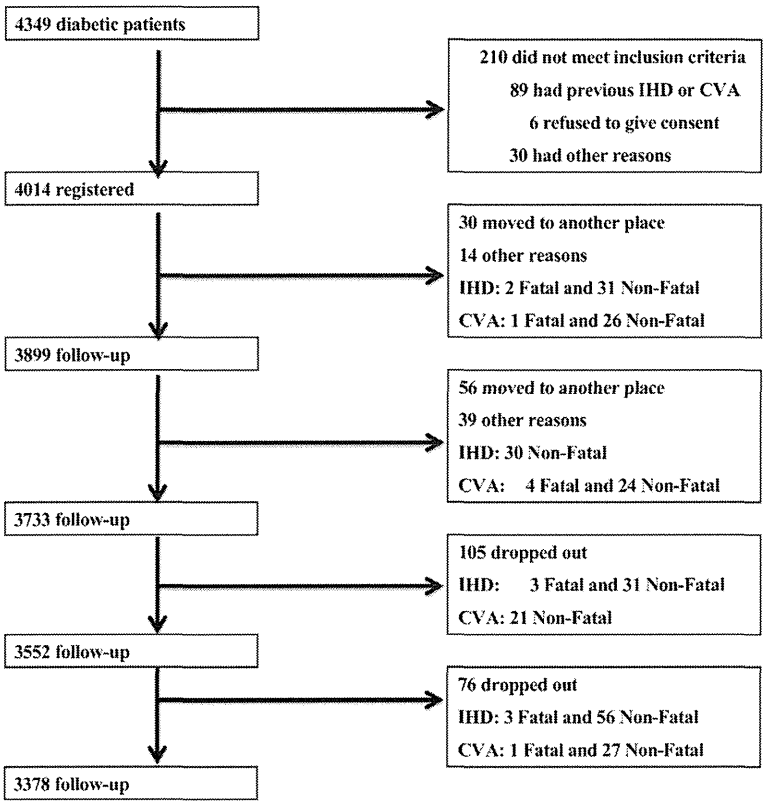

Figure 1 Study profile. "Months" indicates months after March 2005.

Risk factor assessment

Metabolic factors, such as the levels of plasma lipids, fasting plasma glucose (FPG), and HbA1C and BP, were measured at enrollment. The serum LDL-C level was calculated using the Friedewald equation, except in the case of a TG level higher than 400 mg/dl, in which case the LDL-C data were recorded as 'missing.' Information about previous history of IHD and stroke and findings from a 12-lead ECG were obtained for all patients to assess cardiovascular disease at baseline. The study was approved by the institutional review boards and by the safety monitoring board every year. The organizing committee confirmed all cardiovascular events annually. The guidelines of the Japan Atherosclerosis Society (2002) state that the LDL-C level should be less than 120 mg/dl and that the HDL-C level should be higher than 40 mg/dl in diabetic individuals; these clinical guidelines were likely followed by the physicians who were treating these patients at the time of the study [12].

Statistical methods

The results are presented as the means ± SD. All statistical analyses were performed using JMP software (SAS Institute, Inc., Cary, NC). The incidences of IHD and CVA were analyzed in relation to the aforementioned risk factors. Cox multivariate regression analyses were used. Because LDL-C/HDL-C interacts strongly with LDL-C and HDL-C and because non-HDL-C interacts with triglyceride and LDL-C, we analyzed non-HDL-C and LDL-C/HDL-C separately. In other words, common factors (gender, age, duration of diabetes, HbA1C, FPG, systolic BP (SBP), and diastolic BP (DBP)),TG, LDL-C and HDL-C were analyzed first. Then, non-HDL-C and common factors were analyzed. Finally, LDL-C/HDL-C, common factors and TG were analyzed. Values of $P < 0.05$ were considered statistically significant.

Definition of major events. Major events such as IHD and CVA were defined as follows.

1. Definite fatal and nonfatal myocardial infarction (1 or more of the following criteria must be met):

   a) Diagnostic ECG at the time of the event.
   b) Ischemic cardiac pain (and/or unexplained acute left ventricular failure) and diagnostic enzyme levels.
   c) Ischemic cardiac pain and/or unexplained acute left ventricular failure with both equivocal enzyme levels and equivocal ECG.
   d) Diagnostic enzyme levels and equivocal ECG.
   e) Angiographic evidence of major artery occlusion with appropriate ventriculographic wall motion

abnormality where a previous angiogram showed no such abnormality.
f) Postmortem examination.

2. Angina pectoris (stable or unstable, both of the following criteria must be met):

a) Ischemic cardiac pain relieved by nitrates.
b) Equivocal ECG.

3. Ischemic stroke (1 of the following conditions must be met):

a) Rapid onset of focal neurologic deficit lasting at least 24 h or leading to death, plus evidence from neuroimaging (computed tomography or magnetic resonance imaging) showing cerebral/cerebellar infarction or no abnormality, or postmortem examination showing cerebral and/or cerebellar infarction.
b) Rapid onset of global neurological deficit (e.g., coma) lasting at least 24 h or leading to death, plus evidence from neuroimaging showing infarction, or postmortem examination showing infarction.
c) Focal neurological deficit (mode of onset uncertain) lasting at least 24 h or leading to death, plus evidence from neuroimaging showing infarction, or postmortem examination showing infarction.

4 Primary intracerebral hemorrhage (1 of the following conditions must be met):

a) Rapid onset of focal neurological deficit lasting at least 24 h or leading to death, plus neuroimaging or postmortem examination showing primary intracerebral and/or cerebellar hemorrhage.
b) Rapid onset of global neurologic deficit (e.g., coma) lasting at least 24 h or leading to death, plus evidence from neuroimaging or postmortem examination showing primary intracerebral and cerebellar hemorrhage.
c) Focal neurologic deficit (mode of onset uncertain) lasting at least 24 h or leading to death, plus evidence from neuroimaging or postmortem examination showing primary intracerebral and/or cerebellar hemorrhage.

In this study, intracerebral hemorrhage was not included in the variable CVA (stroke) because its pathophysiology is reported to be different from other atherosclerotic diseases, such as stroke and ischemic heart disease.

Results

Subject characteristics

Table 1 presents the following subject characteristics: plasma lipid levels, including LDL-C, TG, and HDL-C; other relevant metabolic measures, such as HbA1C level, FPG level, and SBP and DBP; the duration of diabetes; and the number of patients who were prescribed medications for hypertension, dyslipidemia, and diabetes, as well as the type, upon enrollment. The levels of HbA1C and HDL-C were not different by age group. Dyslipidemia was observed in 79.1% of patients, and anti-hyperlipidemic drugs were prescribed for 57.3% of the total population, of which 83% were HMG-CoA reductase inhibitors (statins). Statins and insulin were prescribed with the same frequency for late elderly patients as for non-elderly patients. Insulin and oral agents for diabetes treatment were prescribed for 23.9% and 70.5% of the late elderly and non-elderly individuals, respectively. Agents for hypertension and diabetes were prescribed more often in late elderly patients than in non-elderly patients. There were also significant differences in several other factors among the age groups.

IHD and CVA incidence

One hundred fifty-three cases of IHD and 104 CVAs occurred during the 5.5 years of the study, which represented incidences of 7.9 and 5.6 per 1,000 patients per year, respectively. The number of deaths was 59 (3.1/1,000 patient-years) over the 5.5 years (Table 2, Figure 1).

The relationships between IHD or CVA and the background factors, such as LDL-C level, in each age group were analyzed by Cox proportional regression analyses (Table 2, Figure 2).

As described in the methods, non-HDL-C and LDL-C/HDL-C were analyzed separately from other lipids, such as LDL-C and triglyceride or HDL-C. However, significant factors were the same in total and in each generation group, although the HR and CI of common factors (gender, age, duration of diabetes, HbA1C, FPG, systolic BP (SBP), and diastolic BP (DBP)) were slightly different in each (data not shown for the HR and CI of common factors in the analyses of non-HDL-C and LDL-C/HDL-C).

In the total patient population, the levels of HbA1C, LDL-C, and HDL-C, and the LDL-C/HDL-C ratio were significantly related to IHD, and only the HDL-C level was significantly related to a CVA. The HbA1C level, SBP, and LDL-C levels were significantly correlated with IHD in patients less than 65 years old, while the variables female gender, short duration of diabetes and HDL-C level were correlated with IHD in patients older than 75. Because the non-HDL-C level and the LDL-C/HDL-C ratio have been proposed as markers representing all types of lipids, we included them in a separate model (excluding LDL-C, triglyceride and HDL-C levels

Table 1 Basic patient profile

n = 4014	Total		<65 years		65-74 years		≥75 years		P1	Male		Female		P2
	n = 4014		n = 1267		n = 1731		n = 1016			n = 2078		n = 1936		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Gender (% male)	51.2	53.1	49.8	49.9	*	100.0	0	-						
Age (yrs,mean/median)	67.9/70	2.0	56.5/58	7.0	70.0/70	2.7	78.8/78	3.5	-	67.0/69	10.0	69.7/70	8.7	**
Duration of DM (months)	177.9	70.0	156.3	64.5	186.1	101.2	190.7	104.3	***	191.6	108.9	169.8	79.7	**
HbA1C (%)	7.70	0.80	7.74	1.38	7.72	1.18	7.62	1.14	0.17	7.61	1.25	7.79	1.20	**
FPG (mg/dl)	149.8	30.3	157.9	53.2	146.4	45.7	145.4	42.0	**	150.9	48.8	146.9	44.9	0.17
SBP (mmHg)	137.3	11.7	133.1	17.3	135.3	16.8	146.0	17.5	**	133.8	17.2	135.9	17.1	*
DBP (mmHg)	74.0	7.3	76.1	11.8	73.5	10.6	72.3	10.7	***	74.5	11.1	73.3	10.9	*
TG (mg/dl)	138.2	53.8	159.4	156.3	128.5	82.0	128.4	73.0	***	142.9	126.3	131.3	83.7	0.18
LDL-C (mg/dl)	118.2	21.3	121.6	33.8	117.6	32.1	115.0	29.3	*	115.0	31.8	121.2	31.4	**
HDL-C (mg/dl)	55.8	10.7	54.8	15.6	55.4	15.5	57.7	15.8	0.38	53.42	15.4	56.78	15.7	**
Non-HDL-C (mg/dl)	145.8	23.4	153.2	40.3	143.3	35.5	141.0	31.8	*	143.6	36.9	147.5	35.6	*
LDL-C/HDL-C	2.31	0.74	2.41	1.17	2.31	1.21	2.27	0.88	*	2.33	0.95	2.33	1.28	0.21
Agents for HT (%)	55.5		49.3		56.0		62.3		**	48.5		62.0		***
ACEI/ARB	39.7		36.9		40.4		41.9		0.65	35.8		43.4		*
CCB	41.2		32.4		43.7		52.6		0.31	36.0		46.5		*
Others	28.3		22.5		31.8		31.4		0.69	28.9		26.7		0.66
Agents for DL (%)	57.3		63.8		54.8		52.6		**	52.1		60.1		***
Strong statins	29.6		31.1		28.3		25.4		0.36	29.7		29.5		0.89
Classical statins	53.3		47.0		58.0		61.6		0.11	51.9		55.0		0.23
Fibrates	8.9		12.0		6.5		6.8		0.13	9.8		7.9		0.18
Others	8.2		9.9		7.2		6.2		0.10	8.6		7.6		0.22
Agents for DM (%)	86.6		76.9		91.6		90.3		**	85.1		88.6		*
Insulin	23.9		24.4		24.6		21.7		0.42	28.0		32.4		*
Sulfonylurea	49.5		41.5		51.3		53.7		0.21	50.0		48.9		0.29
Others	26.1		34.6		21.4		24.5		0.19	26.0		22.0		*
IHD (/1000 year)	9.68		8.84		10.04		9.87		0.97	10.26		9.47		0.32
CVA (/1000 year)	6.78		4.45		7.44		7.56		0.21	7.02		5.72		0.27

P1: Differences in each factor among ages. P2: Differences in each factor between genders. HbA1C:NGSP, *P < 0.05, **P < 0.01, ***P < 0.001.

to avoid the interactive effect on non-HDL-C, or excluding LDL-C and HDL-C levels for the LDL-C/HDL-C ratio). The non-HDL-C level was only correlated with IHD in patients younger than 65. The LDL-C/HDL-C ratio was significantly correlated with IHD in patients of all generations. Age and lower HDL levels were correlated with CVA in patients over 75 years old (Table 2, Figure 2). Subsequently, we evaluated the relationships with IHD and CVA according to the quartile categories for each age group by Kaplan-Meier estimator curves. The HDL-C level was inversely correlated with IHD and CVA, particularly in individuals over 75 (Figure 3). The LDL-C/HDL-C ratio tended to correlate with IHD in all individuals (Figure 3). For the variable current smokers, 6.8% of the total population of subjects smoked. By age category, 9.9, 6.7 and 3.8% of patients younger than 65, patients between 65 and 74, and patients older than 75 smoked, respectively. As the duration of diabetes is pretty long, number of present smokers is not many.

Discussion

Background and discussion points of the study

The numbers of diabetic elderly and their associated net medical costs have drastically increased in recent decades. The mean life expectancy is now approximately an additional 12 and 16 years at age 75 for males and females in Japan, respectively, although the average life span is 78.9 and 85.6 years, respectively. Consequently, the number of late elderly (individuals older than 75) exceeds 13 million, or 10% of the total Japanese population. Diabetes can either develop in the elderly or continue through old age after an earlier onset, and the numbers of diabetic elderly are increasing. In Japan, 55% of diabetic individuals were elderly in 2007, and

Table 2 Risk factors for IHD and CVA by Cox multivariate models in each age group (IHD, upper; CVA, lower)

n = 4014	Total (n = 4014)			<65 years (n = 1267)			65-74 years (n = 1731)			≥75 years (n = 1016)		
IHD	**Adjusted HR**	**95% CI**	**P**	**Adjusted HR**	**95% CI**	**P**	**Adjusted HR**	**95% CI**	**P**	**Adjusted HR**	**95% CI**	**P**
Gender (women vs. men)	1.103	0.972–1.268	0.197	1.044	0.967–1.073	0.456	1.085	0.978–1.210	0.101	**1.132**	**0.992–1.278**	**0.019***
Age (per 10 years)	1.013	0.972–1.066	0.328	1.022	0.977–1.079	0.229	**1.054**	**1.002–1.106**	**0.049***	1.005	0.871–1.139	0.682
Duration of Diabetes (months)	0.995	0.988–1.003	0.053	1.001	0.991–1.008	0.582	**0.993**	**0.985–0.999**	**0.033***	**0.992**	**0.982–0.999**	**0.023***
HbA1C (per 1%)	**1.171**	**1.001–1.356**	**0.047***	**1.327**	**1.025–1.686**	**0.032***	1.219	0.973–1.487	0.083	0.792	0.479–1.059	0.134
FPG (per 10 mg/dl)	1.004	0.997–1.008	0.432	1.005	0.996–1.013	0.355	1.004	0.997–1.009	0.592	0.999	0.987–1.007	0.761
SBP(per 10 mmHg)	1.008	0.995–1.021	0.186	**1.030**	**1.000–1.055**	**0.035***	1.014	0.994–1.037	0.175	0.986	0.954–1.014	0.331
DBP(per 10 mmHg)	0.995	0.978–1.015	0.618	0.982	0.948–1.024	0.386	0.980	0.950–1.011	0.206	1.027	0.986–1.073	0.202
TG (quartile)	1.005	0.889–1.166	0.555	1.002	0.996–1.006	0.502	1.108	0.997–1.220	0.065	1.001	0.961–1.046	0.454
LDL-C (quartile)	**1.318**	**1.103–1.585**	**0.023***	**1.571**	**1.128–2.524**	**0.016***	1.050	0.932–1.176	0.112	1.156	0.998–1.309	0.054
HDL-C (quartile)	**0.751**	**0.611–0.917**	**0.005****	0.828	0.646–1.017	0.072	0.987	0.966–1.008	0.204	**0.629**	**0.401–0.856**	**0.001****
Non-HDL-C (quartile)	1.023	0.981–1.072	0.075	**1.025**	**1.001–1.121**	**0.044***	1.073	0.982–1.161	0.086	0.941	0.791–1.102	0.621
LDL-C/HDL-C (quartile)	**1.583**	**1.298–1.945**	**0.001****	**2.324**	**1.516–3.795**	**0.001****	**1.359**	**1.028–1.824**	**0.021***	**1.407**	**1.015–2.592**	**0.029***
CVA	**Adjusted HR**	**95% CI**	**P**	**Adjusted HR**	**95% CI**	**P**	**Adjusted HR**	**95% CI**	**P**	**Adjusted HR**	**95% CI**	**P**
Gender	1.164	0.985–1.296	0.351	1.014	0.897–1.240	0.655	1.208	0.896–1.526	0.112	0.953	0.912–1.012	0.063
Age	1.015	0.986–1.039	0.282	1.002	0.957–1.076	0.754	1.007	0.916–1.166	0.537	**1.103**	**1.002–1.217**	**0.048***
Duration of Diabetes	0.998	0.992–1.001	0.206	1.003	0.987–1.017	0.709	0.996	0.989–1.001	0.096	0.999	0.991–1.005	0.818
HbA1C	1.001	0.790–1.214	0.128	1.019	0.691–1.401	0.814	0.997	0.855–1.222	0.569	0.928	0.822–1.010	0.059
FPG	1.005	0.995–1.005	0.803	1.003	0.990–1.018	0.741	1.002	0.995–1.008	0.592	0.998	0.986–1.008	0.711
SBP	1.009	0.993–1.024	0.276	1.024	0.988–1.055	0.185	1.015	0.992–1.037	0.206	0.989	0.957–1.018	0.458
DBP	0.998	0.978–1.020	0.846	0.995	0.958–1.046	0.831	0.981	0.948–1.016	0.278	1.024	0.978–1.074	0.317
TG	1.132	0.908–1.302	0.156	1.053	0.658–1.742	0.833	1.253	0.900–1.780	0.184	1.169	0.746–1.853	0.497
LDL-C	1.009	0.912–1.191	0.675	**1.005**	**1.001–1.100**	**0.047***	1.015	0.892–1.136	0.714	0.997	0.982–1.012	0.631
HDL-C	**0.742**	**0.596–0.901**	**0.003****	0.715	0.591–1.191	0.200	**0.750**	**0.494–1.000**	**0.049***	**0.536**	**0.320–0.851**	**0.007****
Non-HDL-C	0.981	0.945–1.019	0.206	**1.021**	**1.003–1.141**	**0.045***	0.942	0.872–1.013	0.172	1.012	0.954–1.077	0.226
LDL-C/HDL-C	1.180	0.951–1.477	0.132	1.271	0.819–2.232	0.263	1.114	0.853–1.582	0.356	1.209	0.803–1.847	0.364

The top panels show the analyses of IHD for subjects aged <65 years (left), 65–74 years (middle) and ≥75 years (right). The lower panels show the incidence of CVA. Bold indicate statistically significant factors. Hazard ratios and 95% CIs are shown. The ratio of males to females was 1. As LDL-C/HDL-C interacts strongly with LDL-C and HDL-C, and non-HDL-C interacts triglyceride and LDL-C, analysis of non-HDL-C and LDL-C/HDL-C were separately shown in methods section.

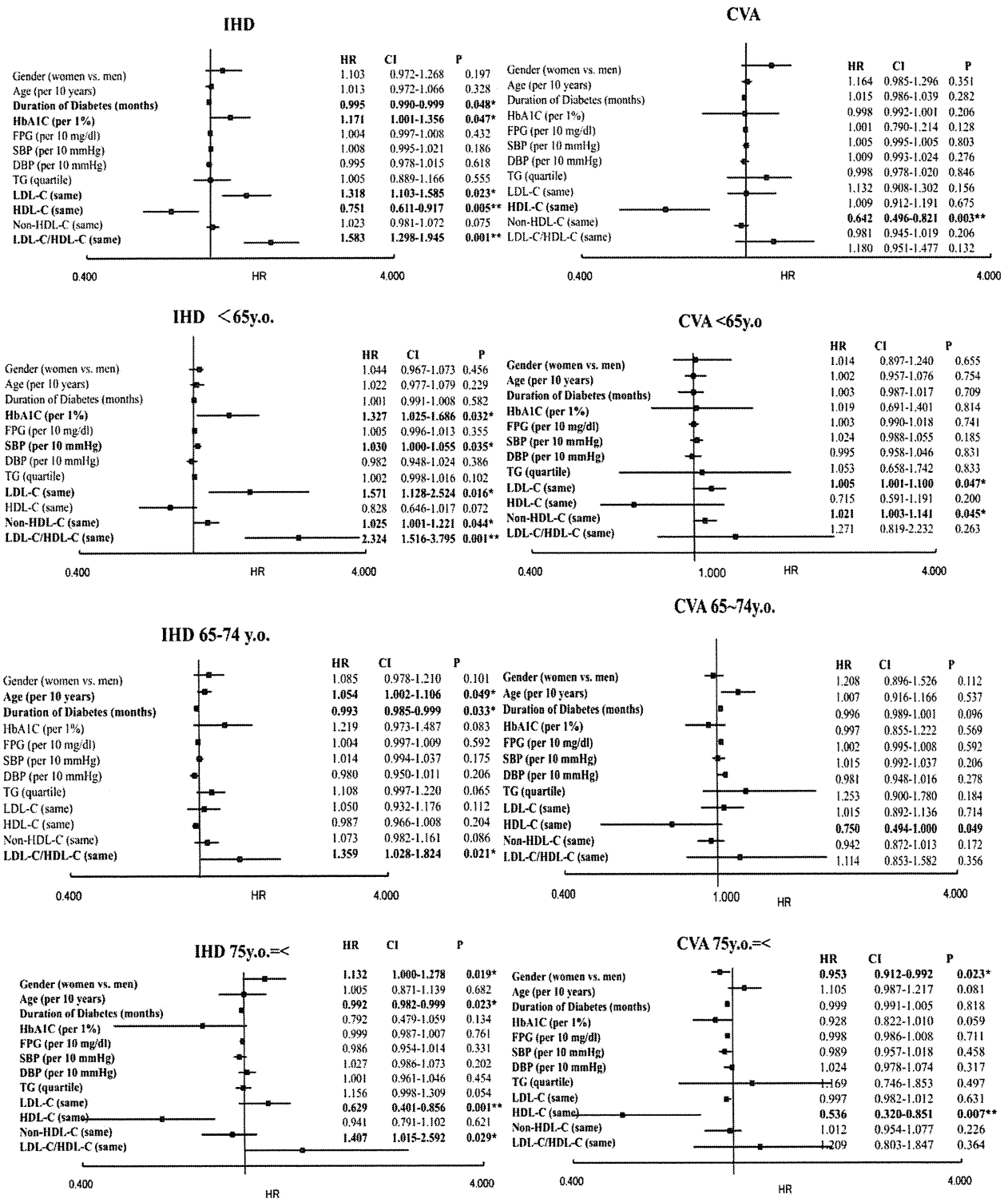

Figure 2 Risk factors for IHD and CVA by Cox multivariate models in representative age groups (IHD, Left; CVA, Right). The upper panels show the analyses of IHD for those younger than 65 years old (Left) and CVA for those younger than 65 years old (Right). The lower panels show the analyses of IHD (Left) and CVA (Right) for those equal to or older than 75 years. HR stands for Hazard Ratio (vertical bar shows 1). Bold characters indicate statistically significant factors. The right side of each figure shows Hazard Ratios and 95% CIs. Because LDL-C/HDL-C interacts strongly with LDL-C and HDL-C and because non-HDL-C interacts with triglyceride and LDL-C, we analyzed non-HDL-C and LDL-C/HDL-C separately. In other words, common factors (gender, age, duration of diabetes, HbA1C, FPG, systolic BP (SBP), and diastolic BP (DBP)), TG, LDL-C and HDL-C were analyzed. Then, non-HDL-C and common factors were analyzed. Finally, LDL-C/HDL-C, common factors and TG were analyzed.

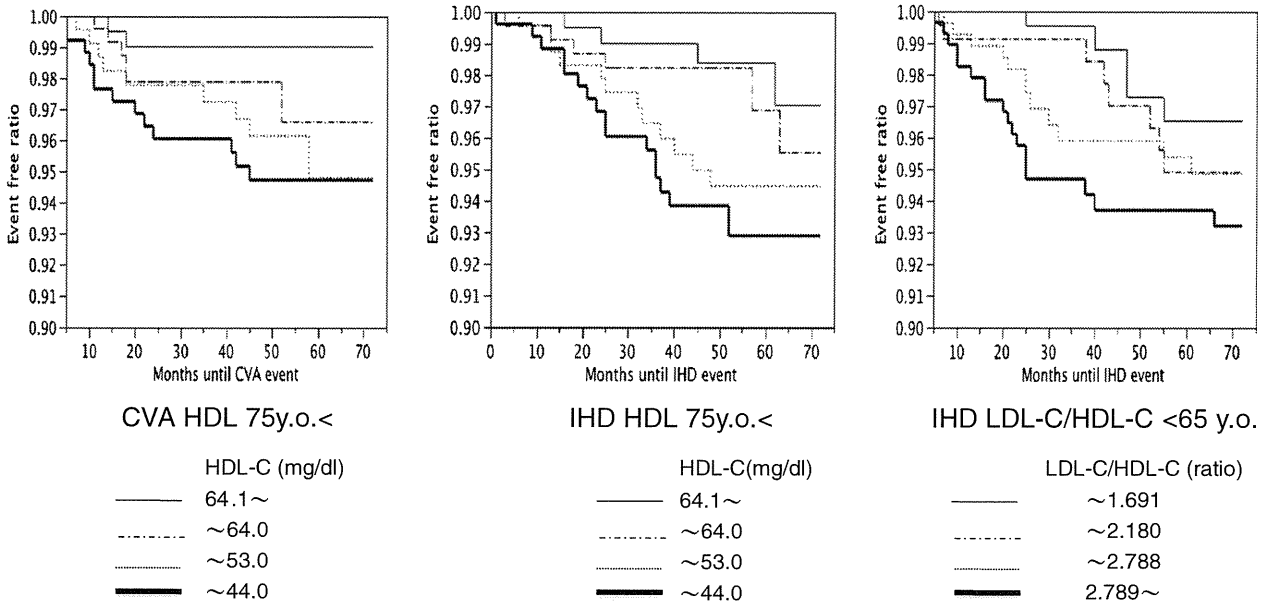

Figure 3 The relationships of HDL-C levels and the LDL-C/HDL-C ratio with IHD and CVA in quartile categories for each age group based on Kaplan-Meier estimator curves. Left: The LDL-C/HDL-C ratio correlates with IHD. The figure shows the data for individuals aged <65 years. **Center:** The HDL-C level was inversely correlated with IHD, particularly in individuals aged >75 years. **Right:** The association between CVA and HDL-C was more prominent in those individuals aged ≥ 75 years. Quartile categories HDL-C: <44.0, 44.1-53.0, 53.1-64.0, >64.1 mg/dl LDL-C/HDL-C: <1.691, 1.692-2.180, 2.181-2.787, >2.789.

approximately 25% were late elderly. These trends are spreading across the world, mainly in developed countries; however, the risk factors for IHD or CVA in late elderly diabetic individuals have not been identified. In the late elderly, atherosclerotic diseases, such as IHD and CVA, are a more frequent cause of death than malignancy. In Canada, diabetic patients are reported to suffer myocardial infarction approximately 14 years earlier than patients without diabetes [13]. However, there is little evidence on the risk and preventive factors for IHD or CVA in the diabetic elderly, and there are no reports on the late elderly [14,15].

Therefore, we organized this study as one of the largest attempts to examine IHD and CVA in middle-aged to elderly diabetic individuals. We defined the age categories as follows: 1) non-elderly: younger than 65, 2) early elderly: from 65 to 74, and 3) late elderly: equal to or older than 75. Sixty-five is usually defined as the threshold for being elderly worldwide [13,16], and 75 is the beginning of the late elderly age in Japan, as defined by health insurance and care insurance systems and the Japan Geriatric Society [12].

The effect of age on IHD and CVA risk factors

One hundred fifty-three cases of IHD and 104 CVAs occurred, which represents 7.8 and 5.7/1,000 people per year, respectively, over this 5.5-year study, although we defined stroke strictly and excluded cerebral and subarachnoid hemorrhages from this definition. IHD occurs 2 to 3 times more frequently in diabetic individuals compared to the normal Japanese population, and CVA also occurs more frequently in diabetic individuals [17]. The prevalence of IHD and CVA is slightly higher than reported in previous Japanese diabetic studies because we targeted relatively older diabetic individuals [16,17]. However, even in diabetic individuals, the combined frequency of IHD and stroke was slightly lower in the Japanese population than among Caucasians [18].

To look for the candidate metabolic markers that may predict IHD and CVA in various age groups, Cox regression analyses were performed. The analyses showed that higher HbA1C and LDL-C levels, SBP and non-HDL-C were significantly correlated with the occurrence of IHD in subjects <65 years old, which is similar to previous reports [14-16]. The ratio of males/females was not significantly different between patients < 65, patients between 65 and 74, and patients ≥75. A relation between diabetes and ischemic stroke was reported. Patients (59.8 ± 7.2 y.o.) having a history of coronary heart disease with diabetes mellitus exhibited a 2.29-fold increased risk for stroke or TIA during the 4.8- to 8.1-year follow-up period than patients without diabetes. Impaired fasting

glucose and hypertension were predictors, while HDL-C was not. These results are fairly consistent with those of the younger patients group (< 65 y.o.) in the present study [19].

In patients ≥75 y.o., a lower HDL-C level was correlated with IHD and CVA. This is a novel finding of the present study. Few data are available on the relationship between elderly type 2 diabetic patients and CVA, particularly among the late elderly [16-18,20]; therefore, the finding of the importance of HDL-C in CVA in the late diabetic elderly may be important. The Kaplan-Meier estimator curves, which are shown in Figure 1, support these findings.

Thus, a lower HDL-C level is an important risk factor for both IHD and CVA among the late elderly diabetic patients in this study. Although the protective effects of higher HDL-C on IHD in the non-elderly are known, the effects on IHD among late elderly diabetics are not known [21]. The CVA and IHD incidences in the late elderly may decrease to the levels found in middle-aged cohorts if higher HDL-C has protective effects on late elderly diabetic individuals and if their levels are easily increased. There are few agents available to increase HDL-C levels, except exercise, and adequate exercise or bodily movement may be necessary even in the elderly. The low HDL-C level may be related to low levels of physical activity in the elderly, which could influence a CVA in many ways that are separate from the HDL-C level. Atherosclerosis is an inflammatory disorder, and HDL-C may preserve endothelial function by increasing endothelial NO [22].

For LDL-C, three large-scale clinical studies on dyslipidemia, which included participants who were up to 75 or 80 years of age, are available [23-25]. Although these studies reported that the reduction in LDL-C by statins decreases IHD (including in diabetic people), the effects were weak in the elderly compared with those in the non-elderly (e.g., Prosper reported that pravastatin, a water-soluble statin, induced a 16% decrease in IHD without any effect on CVA in elderly patients compared to a 21% decrease in non-elderly patients). These data suggest that simply controlling LDL-C may not prevent IHD or CVA in the elderly. There are also no large observational studies on the diabetic elderly older than 75 [26,27]. For example, the international FIELD study analyzed approximately 10,000 patients up to the age of 75 years, with a mean age 63 years [26], and the Swedish NDR-study analyzed 18,673 patients up to 70 years old, with a mean age of 60 years [27]. These large observational studies, analyzing all patients, found LDL-C, non-HDL-C, HDL-C, triglycerides and ratios of LDL-C/HDL-C and total-cholesterol/HDL-C to be significant risk factors for IHD. These data are consistent with our data on participants younger than 65, although those observational studies did not include patients older than 75. To lower LDL-C levels, 57% of the patients in our study had already been prescribed anti-dyslipidemic agents, of which 83% were statins. The average LDL-C level was 120 mg/dl, which matches the guidelines of the Japan atherosclerosis society but not that of the American Heart Association or IDF (100 mg/dl). Although doses and types of anti-dyslipidemic agents were changed often during the study, their effects other than LDL reduction (pleiotropic effects) cannot be evaluated yet.

Our study shows the importance of the LDL-C/HDL-C ratio as well as HDL-C and LDL-C levels, although the strength of these effects is different based on age. The LDL-C/HDL-C ratio was associated with IHD, which may represent the effect of LDL-C levels in the non-elderly and HDL-C levels in the elderly [28]. The non-HDL-C level and the total cholesterol/HDL-C ratio are also proposed markers of atherosclerotic diseases [29,30]. The non-HDL-C level was associated with IHD only among those younger than 65, and the total cholesterol/HDL-C ratio was not significantly associated with IHD (data not shown). We believe that these data are consistent with previous data from non-elderly diabetic individuals because the non-HDL-C level is a reflection of the effect of triglyceride levels, and hyper-triglyceridemia, complicated with metabolic syndrome, occurs more often in non-elderly than in elderly people.

Emerging Risk Factors Collaboration analysis showed the association of non-HDL-C with IHD and CVA. However, in this study, it was associated with CVA only in those younger than 65. The two studies are different in that 1) our cohort consisted only of diabetic patients; 2) in the Collaboration analysis, the mean age was 56.6 y.o., compared to 67.4 y.o. in our study; and 3) in the Collaboration analysis, almost all of the patients were North American or European, whereas our study was Japanese patients only. In the elderly, triglycerides are usually lower than in younger individuals, and non-HDL-C represents triglyceride.

A 1-mg/dl change in HDL-C and/or a 2-mg/dl change in LDL-C reflect a 2% change in the risk for atherosclerotic diseases, and this may be partially consistent within our diabetic elderly study [31]. The LDL-C/HDL-C ratio may reflect the direct effects of both LDL-C and HDL-C levels, which may affect or interact with the progression of atherosclerosis and thrombosis formation more than other lipids, such as chylomicrons and chylomicron remnants, which are represented by the non-HDL-C level or the TC/HDL-C ratio. The fact that elderly individuals have different risk factors than younger individuals could be associated with genetic protection from such events or an accumulation of personal habits that may provide the elderly with protection. For example, differences in single nucleotide polymorphisms (SNP)

may be related to the severity of atherosclerosis and, subsequently, to the different effects of predictors by age and should be evaluated in the future [32].

Interestingly, impaired fasting glucose and hypertension were the strongest predictors of risk for ischemic stroke or TIA in metabolic syndrome, and HbA1c had positive associations with glycemia, TG, HDL-C, and TG/HDL-C but not LDL-C in the study of 118 older adults aged 65–95 years, of whom less than 6.5% had an HbA1c of 93% [19,33]. These data is consistent with our data in diabetic patients younger than 65 [33]. Another study evaluated the predictors of stroke stratified by age (at symptom onset: young; <50 years, older; 51–75 years, and oldest; 75 < years) using data collected over a 4-year period from 3,053 subjects with stroke. The metabolic syndrome was the only predictor among the older patients (OR 1.58) but not in the others. Although most patients were not diabetic, these types of studies should be accumulated to evaluate the effect of age on atherosclerotic diseases [34].

Conclusions

HbA1C, LDL-C, SBP and non-HDL-C in non-elderly diabetic individuals, HDL-C in late elderly diabetic individuals and the LDL-C/HDL-C ratio in all diabetic individuals were associated with IHD in this population. HDL-C was also associated with CVA in late elderly diabetic individuals. The differences in atherosclerotic risk by age must be considered in developing individualized strategies for the prevention of atherosclerotic diseases. Because this was an observational study, we could not analyze the detailed effects of treatment, such as the effect of statins on the risk of IHD or CVA. Although this study targets Japanese, these new findings on metabolic markers in the late elderly could provide additional data for the annotation of cardiovascular risk factors in the diabetic elderly across the world.

Abbreviations

CVA: Cerebrovascular attack; IHD: Ischemic heart disease; LDL-C: LDL-cholesterol; HDL-C: HDL-cholesterol; TG: Triglyceride; FPG: Fasting plasma glucose; HbA1C: Hemoglobin A1C; SBP: Systolic blood pressure; UKPDS: United Kingdom Prospective Diabetes Study.

All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TH and HN wrote the manuscript and researched the data. AA, SK, HS, HW, TO, KY, MT, KK, MN, HN, and KI contributed to the research and reviewed the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We thank the following people for contributing to this research by researching and analyzing the data: I. Sakuma (Kales Sapporo Hospital), H. Iinuma and K. Ohashi (Tokyo University), S. Tanaka (Kyoto University), M. Yoshizumi (Hiroshima University), and H. Umegaki (Nagoya University). This work was supported in part by a grant from The Japanese Ministry of Health, Welfare and Labor. The funding body had no role in its design; in the collection, analysis, and interpretation of data; in the writing of the manuscript; or in the decision to submit the manuscript for publication.

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Received: 10 October 2012 Accepted: 6 January 2013
Published: 9 January 2013

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doi:10.1186/1475-2840-12-10
Cite this article as: Hayashi *et al.*: Metabolic predictors of ischemic heart disease and cerebrovascular attack in elderly diabetic individuals: difference in risk by age. *Cardiovascular Diabetology* 2013 **12**:10.

Original Article
Accepted for publication: October 15, 2013
Published online: December 26, 2013

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.**

J Atheroscler Thromb, 2013; 20:000-000.

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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Received: June 23, 2013
Accepted for publication: October 15, 2013

Advance Publication
Journal of Atherosclerosis and Thrombosis
Accepted for publication: October 15, 2013
Published online: December 26, 2013

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).

Advance Publication
Journal of Atherosclerosis and Thrombosis
Accepted for publication: October 15, 2013
Published online: December 26, 2013

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.

Advance Publication
Journal of Atherosclerosis and Thrombosis
Accepted for publication: October 15, 2013
Published online: December 26, 2013

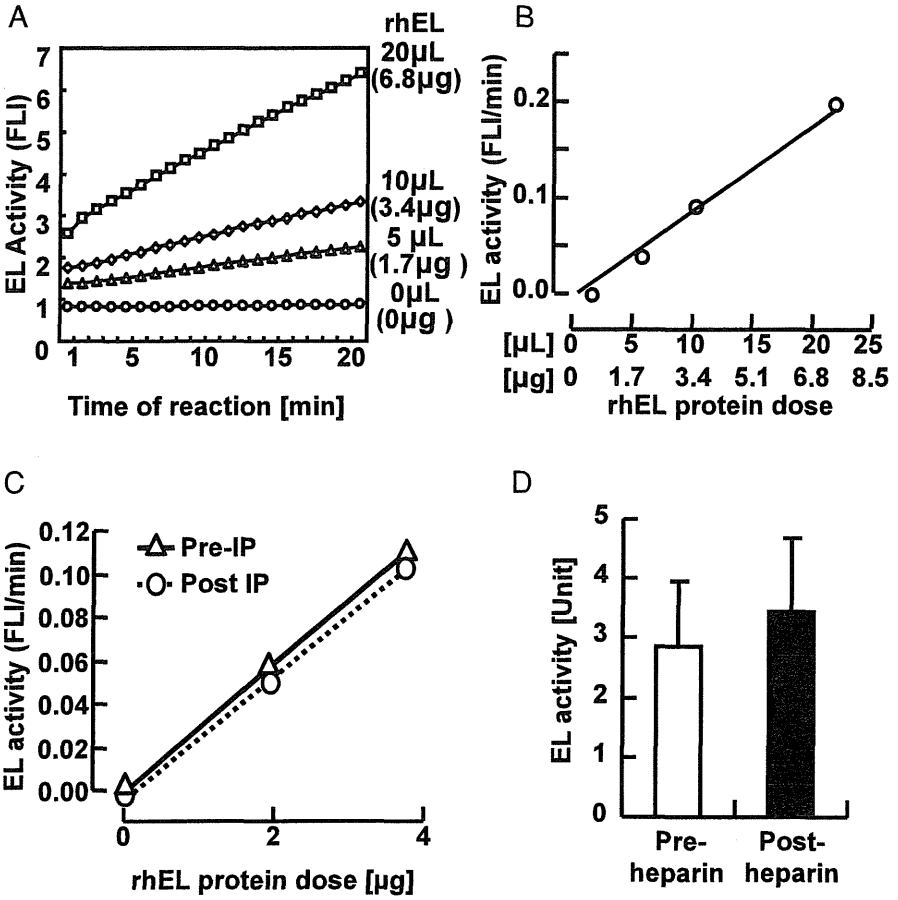

Fig. 1. Validation of the EL activity assay following EL immunoprecipitation

(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.

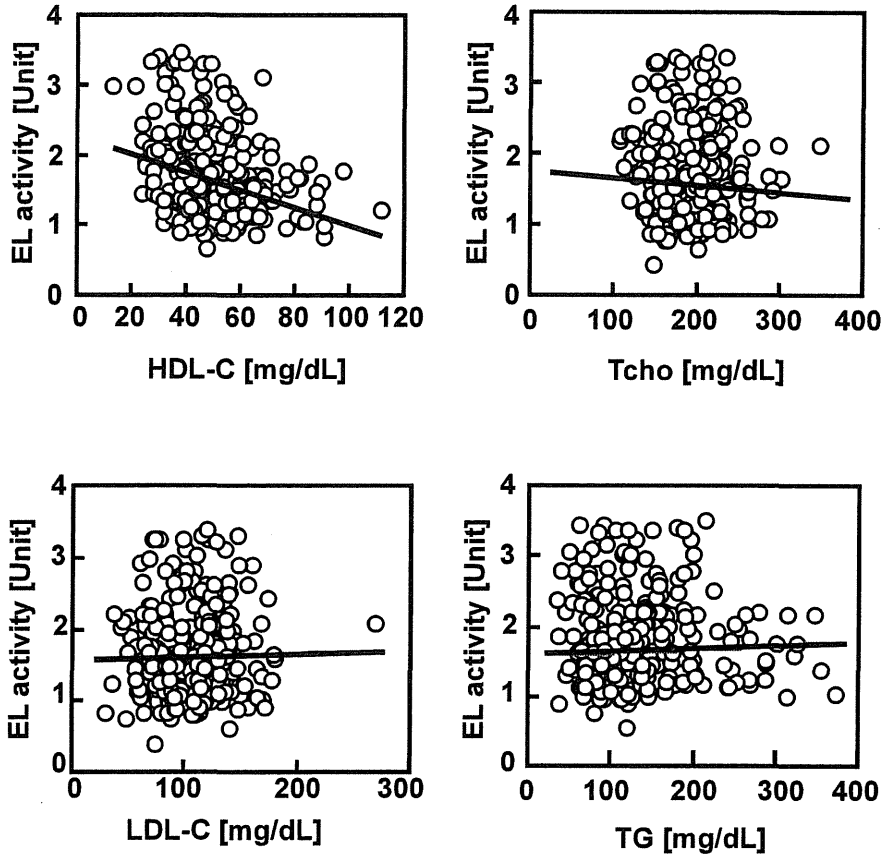

Fig. 2. Plasma EL activity and lipid profiles

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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Journal of Atherosclerosis and Thrombosis
Accepted for publication: October 15, 2013
Published online: December 26, 2013

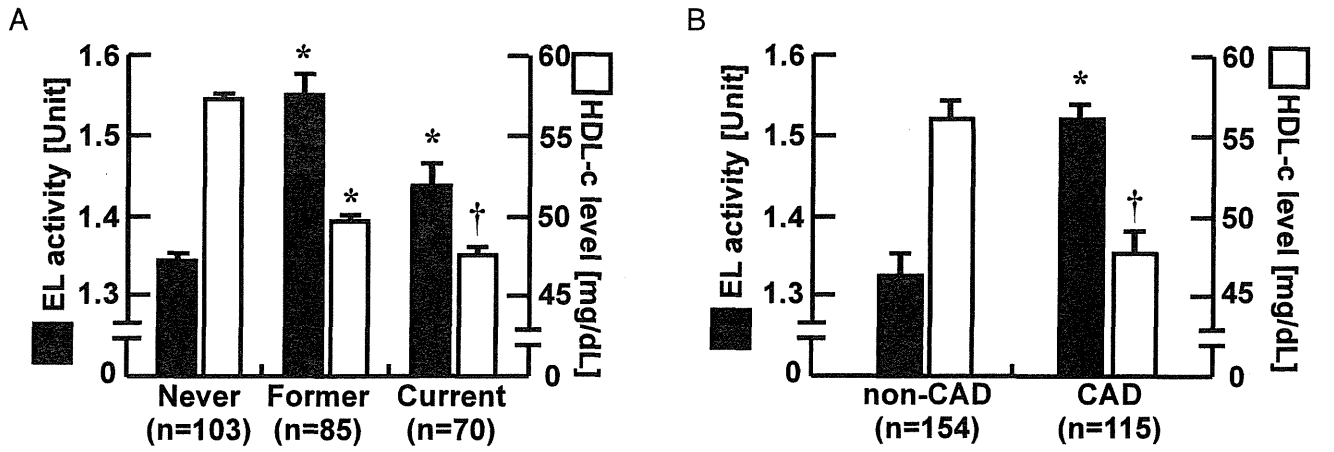

Fig. 3. Plasma EL activity and cardiovascular risks

(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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Accepted for publication: October 15, 2013
Published online: December 26, 2013

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].

Acknowledgements

None.

Notice of Grant Support

This work was supported by Grants-In-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, grants from the SENSHIN Medical Research Foundation and a VRI Research Grant from AstraZeneca.

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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Advance Publication
Journal of Atherosclerosis and Thrombosis
Accepted for publication: October 15, 2013
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Original Article

Pitavastatin Increases HDL Particles Functionally Preserved with Cholesterol Efflux Capacity and Antioxidative Actions in Dyslipidemic Patients

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Aim: Although statins increase the plasma concentration of high-density lipoprotein cholesterol (HDL-C), it has not been elucidated whether the increased HDL particles possess normal antiatherosclerotic properties. Pitavastatin functions to increase the plasma HDL-C level and decrease the low-density lipoprotein cholesterol (LDL-C) level. In the present study, we sought to examine the qualitative changes in HDL during pitavastatin treatment.
Methods: A total of 30 patients with dyslipidemia were treated with 2 mg of pitavastatin for four weeks. The cholesterol efflux capacity and activities of the antioxidative enzymes paraoxonase-1 (PON-1) and platelet-activating factor acetylhydrolase (PAF-AH) were evaluated using polyethethylene glycol-treated HDL fractions before and after pitavastatin treatment.
Results: Pitavastatin treatment decreased the serum LDL-C level by 39% and increased the serum HDL-C level by 9% ($p<0.05$). In addition, pitavastatin increased the phospholipid content of HDL by 7.8% ($p<0.05$). The pitavastatin-induced increase in the HDL-C level coincided with an increase in the cholesterol efflux capacity of the isolated HDL fraction of 8.6% ($p<0.05$). The post-pitavastatin treatment activity of HDL-associated PON-1 (paraoxonase and arylesterase) was increased by 9% ($p<0.05$) and 11% ($p<0.05$), respectively, while the HDL-associated PAF-AH activity was not affected by pitavastatin.
Conclusions: In addition to its LDL-C-lowering effects, pitavastatin elevates the HDL-C level and enhances the cholesterol efflux capacity and antioxidative properties of HDL. Pitavastatin therefore increases the amount of functional HDL without attenuating HDL quality.

J Atheroscler Thromb, 2013; 20:708-716.

Key words: Statin, HDL, Cholesterol efflux, PON-1

Introduction

It has been established that low-density lipoprotein cholesterol (LDL-C) is a central therapeutic target for coronary artery disease (CAD)[1]. Although statins reduce the risk of CAD by 30-40%, cardiovascular events, known as "residual risks"[2], can continue to occur during intensive LDL-lowering therapy, and research aims to identify new therapeutic targets beyond LDL-C. The well-characterized residual risks following statin treatment include a high level of triglycerides (TGs), a low level of high-density lipoprotein cholesterol (HDL-C), uncontrolled diabetes mellitus, hypertension, obesity and lifestyle factors, such as smoking and inactivity. Such factors are not controlled with statin treatment. There is an inverse correlation between the circulating HDL-C level and the risk of CAD[3]. Even after statin treatment, the inverse correlation between the incidence of cardiovascular

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Received: November 27, 2012
Accepted for publication: April 4, 2013

events and the circulating HDL-C level still holds[4]. HDL exhibits a variety of cardiovascular protective effects by promoting reverse cholesterol transport (RCT) from the vascular wall to the liver[5]. HDL particles also possess several antiatherogenic functions, such as anti-inflammatory, antiapoptotic, antithrombotic and immunomodulating effects[6]. Therefore, it is believed that HDL-raising therapy can reduce the incidence of cardiovascular events. However, large clinical trials using cholesteryl ester transfer protein (CETP) inhibitors have failed to document the efficacy of HDL-raising therapy, despite a marked increase in the serum HDL-C level and a decrease in the serum LDL-C level[7]. Therefore, both raising the HDL level and improving the quality of functional HDL have become interesting topics of discussion in HDL-targeting therapy. Under pathological conditions, HDL particles have been reported to lose their antiatherogenic function and convert into dysfunctional HDL, which promotes inflammation and oxidation[8,9].

The HDL-raising effects of statins are variable in contrast to their comparable LDL-lowering effects[10]. Pitavastatin is a unique statin that increases the HDL-C level more significantly than other strong statins[10-13]. Furthermore, pitavastatin improves event-free survival following percutaneous coronary intervention[13]. The mechanisms by which pitavastatin increases the HDL-C level[14-16] are partially understood; however, the effects of pitavastatin on the quality of HDL remain unknown. In this study, we investigated qualitative and quantitative changes in HDL during pitavastatin treatment. To this end, we evaluated the cholesterol efflux capacity of HDL, the first step in RCT, and the antioxidant properties of the paraoxonase-1 (PON-1) and platelet-activating factor acetylhydrolase (PAF-AH) activities.

Materials and Methods

Patients

This investigation conformed to the principles outlined in the Declaration of Helsinki. All patients gave their written informed consent, and the clinical study was approved by the Institutional Review Board of Kobe University Graduate School of Medicine. A total of 30 patients with dyslipidemia, as defined by the Japan Atherosclerosis Society guidelines[17], were enrolled in this study. The backgrounds of the participants are described in **Table 1**. The patients were treated with 2 mg of pitavastatin daily for four weeks, and serum was obtained pre- and post-pitavastatin treatment. Blood samples were collected after overnight fasting, and the serum was stored at −80℃ until the analysis.

Table 1. The background of the enrolled dyslipidemia patients

Variable	Patients with Dyslipidemia (n=30)
Male, n (%)	22 (73.3)
Age (year)	59.6 ± 15.9
Body mass index (kg/m^2)	24.1 ± 3.0
Smoking status	
Never smoked, n (%)	18 (60.0)
Former smoker, n(%)	10 (33.3)
Current smoker, n (%)	2 (6.6)
Alcohol consumption	
None, n (%)	14 (46.7)
Moderate, n (%)	13 (43.3)
Excessive, n (%)	3 (10.0)
Past history	
CAD history, n (%)	22 (73.3)
Hypertension, n (%)	10 (33.3)
Diabetes mellitus, n (%)	10 (33.3)
Dyslipidemia, n (%)	30 (100)
Stroke, n (%)	0 (0)
Family history of CAD and CAD risk factor, n (%)	7 (23.3)

CAD; coronary artery disease, data represent mean ± SD

Preparation of the HDL and LDL Fractions

The serum samples were thawed on ice and incubated with 20% polyethethylene glycol (PEG) (Sigma-Aldrich, MO, USA) solution in 200 mmol/L of glycine buffer to remove apolipoprotein B (apoB)-containing lipoproteins, as previously described[18]. In brief, each serum sample was mixed with PEG solution (100:40) and incubated for 15 minutes at room temperature. The samples were then centrifuged at 4,000 rpm for 20 minutes to precipitate all apoB-containing lipoproteins, and the supernatant was kept as the HDL fraction (PEG-HDL). Phosphate-buffered saline (PBS) was incubated with PEG solution and centrifuged according to the same methods as the serum, and the supernatant was used as a control (PEG-PBS). LDL was isolated from pooled human plasma using ultracentrifugation (1.020-1.063)[19]. Acetyl LDL was generated by incubating LDL with acetic anhydride[20], then used in the cholesterol efflux assay.

Lipid Analysis

The serum lipid levels were measured enzymatically using a commercially available kit from WAKO (Osaka, Japan). The serum and HDL-associated apolipoprotein A-I (apoA-I), apoA-II and apoE levels were