variations in the EL genomic *LIPG* locus such as T111I, G26S, and N396S are linked to differences in circulating HDL-C concentrations or CVD *(17–21)*, although recent studies with a large number of subjects have established associations between *LIPG* single-nucleotide polymorphism

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[6] Nonstandard abbreviations: HDL-C, HDL cholesterol; CVD, cardiovascular disease; LDL-C, LDL cholesterol; TG, triglyceride; EL, endothelial lipase; hEL, human EL; CHO, Chinese hamster ovary; FBS, fetal bovine serum; LPL, lipoprotein lipase; HTGL, hepatic triglyceride lipase; HRP, horseradish peroxidase.