$n=4$) than in non-disrupted plaques (1.11 ± 0.12, $n=6$, $p<0.05$), despite the equal expression of an internal marker protein, endothelin-1 receptor (Fig. 2A). Interestingly, the amount of active form MMP-9 determined by zymography was significantly higher in the disrupted (2.62 ± 0.12) than in non-disrupted plaques (0.72 ± 0.07, $p<0.05$), although pro MMP-9 activity was not significantly different in disrupted (1.8 ± 0.10) and non-disrupted plaques (1.4 ± 0.11) (Fig. 2B). There were no significant differences between the levels of pro and active forms of MMP-2, as demonstrated in its mRNA expression.

3.3. Immunohistochemistry

In the adjacent control regions (Fig. 3A), there was mild atherosclerosis where a few CD-68 positive macrophages existed. Under these conditions, MMPs and TIMPs were scatteringly positive. In contrast, TFPI-2 was diffusely positive in the intima and media. Plaque regions mainly consisted of lipid-rich core and fibrous tissue (Fig. 3B) where CD-68 positive macrophages were accumulated particularly in the shoulder regions of atheroma and all MMPs and TIMPs were strongly positive. It was interesting that, under these conditions, TFPI-2 was regionally positive in the plaque regions. Because of small number of examined plaques, we could not correlate expression of MMPs, TIMPs and TFPI-2 to the stage of plaque development.

4. Discussion

4.1. Gene expression of MMPs, TIMPs and TFPI-2 in plaque

One of the striking findings of the present study was that with a decreased TFPI-2 gene expression, the MMP-9 gene together with the MMP-9 protein was significantly upregulated in plaques, particularly in plaques with disrupted fibrous cap. Increased production of MMP-9 is thought to contribute to the progressive deterioration of the elastic lamellae associated with vessel remodeling, which could be closely related to the occurrence of plaque disruption [15]. Indeed, previous studies indicated that MMP-9 was present in the coronary plaque from unstable angina [16] and carotid plaque from

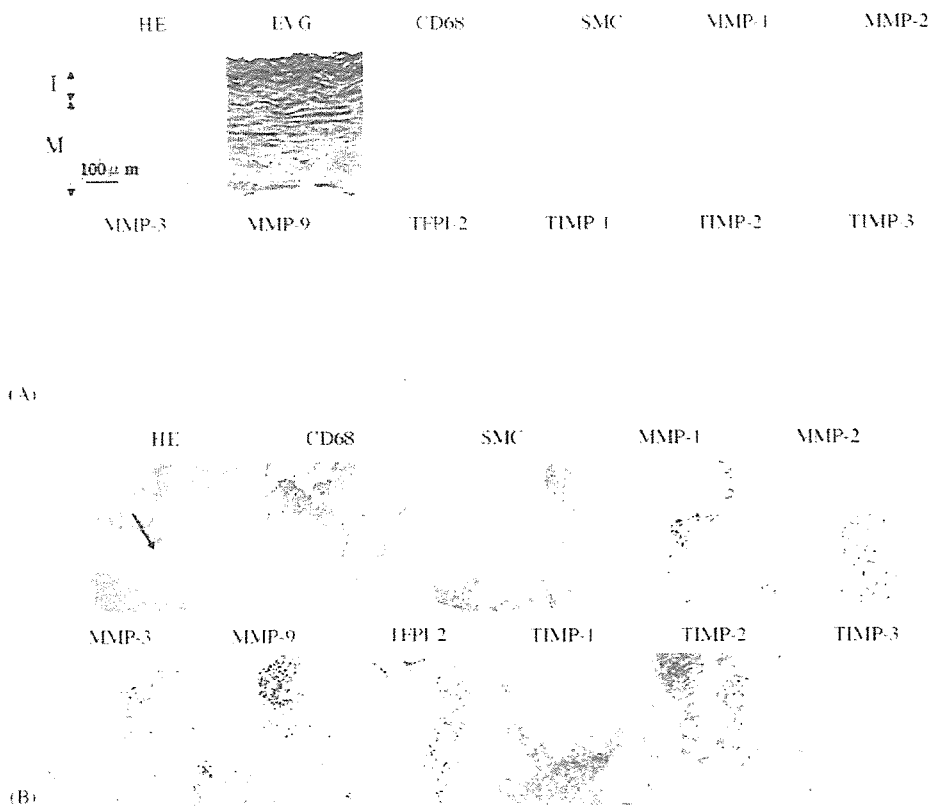

Fig. 3. Histologic and immunohistologic findings (with original magnification of ×25). (A) In the control tissues, there existed mild atherosclerotic lesion where a few CD-68 positive macrophages was found. Under these conditions, tissue factor pathway inhibitor (TFPI)-2 was diffusely positive in the intima and media, although matrix metalloproteinases (MMPs) and tissue inhibitor of MMPs (TIMPs) were scatteringly positive. An arrow indicates boundary between intima and media. (B) In plaque lesions with a lipid-rich core where CD-68 positive macrophages were accumulated, all MMPs and TIMPs were strongly positive particularly in the shoulder regions of atheroma (arrow). It was interesting that, under these conditions, TFPI-2 was regionally positive in this lesion. EVG, elastica van Gieson; HE, hematoxylin–eosin; I, intima; M, media; SMC, smooth muscle cell.

symptomatic patients [17]. We in fact demonstrated greater upregulation and function of MMP-9 in plaques with fissured fibrous cap at the mRNA as well as protein level, based on histological findings.

Simultaneous upregulation of the MMP-1 and -3 genes was also observed, as previously reported [18,19]. MMP-1 specifically cleaves collagen types I and III, which are key components of the extracellular framework of the arterial wall and major constituents of human atherosclerotic plaques, and activate other MMPs [6] that degrade denatured collagen, gelatin and elastin. MMP-3 has the widest substrate repertoire of all MMPs, showing activity against most of the extracellular proteins and proteoglycans [20]. However, unlike MMP-9, there were no differences in the expression of MMP-1 and -3 genes between plaques with and without rupture. This suggests that simultaneous upregulation of these MMPs is a plausible phenomenon in the development of atherosclerotic plaques.

This study demonstrates diminished gene expression of TFPI-2 in plaques that contain abundant MMPs. TFPI-2, originally considered as a serine proteinase inhibitor, is known to be highly expressed in smooth muscle cells of the relatively non-diseased tissue favoring ECM stability by inactivating collagenases such as MMP-1 as well as gelatinases probably through direct protein/protein interactions. Indeed, Herman et al. [7] demonstrate inverse relation between TFPI and MMP activity in atherosclerotic tissue. Thus, decreased TFPI-2 gene expression in plaques, as observed in the present study, might allow increased matrix degradation by MMP-1, -3 and -9 in plaques, enhancing their susceptibility to plaque development. It is interesting, under these conditions, TIMP-1 exhibited significantly higher expression in plaques than in controls. The combined deletion of TIMP-1 and ApoE in mice leads to a reduction in atherosclerotic plaque size [21], whereas overexpression of TIMP-1 induced by adenovirus-mediated transfer in ApoE-deficient mice leads to a decrease in plaque size and an increase in collagen content [22]. Taken together, under the condition where TFPI-2 was diminished to express, upregulation of TIMP-1 seems to counteract overexpression of MMPs, to exert an inhibitory effect on the development of atherosclerotic plaque.

However, the expression ratios of MMPs to TIMP-1 were still higher in the plaque compared with the control regions. Compensatory expression of TIMP-1 might not be sufficient to counteract the degenerative role of MMPs in the plaque, thus contributing to the development of atherosclerotic plaque. Particularly, the MMP-9/TIMP-1 ratio was significantly higher in plaques with disruption than in those without disruption. This suggests the functional significance of the imbalance of expression of these genes in the occurrence of plaque disruption. It would be of interest to examine which can play a more important role, TFPI-2 or TIMP-1, for the regulation of MMP activity, since compartmentalization might result in distinct microenvironments with corresponding variations in MMP/inhibitor ratios.

4.2. Clinical implications and limitations

A recent experimental study in which local MMP-9 was upregulated by gene transfection resulted in enhanced formation of local thrombus [23]. On the contrary, manipulation to augment expression of expression of TIMPs prevented the occurrence of plaque disruption [22]. Therefore, one might speculate that the altered balance of MMP-9/TIMP-1 with decreased TFPI-2 observed in the present study contributes to plaque disruption associated with or without regional thrombosis.

The carotid plaques examined in the present study were obtained from highly stenotic lesion probably representing the final stage of plaque development and destabilization. In acute coronary syndrome, however, atherosclerotic plaque disruption is known to occur at the sites of mild to moderate stenotic lesions [24] that were not examined in the present study. Although preliminary results indicate that in coronary plaques related to acute coronary syndrome MMP-9 gene was highly expressed in comparison with that in plaques from stable coronary disease [25], further study will need to confirm gene expression in carotid plaque from mild to moderate stenotic lesion.

The present study has a limitation regarding histological assessment of the presence of plaque disruption. Only a small portion of each plaque was examined histologically, and it may well be that features were missed in some patients. Several reports suggest that vulnerability to plaque rupture is a multifocal phenomenon particularly at the time of acute presentation [26,27]. Conversely, one might argue that we did not necessarily determine mRNA expression levels in the part of the plaque where histological analysis was performed. Even under these conditions, imbalanced expression of MMPs/TIMPs with reduction of TFPI-2 was observed in plaques, particularly in those associated with disruption. That the control regions were obtained from adjacent to the culprit lesion is another limitation. However, there was no histological evidence for plaque disruption in the control regions used for present study even in the presence of mild atherosclerosis. It can not be excluded, however, that the disruption of the fibrous cap could be resulted from surgical procedure, although we carefully examined the part of plaque where surgical procedures was not affected.

Whether upregulation of MMPs is the cause or result of plaque disarrangement is unclear. A recent study suggested that MMP-9 might have a protective effect against plaque development in double ApoE and MMP-9 knockout mice [28]. Thus, a causal relationship cannot be concluded until a controlled trial with a specific MMP-9 inhibitor is performed. Recently, MMP-8, traditionally associated only with neutrophils, which enhanced matrix breakdown by activating MMPs and/or by inactivating TIMP-1, was found to be highly expressed in macrophages in disrupted plaques [29]. Reduced expression of TFPI-2 might be related to the enhanced expression of neutrophil elastase in plaques, although MMP-

8 gene expression was not determined in the present study.

In the present study, we used real-time RT-PCR, which gives an estimate of mRNA expression instead of protein level for each enzyme and inhibitor, because it is still difficult to extract some proteases such as MMP-1, which binds strongly to connective tissue and to quantitatively assay enzyme activities [30]. However, it is important to determine the activity of TIMPs in protein level, since determination of gene expression can sometimes misinterpret the actual change of protein expression [31]. Therefore, evaluation of mRNA expression of multiple genes by the present real-time RT-PCR method in combination with determination of protein should be done for systematic evaluation of the activities of MMPs, TIMPs and TFPI-2 in clinical tissue samples.

Finally, the precise mechanism of the sustained overexpression of MMPs and TIMPs with reduction of TFPI-2 in advanced atherosclerotic plaque is still unclear. Our preliminarily report indicate that CXCR-2, a chemokine receptor, gene was highly upregulated in accordance with MMP expression in macrophages [32]. This suggests that overexpression of MMPs could be related to a continuous inflammatory reaction, although there was no difference in serum levels of hs-CRP between patients with ruptured and non-ruptured plaques. Further study of the regulatory mechanisms of chemokine and cytokine systems with transcription factors that also play a crucial role in MMP expression [33] may demonstrate a significant pathway for the expression and activation of proteinases and their inhibitors in human atherosclerotic lesions.

5. Conclusion

We applied a real-time RT-PCR method to quantitate mRNA expression in small samples of human carotid plaque. Levels of MMP-1, -3, -9 and TIMP-1 mRNAs were significantly upregulated in human carotid plaque where TFPI-2 mRNA was decreased to be expressed. The particular upregulation of MMP-9 and resultant imbalance of MMP-9/TIMP-1 expression could play a pivotal role in plaque disruption.

Acknowledgements

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Usefulness of Combined White Blood Cell Count and Plasma Glucose for Predicting In-Hospital Outcomes After Acute Myocardial Infarction

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Admission white blood cell (WBC) count and plasma glucose (PG) have been associated with adverse outcomes after acute myocardial infarction (AMI). This study investigated the joint effect of WBC count and PG on predicting in-hospital outcomes in patients with AMI. WBC count and PG were measured at the time of hospital admission in 3,665 patients with AMI. Patients were stratified into tertiles (low, medium, and high) based on WBC count and PG. Patients with a high WBC count had a 2.0-fold increase in in-hospital mortality compared with those with a low WBC count. Patients with a high PG level had a 2.7-fold increase in mortality compared with those with a low PG level. When a combination of different strata for each variable was analyzed, a stepwise increase in mortality was seen. There was a considerable number of patients with a high WBC count and low PG level or with a low WBC count and high PG level. These patients had an intermediate risk, whereas those with a high WBC count and high PG level had the highest risk, i.e., 4.8-fold increase in mortality, compared with those with a low WBC count and low PG level. Multivariate analysis was performed to assess the predictor for in-hospital mortality using WBC count and PG level as continuous variables and showed that WBC count and PG level were independently associated with in-hospital mortality. These findings suggested that a simple combination of WBC count and PG level might provide further information for predicting outcomes in patients with AMI. (Am J Cardiol 2006;97:1558–1563)

An increase in white blood cell (WBC) count and a high plasma glucose (PG) level are frequently observed in patients with acute myocardial infarction (AMI).[1] WBC count is a simple marker of inflammation, which plays an important role in acute coronary syndrome.[2] Numerous studies have demonstrated that a high WBC count is associated with a large infarct, impaired left ventricular function, and mortality after AMI.[3–8] The potential roles of WBC count in promoting blood coagulation, mediating microvascular no reflow, and causing myocyte dysfunction have been reported.[9] In addition, a high PG level has been associated with an increased risk of mortality and morbidity in patients with AMI, regardless of diabetic status.[10–15] Acute hyperglycemia has been reported to induce oxidative stress and activate coagulation, endothelial dysfunction, and inflammation.[16] Therefore, inflammation and hyperglycemia seem to have at least partly similar pathogenetic mechanisms that might increase myocardial injury. WBC count and PG level are inexpensive risk markers that are routinely assessed at the time of hospital admission in clinical practice. This study investigated the joint effects of admission WBC count and PG level on predicting in-hospital outcomes of patients with AMI.

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The Japanese Acute Coronary Syndrome Study (JACSS) is a retrospective, observational, multicenter study that was conducted at 35 medical institutions.[8,15] Between January 2001 and December 2003, consecutive patients

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Table 1
Baseline characteristics of patients with low, medium, and high white blood cell counts

Variable	WBC Count			p Value
	Low (n = 1,220)	Medium (n = 1,236)	High (n = 1,209)	
Age (yrs)	71 ± 11	67 ± 12	64 ± 13	<0.001
Men	782 (64%)	879 (71%)	963 (80%)	<0.001
Diabetes mellitus	331 (27%)	399 (32%)	438 (36%)	<0.001
Hypertension	748 (61%)	705 (57%)	648 (54%)	<0.001
Smoker	420 (34%)	562 (45%)	670 (55%)	<0.001
Previous infarction	188 (15%)	152 (12%)	131 (11%)	0.003
Killip's class ≥II	162 (13%)	212 (17%)	259 (21%)	<0.001
Time to admission (h)	6.5 ± 8.6	6.5 ± 8.6	7.0 ± 9.4	0.29
ST-segment elevation	1,008 (83%)	1,084 (88%)	1,105 (91%)	<0.001
Coronary intervention	870 (71%)	919 (74%)	918 (76%)	0.03
Thrombolysis	163 (13%)	214 (17%)	221 (18%)	0.002

Table 2
Baseline characteristics of patients with low, medium, and high plasma glucose levels

Variable	PG Level			p Value
	Low (n = 1,206)	Medium (n = 1,225)	High (n = 1,234)	
Age (yrs)	67 ± 13	68 ± 12	68 ± 12	0.07
Men	901 (75%)	883 (72%)	840 (68%)	0.001
Diabetes mellitus	145 (12%)	271 (22%)	752 (61%)	<0.001
Hypertension	631 (52%)	734 (60%)	736 (60%)	<0.001
Smoker	564 (47%)	561 (46%)	527 (43%)	0.11
Previous infarction	159 (13%)	135 (11%)	177 (14%)	0.04
Killip's class ≥II	132 (11%)	174 (14%)	327 (27%)	<0.001
Time to admission (h)	8.2 ± 10.1	5.9 ± 8.0	5.9 ± 8.1	<0.001
ST-segment elevation	1,025 (85%)	1,074 (88%)	1,098 (89%)	0.01
Coronary intervention	824 (68%)	946 (77%)	937 (76%)	<0.001
Thrombolysis	177 (15%)	216 (18%)	205 (17%)	0.13

who were admitted to the participating institutions within 48 hours after the onset of AMI were enrolled in the JACSS. WBC count and PG level were measured at the time of hospital admission in 3,665 patients who constituted the present study group. PG level was the nonfasting glucose level measured at the time of admission. AMI was defined by a combination of 2 of 3 characteristics, i.e., chest pain consistent with ongoing myocardial ischemia that persisted >30 minutes, ischemic electrocardiographic changes, and peak creatine kinase values >2 times the upper limit of normal. The study protocol was reviewed and approved by the ethical committee of each participating institution.

Creatine kinase was measured every 2 to 4 hours after admission and peak creatine kinase was obtained in 3,477 patients (95%). Contrast left ventriculography was performed at the time of predischarge catheterization and left ventricular ejection fraction was obtained in 1,980 patients (54%).

Blood samples for measurements of WBC count and PG level were obtained at the time of hospital admission. Patients were stratified into tertiles based on admission WBC count (low <8,300/mm^3, n = 1,220; medium 8,300 to 11,000/mm^3, n = 1,236; high >11,000/mm^3, n = 1,209). Patients also were stratified into tertiles based on admission PG level (low <133 mg/dl, n = 1,206; medium 133 to 182 mg/dl, n = 1,225; high >182 mg/dl, n = 1,234).

Statistical analysis was performed with the chi-square test for categorical variables. The *t* test and analysis of variance were used for continuous variables. The nominal regression model was used to obtain odds ratios and 95% confidence intervals for in-hospital mortality. In this model, WBC count and PG level were used as continuous variables. Multivariate analysis was performed after adjusting for age, gender, diabetes mellitus, hypertension, smoking, previous infarction, time to admission, Killip's class, ST-segment elevation, and use of reperfusion therapy. Reperfusion therapy included percutaneous coronary intervention and thrombolysis. Differences were considered statistically significant at $p < 0.05$.

This study examined 3,665 patients (mean age 68 ± 12 years). There were 2,624 men (72%), 1,168 diabetics (32%), 2,101 hypertensives (57%), and 1,652 smokers (45%). Previous infarction was present in 471 patients (13%) and a Killip's class ≥II was present in 633 patients (17%). Mean time to admission was 6.7 ± 8.8 hours;

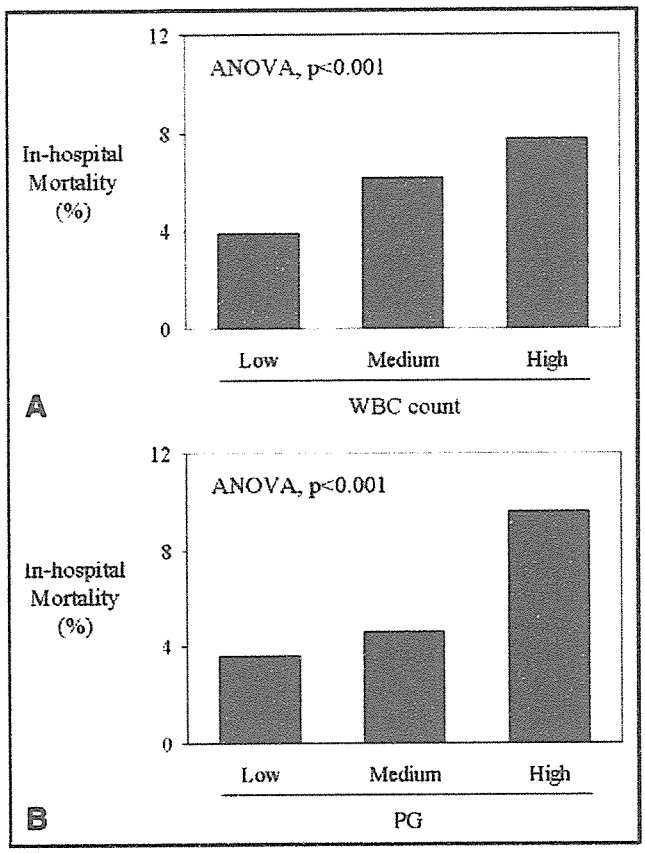

Figure 1. In-hospital mortality increased as tertiles of *(A)* WBC count and *(B)* PG level increased. ANOVA = analysis of variance.

2,631 patients (72%) were admitted to the hospital within 6 hours after the onset of AMI. Electrocardiography on admission showed ST-segment elevation in 3,197 patients (87%). Percutaneous coronary intervention was performed in 2,707 patients (74%), and coronary stents were used in 2,243 patients (61%). Baseline characteristics of patients with low, medium, and high WBC counts are listed in Table 1. Baseline characteristics of patients with low, medium, and high PG levels are presented in Table 2.

There were 218 deaths (5.9%) during hospitalization. In-hospital mortality was more likely to be higher in patients in a higher tertile of WBC count (3.9% in low, 6.2% in medium, and 7.8% in high WBC tertile, p <0.001; Figure 1). Peak creatine kinase concentrations were 2,244 ± 2,282 IU/L in the low WBC tertile, 2,851 ± 2,756 IU/L in the medium tertile, and 3,663 ± 3,117 IU/L in the high tertile (p <0.001; Figure 2). Predischarge left ventricular ejection fractions were 56 ± 12% in the low WBC tertile, 55 ± 13% in the medium tertile, and 52 ± 13% in the high tertile (p <0.001).

A higher PG tertile was also associated with higher in-hospital mortality (3.6% in low PG tertile, 4.6% in medium tertile, and 9.6% in high tertile, p <0.001; Figure 1). Peak creatine kinase concentrations were 2,444 ± 2,403 IU/L in the low PG tertile, 3,025 ± 2,820 IU/L in the medium tertile, and 3,270 ± 3,061 IU/L in the high tertile (p <0.001; Figure 2). Predischarge left ventricular ejection fractions were 56 ± 13% in the low PG tertile, 54 ± 12% in the medium tertile, and 52 ± 12% in the high tertile (p <0.001).

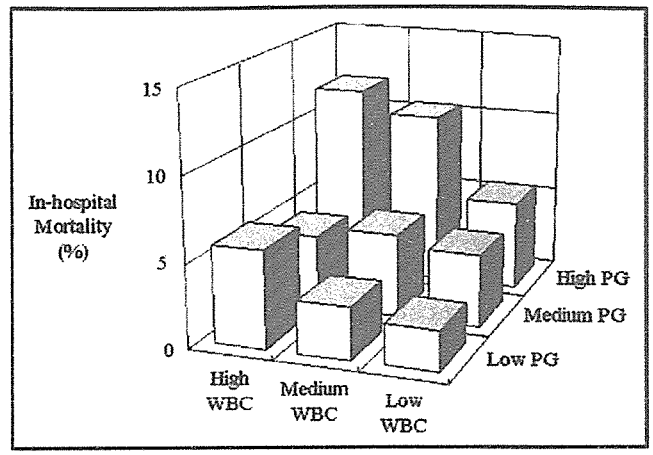

Figure 2. In-hospital mortality across tertiles of WBC count and PG level.

Table 3
Mulitivariate analysis assessing predictors for in-hospital mortality

	Chi-square	Odds Ratio	95% CI	p Value
Killip's class ≥II	115.9	5.81	4.22–8.00	<0.001
Age	57.4	1.07	1.05–1.09	<0.001
WBC count	33.1	1.17	1.11–1.24	0.001
PG	13.8	1.06	1.03–1.10	0.001
Previous infarction	8.7	1.75	1.21–2.54	0.003
Reperfusion therapy	6.9	0.63	0.45–0.89	0.009
Smoker	5.2	0.65	0.45–0.94	0.02
Diabetes mellitus	0.4	0.89	0.61–1.29	0.53
ST-segment elevation	0.3	0.89	0.58–1.36	0.59
Hypertension	0.2	0.93	0.67–1.28	0.65
Time to admission	<0.1	1.00	0.98–1.02	0.91
Men	<0.1	1.01	0.77–1.35	0.94

CI = confidence interval.

Influence of diabetes mellitus on in-hospital outcomes was also investigated. There was only a marginally significant difference in in-hospital mortality between diabetic and nondiabetic patients (7.1% vs 5.4%, p = 0.05). Peak creatine kinase level was lower in patients with diabetes mellitus than in those without (2,755 ± 2,673 vs 2,992 ± 2,852 IU/L, p = 0.02). There was no significant difference in predischarge left ventricular ejection fraction (54 ± 12% vs 55 ± 13%, p = 0.16).

In univariate analysis, WBC count (odds ratio 1.12 per 1,000/mm^3 increase, 95% confidence interval 1.09 to 1.11, p <0.001) and PG level (odds ratio 1.11 per 18 mg/dl [1 mmol] increase, 95% confidence interval 1.09 to 1.14, p <0.001) were associated with in-hospital mortality. Multivariate analysis showed that WBC count and PG level were independent predictors for in-hospital mortality and that diabetes mellitus was not (Table 3).

To examine possible interactions between WBC count and PG level, a combination of different strata for each

Table 4
Joint effect of WBC count and PG on in-hospital mortality, peak creatine kinase, and predischarge left ventricular ejection fraction

Variable	Low WBC Count			Medium WBC Count			High WBC Count			p Value for Trend
	Low PG (n = 491)	Medium PG (n = 398)	High PG (n = 331)	Low PG (n = 387)	Medium PG (n = 436)	High PG (n = 413)	Low PG (n = 328)	Medium PG (n = 391)	High PG (n = 490)	
In-hospital mortality (n = 3,665)	2.40%	4.30%	5.4%‡	3.10%	5.0%‡	10.4%*	5.8%‡	4.30%	11.8%*	<0.001
Peak creatine kinase (IU/L) (n = 3,476)	1,891 ± 2,048	2,435 ± 2,190*	2,538 ± 2,631*	2,409 ± 2,126*	2,946 ± 3,158*	3,162 ± 2,771*	3,327 ± 2,912*	3,700 ± 2,846*	3,854 ± 3,429*	<0.001
Predischarge left ventricular ejection fraction (%) (n = 1,979)	57 ± 12	56 ± 13	54 ± 12	57 ± 12	54 ± 13‡	53 ± 12†	53 ± 14†	53 ± 12*	50 ± 13*	<0.001

* p <0.001; † p <0.01; ‡ p <0.05 versus low WBC and low PG.

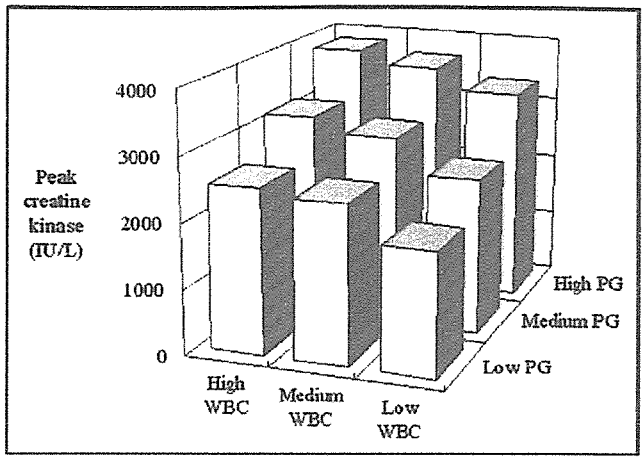

Figure 3. Peak creatine kinase across tertiles of WBC count and PG level.

variable was analyzed (Table 4) and showed a stepwise increase in in-hospital mortality, i.e., 4.8-fold increase for patients with a high WBC count and high PG level compared with those with a low WBC count and low PG level (Figure 3). A stepwise increase in peak creatine kinase level and a stepwise decrease in predischarge left ventricular ejection fraction were also observed.

• • •

This study showed that a high WBC count and high PG level were strong, graded, and independent predictors for in-hospital mortality after AMI. Infarct enlarged and left ventricular function decreased in a stepwise manner as WBC count and PG level increased. These findings suggested that a combination of WBC count and PG level provide further information for predicting outcomes of patients with AMI.

The JACSS is a Japanese multicenter registry. This study assessed 3,665 patients with AMI who were admitted to the participating medical institutions during the first 3 years of the new millennium. Seventy-five percent of patients underwent percutaneous coronary intervention as reperfusion therapy and coronary stents were used in most patients. Thus, the JACSS has data of patients with AMI in the contemporary era of percutaneous coronary intervention.

It remains unclear as to whether a high WBC count results in a larger infarct or is merely a consequence. However, recent clinical studies have suggested that a high WBC count is not a simple reflection of a larger infarct before reperfusion therapy. Barron et al[4] reported that there was no significant relation between WBC count and baseline creatine kinase (a surrogate of infarct size on presentation), anterior infarct location (which is associated with a large infarction), or symptom duration. Several experimental studies have suggested that inflammation and WBC count may directly contribute to a larger infarct.

Recent studies have demonstrated that acute hyperglycemia is a strong predictor for adverse outcomes after AMI in diabetic and nondiabetic patients.[10–15] Stranders et al[13] re-

ported that in-hospital mortality was higher in patients with admission hyperglycemia, but there was no significant difference in in-hospital mortality between diabetic and nondiabetic patients; diabetes mellitus was associated with higher long-term mortality. The present study associated a high PG level with a larger infarct, impaired left ventricular function, and high mortality after AMI. There was only a marginally significant difference in mortality between diabetic and nondiabetic patients. Multivariate analysis showed that hyperglycemia was an independent predictor for in-hospital mortality and that diabetes mellitus was not. In some cases, a high PG level could be a marker of preexisting, undiagnosed diabetes mellitus. However, hemoglobin A1c has been reported to not be a determinant of acute hyperglycemia and admission PG level was associated with high mortality after AMI even after adjustment for hemoglobin A1c.[17–19] These findings suggest that PG level is a more important predictor than diabetes mellitus for in-hospital outcomes after AMI in the contemporary era of percutaneous coronary intervention.

This study has the limitations of all retrospective investigations. However, assessment of admission WBC count and PG level on outcomes after AMI was 1 of the main purposes of the JACSS.[8,15] Differential WBC count might have provided further information but we did not assess WBC subtypes.

Appendix

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CETP (cholesteryl ester transfer protein) promoter −1337 C > T polymorphism protects against coronary atherosclerosis in Japanese patients with heterozygous familial hypercholesterolaemia

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ABSTRACT

CETP (cholesteryl ester transfer protein) and HL (hepatic lipase) play a role in the metabolism of plasma lipoproteins, but the effects of *CETP* and *LIPC* (gene encoding HL) genotypes on coronary atherosclerosis may be dependent on LDL (low-density lipoprotein)-receptor activity. Recently, the −1337 C > T polymorphism in the *CETP* gene has been reported in REGRESS (Regression Growth Evaluation Statin Study) to be a major determinant of promoter activity and plasma CETP concentration. In the present study, we have investigated the effects of the *CETP* promoter −1337 C > T and *LIPC* promoter −514 C > T polymorphisms on serum lipid profiles and risk of coronary atherosclerosis in 206 patients (154 males) with heterozygous FH (familial hypercholesterolaemia). To evaluate coronary atherosclerosis, we used CSI (coronary stenosis index) calculated from coronary angiograms. The *CETP* −1337 T allele was less frequent in subjects with a CSI ⩾ 14 (mean value) in the group with coronary artery disease ($P = 0.04$, as determined by χ^2 test). ANOVA revealed that HDL-C (high-density lipoprotein-cholesterol) and triacylglycerol (triglyceride) levels were not significantly higher in the presence of the *CETP* promoter −1337 T allele. Combined with *LIPC* promoter polymorphisms, HDL-C levels were highest and CSI were lowest with *CETP* −1337 CT+TT and *LIPC* −514 CC genotypes, but a significant interaction was not shown. A multiple logistic regression analysis revealed that, in patients with coronary atherosclerosis, the *CETP* −1337 CC genotype was a significant genetic risk factor in FH (odds ratio = 2.022; $P = 0.0256$). These results indicate that the *CETP* promoter −1337C > T polymorphism is associated with the progression of coronary atherosclerosis in Japanese patients with FH, independent of HDL-C and triacylglycerol levels.

Key words: cholesteryl ester transfer protein (CETP), coronary artery disease, familial hypercholesterolaemia, hepatic lipase, single nucleotide polymorphism.

Abbreviations: AP, angina pectoris; Apo, apolipoprotein; BMI, body mass index; CAD, coronary artery disease; CETP, cholesteryl ester transfer protein; CSI, coronary stenosis index; FH, familial hypercholesterolaemia; HDL, high-density lipoprotein; HDL-C, HDL-cholesterol; HL, hepatic lipase; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LDL-C, LDL-cholesterol; MI, myocardial infarction; NCBI, National Center for Biotechnology Information; REGRESS, Regression Growth Evaluation Statin Study.

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INTRODUCTION

CETP (cholesteryl ester transfer protein) is a key player in the metabolism of major plasma lipoproteins. CETP mediates the transfer of cholesteryl esters from HDL (high-density lipoprotein) to Apo (apolipoprotein) B-containing lipoproteins in exchange for triacylglycerols (triglycerides) [1]. CETP activities are known to be highly affected by genetic factors. For example, individuals with homozygous CETP deficiency have high HDL-C (HDL-cholesterol) levels and low LDL-C [LDL (low-density lipoprotein)-cholesterol] levels, and have no evidence of premature atherosclerosis [2]. Also, *CETP* gene polymorphisms, especially the *Taq*IB polymorphism identified in intron 1, is reported to be highly associated with plasma CETP concentrations and HDL-C levels. Moreover, recent meta-analyses revealed that this polymorphism is associated with the incidence of CAD (coronary artery disease) [3–7]. However, this polymorphism is unlikely to be functional by itself, instead representing a surrogate marker of functional variants of the *CETP* gene [8]. Indeed, previous studies have shown that the *CETP* promoter −629 A > C polymorphism has almost complete linkage disequilibrium with the *Taq*IB polymorphism [9,10], and that this polymorphism is associated with CAD [11]. On the other hand, we have reported [8] that the haplotype block consisting of −2668 G/A, −2505 C/A, −1337 C/T and the shortest (gaaa) repeat had a stronger association than *Taq*IB2 or −629 A/C with low plasma CETP concentrations and high HDL-C levels in healthy Japanese males. Moreover, functional interaction between −629 C/A, −971 G/A and −1337 C/T polymorphisms in the *CETP* gene is a major determinant of promoter activity and plasma CETP concentration in REGRESS (Regression Growth Evaluation Statin Study) [12].

In addition to CETP, HL (hepatic lipase) also plays a crucial role in the metabolism of plasma lipoproteins. HL is involved in the hydrolysis of triacylglycerol and phospholipids in IDL (intermediate-density lipoprotein) and large LDL particles to form smaller and denser LDL particles, and also plays a major role in promoting the conversion from HDL_2 into HDL_3 particles [13]. The effects of the *LIPC* genotype (the gene encoding HL) on atherosclerosis have been controversial [14], and may be dependent on LDL-receptor activity.

FH (familial hypercholesterolaemia) is an autosomal-dominant disorder characterized by primary hypercholesterolaemia with tendon xanthomas and premature CAD caused by mutations in the LDL receptor [15,16]. Mortality from CAD is reported to be several times higher in subjects with heterozygous FH than in the general population [15,16]. There are several reports that polymorphisms or mutations in the *CETP* gene influence the clinical characteristics of FH subjects [17,18]. Carmena-Ramon et al. [17] reported that in FH the *Taq*IB2 allele was associated with higher HDL-C and ApoAI levels. On the other hand, our previous study [18] showed that increased HDL-C levels caused by a heterozygous CETP deficiency was insufficient to prevent CAD in FH.

With this background, the present study investigated the effects of *CETP* promoter −1337 C > T and *LIPC* promoter −514 C > T polymorphisms on coronary atherosclerosis in Japanese patients with heterozygous FH.

METHODS

Study participants

We enrolled 206 consecutive Japanese patients with heterozygous FH (26–83 years old; 154 males) who attended our hospital. FH was diagnosed when one of the following two criteria was met: (i) primary hypercholesterolaemia [> 5.96 mmol/l (> 230 mg/dl) in any age group] in a patient with tendon xanthomas, or (ii) primary hypercholesterolaemia with a definitive diagnosis of FH in any first-degree relative [19]. All the females were postmenopausal, as defined by the absence of menstruation for > 6 months or having attained an age of ⩾ 60 years. Those with surgical menopause were excluded. For patients with MI (myocardial infarction), the age at the first event was recorded, whereas for patients with AP (angina pectoris), the age at which coronary angiography was performed was recorded. Inclusion criteria for this study were FH patients who were examined by coronary angiography because of chest symptoms and/or a positive exercise test before lipid-lowering therapy was initiated. Individuals who had thyroid disease, levels of triacylglycerol ⩾ 4.52 mmol/l (> 400 mg/dl) or who received lipid-lowering agents, corticosteroid or oestrogen hormone replacement therapy were excluded. All patients provided informed consent for participation in the present study, which was approved by the Ethical committee of Kanazawa University Graduate School of Medical Science.

Assessment of CAD

For the evaluation of CAD, we used CSI (coronary stenosis index) to quantify the severity of coronary atherosclerosis. The severity of stenotic changes was assessed by a score assigned to each of the 15 segments according to the classification of the American Heart Association Grading Committee. A normal coronary angiogram was graded as 0, stenosis of < 25 % was graded as 1, 25–50 % stenosis was graded as 2, 50–75 % stenosis was graded as 3, and > 75 % stenosis was graded as 4. CSI was defined as the sum of these scores in all 15 segments, producing a maximal value of 60 [15]. In the present study, MI was diagnosed in 56 subjects with

heterozygous FH (48 male), and AP was diagnosed in 53 subjects with heterozygous FH (all male). The mean CSI was 14.0 ± 11. The mean CSI in subjects who were diagnosed with MI and AP was 20, whereas the mean CSI in those subjects who were without clinical symptoms of CAD was 8. In our previous study [15], we observed that the age of coronary artery stenosis detectable by angiogram occurs after 17–25 years of age in male and female subjects with heterozygous FH. In the present study, 86 % of the subjects with MI and AP had a CSI > 14, whereas 80 % of subjects without clinical symptoms of CAD had a CSI < 14. Therefore we diagnosed CAD as being present when CSI was > 14.

Assessment of conventional risk factors

Data for BMI (body mass index), smoking history, alcohol drinking, blood pressure, diabetes status and lipid profile were collected. Hypertension was considered to be present if any antihypertensive treatment had been instituted, if systolic blood pressure was > 160 mmHg or diastolic blood pressure > 95 mmHg. Diabetes mellitus was diagnosed if fasting plasma glucose was ⩾ 6.70 mmol/l (> 120 mg/dl) or ⩾ 11.10 mmol/l (> 200 mg/dl) at 120 min after 75 g of oral glucose loading, or if HbA_{1c} (glycated haemoglobin) was ⩾ 6.5 %. For smoking status, we defined subjects who smoked ⩽ 10 cigarettes/day as non-smokers, past smokers as ex-smokers and current smokers.

Laboratory analysis

Blood samples were collected from subjects after 12 h of fasting before starting lipid-lowering agents. Total cholesterol, triacylglycerols and HDL-C levels were determined by standard enzymatic methods. LDL-C levels were calculated using the Friedewald formula [20]. Plasma CETP levels were determined by sandwich ELISA, as described previously [21].

Determination of *CETP* and *LIPC* promoter polymorphisms

Genomic DNA was isolated and purified from peripheral white blood cells. The *CETP* promoter −1337 C > T polymorphism and the *LIPC* promoter −514 C > T polymorphism (−480 in older reports) were analysed by PCR-RFLP (restriction-fragment-length polymorphism) methods, as described previously [8,22]. Accession numbers are as follows: *CETP*, gene ID 1071 [NCBI (National Center for Biotechnology Information) Entrez Gene database], nulceotide sequence NM_000078 (NCBI Entrez Nucleotide database) and −1337C/T SNP rs17231506 (NCBI SNP database); and *LIPC*, gene ID 3990 (NCBI Entrez Gene database), nucleotide sequence NM_000236 (NCBI Entrez Nucleotide database), −514 C/T SNP rs1800588 (NCBI SNP database) and −514 C/T USF binding site ccttttgaca(c/t)gggggtgaag.

Table 1 Characteristics of subjects in this study

Values are means ± S.E.M. HDL-C* was adjusted by multiple linear regression analysis, including gender, alcohol intake, smoking and BMI.

Parameter	CAD	non-CAD	*P* value
Gender (male/female)	77/17	77/35	0.0303
Age (years)	52 ± 12	50 ± 12	0.3001
BMI (kg/m^2)	23.7 ± 3.0	23.9 ± 2.7	0.5000
Total cholesterol (nmol/l)	8.34 ± 1.74	8.37 ± 1.63	0.9131
Triacylglycerol (nmol/l)	1.64 ± 0.69	1.65 ± 0.80	0.8618
HDL-C (nmol/l)	1.04 ± 0.28	1.09 ± 0.34	0.2052
HDL-C* (mmol/l)	1.17 ± 0.28	1.22 ± 0.31	0.3888
LDL-C (nmol/l)	6.55 ± 1.79	6.53 ± 1.66	0.8658
ApoAI (g/l)	1.01 ± 0.25	1.08 ± 0.24	0.1222
ApoB (g/l)	1.77 ± 0.53	1.78 ± 0.44	0.9052
ApoE (g/l)	0.06 ± 0.03	0.06 ± 0.02	0.7330
Hypertension (*n*)	32 (34.0 %)	20 (17.9 %)	0.0070
Diabetes mellitus (*n*)	34 (36.2 %)	22 (19.6 %)	0.0079
Smokers (*n*)	54 (57.4 %)	63 (56.2 %)	0.8629
Alcohol drinkers (*n*)	36 (38.3 %)	44 (39.3 %)	0.8848
CSI	23.7 ± 7.4	5.8 ± 4.3	< 0.0001

Statistical analyses

All values are expressed as means ± S.D. unless otherwise noted. The allele frequency was estimated by gene counting. One-way ANOVA was performed, followed by multiple comparisons using Fisher's protected least significant difference. Serum HDL-C was adjusted by multiple linear regression analysis. The prevalence of patients with hypertension, diabetes mellitus, current and past smoking, and alcohol drinking were compared between different groups using a χ^2 test. A multiple logistic regression analysis was used to predict CAD from the genotype of polymorphism, with conventional risk factors as covariates. A probability value of $P < 0.05$ was considered to be significant. All tests were performed with StatView software (version 5.0; SAS Institute).

RESULTS

Characteristics of study subjects

The clinical and biochemical characteristics of the study population either with CAD or without CAD (non-CAD) are summarized in Table 1. A total of 94 the subjects with heterozygotes FH were suffering from CAD. There were significantly more males and subjects with hypertension and diabetes mellitus in the CAD group compared with the non-CAD group.

Association between −1337 C > T polymorphism and CSI

The frequency of the *CETP* promoter −1337 T allele was 0.20 in both males and females; lower than in Caucasians [12]. A few subjects in the present study had

Table 2 *CETP* −1337 C > T polymorphism and plasma CETP levels

P value was determined using χ^2 test.

	CETP genotype				
	−1337 CC		−1337 CT + TT		
	n	CETP (μg/ml)	*n*	CETP (μg/ml)	*P* value
All	31	3.1 ± 1.1	13	2.6 ± 0.6	0.1364
Male	17	2.6 ± 0.6	8	2.4 ± 0.6	0.3139
Female	14	3.6 ± 1.3	5	3.0 ± 0.5	0.2831

the *CETP* promoter −1337 TT genotype (11 males and two females), and the T allele was less frequent in subjects with a CSI ⩾ 14. The distribution of the *CETP* promoter −1337 CC genotype differed significantly between those with a CSI ⩾ 14 and those with a CSI < 14 ($P = 0.0426$, as determined by a χ^2 test).

CETP promoter polymorphism and CETP concentrations

We compared plasma CETP concentrations between the −1337 CC and −1337 CT + TT genotypes in a subset of 44 subjects (25 males; Table 2). The CETP concentration tended to be lower in the presence of the T allele ($P = 0.14$).

CETP promoter polymorphism, lipid profile and development of CAD

The characteristics of subjects according to *CETP* promoter polymorphism are summarized in Table 3. As there were only two females with the TT genotype, we analysed men and women combined. HDL-C levels were not significantly higher in TT genotype, and the CSI tended to be lower in patients carrying the T allele ($P = 0.19$).

Effects of *CETP* and *LIPC* promoter polymorphisms on lipid profile and CSI

The frequency of the *LIPC* promoter −514 T allele was 0.53 in males and 0.50 in females, which is similar to the frequencies previously reported in Japanese subjects, but higher than those in Caucasians [22,23]. To investigate the effects of *CETP* and *LIPC* promoter polymorphisms on lipid profile, we compared four subgroups stratified by high *CETP* genotype CC compared with low CETP CT + TT, and high *LIPC* genotype CC compared with low *LIPC* genotype CT + TT. Figure 1 shows that the HDL-C level was significantly higher in −514 CC/−1337 CT + TT than in −514 CC/−1337 CC [1.22 ± 0.36 mmol/l (47 ± 14 mg/dl) compared with 0.98 ± 0.30 mmol/l (38 ± 10 mg/dl) respectively; $P < 0.02$], and it was significantly higher in −514 CC/−1337 CT + TT than in −514 CT + TT/−1337 CC or in both CT + TT ($P < 0.05$). LDL-C levels did not differ significantly between the four groups. CSI was significantly lower in −514 CC/−1337 CT + TT than in −514 CC/−1337 CC (9.6 compared with 17.2 respectively; $P = 0.02$), suggesting an interaction between *CETP* and *LIPC* genotype on CSI.

Table 3 Characteristics of the subjects according to *CETP* genotype status

Values are means ± S.E.M. HDL-C* was adjusted by multiple linear regression analysis, including gender, alcohol intake, smoking and BMI.

	CETP genotype		
	CC	CT	TT
n	127	66	13
Total cholesterol (nmol/l)	8.50 ± 1.71	8.18 ± 1.66	7.87 ± 1.27
Triacylglycerol (nmol/l)	1.62 ± 0.72	1.73 ± 0.82	1.48 ± 0.57
HDL-C (nmol/l)	1.04 ± 0.28	1.09 ± 0.37	1.17 ± 0.37
HDL-C* (mmol/l)	1.19 ± 0.28	1.22 ± 0.37	1.23 ± 0.37
LDL-C (nmol/l)	6.71 ± 1.81	6.29 ± 1.61	6.03 ± 1.24
ApoAI (g/l)	1.02 ± 0.25	1.09 ± 0.25	1.08 ± 0.21
ApoB(g/l)	1.81 ± 0.50	1.76 ± 0.47	1.66 ± 0.36
ApoE(g/l)	0.07 ± 0.03	0.07 ± 0.03	0.05 ± 0.02
Age (years)	50 ± 11	53 ± 13	48 ± 11
BMI (kg/m^2)	23.7 ± 2.7	24.3 ± 3.2	22.8 ± 2.5
Smokers (%)	69 (54.3)	38 (57.6)	10 (76.9)
Hypertension (%)	30 (23.6)	18 (27.3)	4 (30.8)
Diabetes mellitus (%)	35 (27.6)	19 (28.8)	2 (15.4)
CSI	15.0 ± 10.7	12.4 ± 10.6	11.6 ± 9.6

Multiple logistic regression analysis

A multiple logistic regression analysis was performed to determine the association of CAD and *CETP* promoter polymorphism and other conventional risk factors. Gender, hypertension, diabetes mellitus and *CETP* −1337 CC genotype exhibited significantly higher odds ratios; however, age, smoking, HDL-C and triacylglycerol levels, and the presence of *LIPC* −514 C > T were not significant variates (Table 4).

DISCUSSION

The present study investigated the effects of *CETP* and *LIPC* promoter polymorphisms on serum lipid profiles and risk of coronary atherosclerosis in subjects with heterozygous FH. None of the other coronary risk factors differed significantly between *CETP* genotypes; however, multiple logistic regression analysis revealed that coronary atherosclerosis was associated with the *CETP* −1337 CC genotype. An interaction between the *CETP* and *LIPC* genotypes for plasma HDL-C and CAD has also been shown.

To our knowledge, this is the first study on the effects of the *CETP* promoter −1337 C > T polymorphism

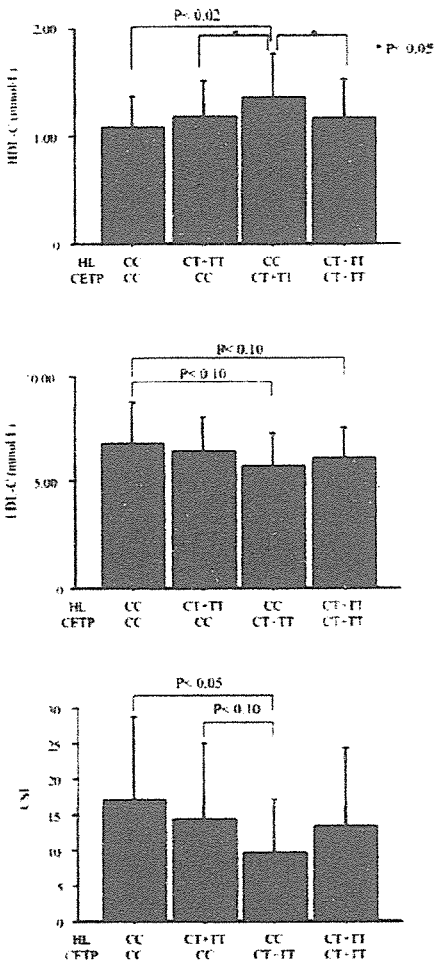

Figure 1 Effects of *CETP* and *LIPC* promoter polymorphisms on LDL-C, HDL-C and CSI

Subjects: −514 CC/−1337 CT+TT ($n=29$); −514 CT+TT/−1337 CC ($n=90$); −514 CC /−1337 CT+TT ($n=17$); −514 CT+TT/−1337 CT+TT ($n=62$). The HDL-C level was significantly higher in −514 CC/−1337 CT+TT than in −514 CC/−1337 CC (1.22±0.36 compared with 0.98±0.03 mmol/l; $P<0.02$), and it was significantly higher in −514 CC/−1337 CT+TT than in −514 CT+TT/−1337 CC or CT+TT ($P<0.05$). The LDL-C levels did not differ significantly between the four groups. CSI was significantly lower in −514 CC/−1337 CT+TT subjects than in −514 CC/−1337 CC subjects (9.6 compared with 17.2; $P=0.02$).

in coronary atherosclerosis and, therefore, the first to suggest that the *CETP* promoter −1337 C>T polymorphism is associated with the severity of coronary atherosclerosis in heterozygous FH. In a previous study [8], this polymorphism was associated with low plasma CETP concentrations and high HDL-C levels more strongly than with the *Taq*IB2 allele in elderly Japanese males and, recently, this polymorphism has been reported to be a major determinant of promoter activity and plasma CETP concentration in REGRESS [12]. Therefore we

Table 4 Multivariate adjusted relative prevalence odds ratio of coronary atherosclerosis by multiple logistic regression analysis

For sex, male = 1 and female = 0; for hypertension, yes = 1 and no = 0; for diabetes mellitus, yes = 1 and no = 0; for *CETP* −1337 C>T polymorphism, CC = 1 and T+ = 0; for *LIPC* −514 C>T polymorphism, CC = 2, CT = 1 and TT = 0.

Variate	Odds ratio	*P* value
Age	1.021 (0.993–1.050)	0.1431
Sex	4.283 (1.788–10.259)	0.0011
Hypertension	2.628 (1.252–5.519)	0.0107
Diabetes mellitus	2.136 (1.081–4.218)	0.0289
Smoking	0.992 (0.969–1.015)	0.3261
CETP −1337 C>T polymorphism	2.022 (1.090–3.754)	0.0256
LIPC −514 C>T polymorphism	0.856 (0.562–1.305)	0.4698

investigated this −1337 site rather than the well-known *Taq*IB polymorphism. As subjects with FH have a high risk of premature CAD, we determined the existence of early stage coronary atherosclerotic changes by using CSI. Our present data suggest that the association of the *CETP* genotype with cardiovascular risk is independent of serum HDL-C levels. As indicated in Table 3, there was no significant difference in HDL-C/adjusted HDL-C levels between *CETP* genotypes. The *CETP Taq*IB2 allele was associated with HDL-C, especially HDL_2-C, in Japanese subjects [24] and, therefore, if we had assessed HDL_2-C, this might have revealed a significant difference between the genotypes.

There are conflicting reports as to whether CETP is pro- or anti-atherogenic. Humans with homozygous CETP deficiency have markedly high HDL-C levels and decreased LDL-C levels, with no clear evidence of premature atherosclerosis [2]. A *CETP* gene mutation (D442G) was shown to be associated with increased LDL particle size [25], suggesting that CETP is pro-atherogenic. In contrast, Hirano et al. [26] have reported that the prevalence of CETP deficiency was lower in individuals older than 80 years of age residing in a district of northern Japan, suggesting that CETP deficiency is not association with longevity, and the same investigators have shown that reduced CETP activity in conjunction with reduced HL activity is associated with an increased risk of CAD [27]. On the other hand, Moriyama et al. [28] found in a cross-sectional analysis that HDL-C elevation (≥80 mg/dl) was protective against coronary heart disease, regardless of *CETP* genotype, in 19044 male and 29487 female Japanese subjects. In addition, a recent prospective study in the Honolulu Heart Program has shown the protective effects of heterozygous CETP deficiency against CAD, although the effect was not statistically significant [29].

At lower CETP concentrations, LDL-receptor activity is up-regulated, causing a reduction in serum LDL levels and leading to atheroprotection. Lowering CETP activity may be beneficial in an affluent environment, where high-fat and cholesterol-rich diets increase plasma LDL-C levels and down-regulate hepatic LDL-receptors, such as in FH. We presume that individuals with FH have higher CETP activity or concentration than normolipidaemic controls [30,31], which would be less pro-atherogenetic when they carry the *CETP* promoter −1337 T allele. De Grooth et al. [32] reported a significant positive correlation between carotid intima-media thickness and CETP levels in FH, suggesting that plasma CETP would be pro-atherogenic in FH. There are also some reports on the *CETP TaqI*B polymorphism and impaired glucose tolerance [33], suggesting that CETP could be pro-atherogenic independently of lipid metabolism. In the present study, however, there was no significant difference between *CETP* promoter −1337 C > T polymorphism and serum glucose levels (5.99 ± 1.94 mmol/l in −1337 CC compared with 5.72 ± 1.33 mmol/l in −1337 CT + TT; $P = 0.20$), and no difference in diabetes prevalence (results not shown).

In addition to CETP, HL also plays a crucial role in the metabolism of plasma lipoproteins, but the effects of CETP and HL activity on lipid profile and CAD are unclear [14,34]. The present study found no association between the *LIPC* promoter −514 C > T polymorphism and CAD and HDL-C levels; however, CSI with the *CETP* −1337 T allele and *LIPC* −514 CC was lowest in the subgroup. In another study from our laboratory (M. Takata and A. Inazu, unpublished work), HL activity was significant higher in −514 CC than CT + TT (0.282 ± 0.011 compared 0.231 ± 0.005 mmol/l respectively, $P < 0.001$) in hyperlipidaemic patients ($n = 325$, of which 183 were male). In human studies, HL activity tends to be elevated in the presence of smoking [35], insulin resistance in Type II diabetes mellitus [36], in females with omental fat mass [37] and males in general. These reports suggest that HL is pro-atherogenic. On the other hand, it has been reported that HL activity is lower in patients with CAD than in those without CAD [38]. Another group found that HL activity did not differ between subjects with and without CAD in REGRESS [39]. In an environment of low HL activity, IDL increases and it may be pro-atherogenic [40]. HL also promotes the formation of small and dense atherogenic LDL particles [13]. Lowering HL activity in hypertriglyceridaemia may decrease the pro-atherogenic risk due to an improved lipid profile, notably an increased LDL size [14]. In conditions where LDL-receptor activity is low, as in FH, HL activity appears to be inversely associated with CAD in subjects with low CETP concentrations (Figure 1), suggesting that the flux of cholesterol through the system of HDL-C transport may be more important in preventing atherosclerosis.

The main limitations of the present study were the relatively small sample size and the absence of data on HDL subclass and LDL particle size.

In conclusion, the *CETP* promoter −1337 C > T polymorphism is associated with the progression of coronary atherosclerosis in Japanese patients with FH, independent of HDL-C and triacylglycerol levels. We believe that this genetic variant of the *CETP* gene promoter could be an important determinant of coronary atherosclerosis in FH, and genotype differences between promoter variants and missense mutations need to be clarified in future investigations.

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Original Article

Effect of Walking with a Pedometer on Serum Lipid and Adiponectin Levels in Japanese Middle-aged Men

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Objective: To clarify the effects of walking with a pedometer on metabolic parameters, including adiponectin (APN).
Methods: We recruited 44 male Japanese volunteers (age, 37 ± 9 yrs; body mass index (BMI), 24.2 ± 2.9 kg/m^2; fasting plasma glucose (FPG), 96 ± 11 mg/dL; total cholesterol (TC) 190 ± 26 mg/dL; triglycerides (TG) 119 ± 80 mg/dL; HDL-C56 ± 14 mg/dL). Subjects were instructed to walk with a pedometer and record the number of steps they walked every day for 50 days. Serum adiponectin (APN) levels were measured by enzyme immunoassay. Treatment effects were examined by Wilcoxon's rank test.
Results: The average number of steps was 8211 ± 2084 per day. There were significant reductions in BMI, sBP, TG and TNF-α levels after 50 days, but no changes in adiponectin levels. We then divided the subjects into 2 groups according to the steps walked per day, namely, more than 8000 steps (MT group, n=22) and less than 8000 steps (LT group, n=22) and found that the reduction in TG and BP was observed only in the MT group.
Conclusions: Walking with a pedometer is effective for improving metabolic parameters, such as TG and blood pressure, but is not sufficient to increase adiponectin levels in Japanese men.

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Key words; Waist circumference, Percent body fat, Triglycerides, HOMA-R, Adiponectin

Introduction

To prevent lifestyle-related disease, such as dyslipidemia, hypertension, type 2 diabetes (T2DM) and cardiovascular disease, it is important to exercise daily. Walking with a pedometer is easy and can be incorporated in to daily life even for individuals with a busy schedule. It is generally accepted that regular exercise, including walking, is beneficial for preventing life-related disease, such as type 2 diabetes, hypertension and hyperlipidemia[1-3].

Adiponectin (APN), an adipocytokine, is a plasma protein expressed exclusively in adipose tissue[4-6], the plasma levels of which are linked to insulin sensitivity[7-13]. APN mRNA and its plasma concentrations are known to be reduced in T2DM and atherosclerotic disease[14, 15]. Several studies in humans, monkeys and rodents have shown that APN is an insulin-sensitizing cytokine and exhibited anti-atherogenic moieties[16].

Several studies have been conducted on the effects of exercise using a bicycle ergometer and treadmill walking on plasma APN levels in obese or overweight subjects[17-19], and they showed that exercise did not change APN levels. However, to our knowledge, no study has investigated the effects of walking with a pedometer, a practical form of daily exercise, on APN levels in individuals of normal body weight.

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With this background, the aim of this study was to clarify the effects of daily walking with a pedometer on APN levels as well as other metabolic parameters connected to life-style-related disease.

Subjects and Methods

We recruited 44 male volunteers (age, 37 ± 9 yrs) with a sedentary lifestyle who were instructed to wear a pedometer (TANITA FB-714) from the time they got up until they went to bed for 50 consecutive days and to record the number of steps they walked every day. All subjects were employees of a food company in Kanazawa city, Japan. We did not perform dietary therapy. Exclusion criteria included: abnormal liver or muscle enzymes, creatinemia, habitual alcohol intake >3 standard drinks/day or endocrinological disorder. Subjects who already walked daily using a pedometer were also excluded. Two subjects were on medication for hypertension (A-II receptor antagonist), three for hyperlipidemia (statins) and one for hyperuricemia (allopurinol). The dosage for these drugs did not change during the study period, and they had been on those medications for at least a few months before starting this study. Drinkers (consuming more than 30 grams of alcohol per day) and smokers (smoking more than 10 cigarettes) were also excluded from this study, because these factors are known to considerably affect plasma lipoprotein metabolism. Percent body fat (PBF) was determined from bioelectrical impedance analyses (BIA) using TANITA BC-118D (TANITA Corporation, Tokyo, Japan). Venous blood was obtained after a 12-h overnight fast. Serum total- and high-density lipoprotein (HDL) cholesterol and triglyceride levels were determined before and after 50 days. Serum cholesterol and triglyceride levels were measured by enzymatic methods. HDL-cholesterol was measured by a polyanion-polymer/detergent (PPD) method (Daiichi, Tokyo). Serum insulin concentrations were determined using a commercial enzyme immunoassay kit (Eiken, Tokyo, Japan). The homeostasis model assessment of insulin resistance (HOMA-R), a surrogate measure for insulin sensitivity[20], was calculated as fasting insulin X PG (mmol/L)/22.5. Serum levels of adiponectin [Otsuka Chemical] and TNF-α [R&D Systems] were measured by ELISA. A statement of institutional approval was granted for this study in accordance with the Declaration of Helsinki and informed consent was obtained from all participants.

Statistical Analysis

Statistical evaluation was performed using StatView-J 5.0 software (SAS Institute, Cary, NC, on a Macintosh Computer). All results are presented as the mean ± SD. Wilcoxon's rank test was used for evaluation of the significance of differences between before and after 50 days of walking with a pedometer.

Table 1. Baseline characteristics and changes in metabolic parameters in all subjects (n=44)

Variables	Before	After	*P* value
Body mass index, kg/m^2	24.2 ± 2.9	23.9 ± 2.9	0.0009
Percent body fat, %	22.7 ± 5.3	22.4 ± 5.4	0.21
Waist, cm	85.1 ± 7.7	84.7 ± 8.1	0.27
sBP, mmHg	122 ± 11	117 ± 10	0.0089
dBP, mmHg	77 ± 9	75 ± 9	0.176
TC, mg/dL	190 ± 26	189 ± 24	0.99
TG, mg/dL	119 ± 80	101 ± 52	0.019
HDL-C, mg/dL	56 ± 14	57 ± 13	0.25
FPG, mg/dL	96 ± 11	95 ± 11	0.36
Glycoalbumin, %	14.4 ± 1.0	14.3 ± 1.1	0.091
HOMA-R	1.87 ± 1.20	1.68 ± 1.15	0.19
TNF-α, pg/mL	1.58 ± 0.49	1.48 ± 0.51	0.023
Adiponectin, μg/mL	6.8 ± 2.3	6.6 ± 2.3	0.32

sBP, systolic blood pressure; dBP, diastolic blood pressure
TC, total cholesterol; TG, triglycerides; HDL-C, HDL-cholesterol
FPG, fasting plasma glucose; HOMA-R, homeostasis model assessment of insulin resistance

Results

Baseline Characteristics and Changes in Metabolic Parameters in All Subjects (Table 1)

The average number of steps of the 44 men over the 50-day period was 8211 ± 2084 steps per day. There was a subtle but significant reduction in body mass index (BMI), but no significant changes in PBF and waist circumference. There were significant reductions in systolic blood pressure (sBP), serum triglycerides (TG) and TNF-α levels; however, APN did not show a significant change during this period.

Changes in Metabolic Parameters in MT and LT Groups (Tables 2 and 3)

We divided the subjects into 2 groups according to the steps they walked per day, namely, more than 8000 steps (MT group, n=22) and less than 8000 steps (LT group, n=22). The average number of steps in the MT and LT groups was 9960 ± 1100 and 6462 ± 1128 steps per day, respectively. In the MT group, TG and sBP levels fell considerably after 50 days, whereas in the LT group none of these metabolic parameters showed a considerable change after 50 days of walking.

Table 2. Changes in metabolic parameters in subjects walking more than 8000 steps per day (n=22)

	Before	After	*P* value
Body mass index, kg/m^2	23.9 ± 2.3	23.7 ± 2.2	0.0018
Percent body fat, %	22.0 ± 4.5	21.9 ± 4.1	0.52
Waist, cm	84.4 ± 7.3	84.0 ± 7.2	0.41
sBP, mmHg	124 ± 11	114 ± 11	0.0005
dBP, mmHg	78 ± 10	74 ± 9	0.085
TC, mg/dL	189 ± 26	187 ± 25	0.71
TG, mg/dL	125 ± 75	102 ± 61	0.034
HDL-C, mg/dL	53.0 ± 9.8	53.1 ± 10.6	0.45
FPG, mg/dL	96 ± 10	95 ± 8.5	0.42
Glycoalbumin, %	14.4 ± 1.0	14.3 ± 1.0	0.15
HOMA-R	1.78 ± 1.06	1.46 ± 0.64	0.11
TNF-α, pg/mL	1.5 ± 0.31	1.38 ± 0.26	0.09
Adiponectin, μg/mL	6.7 ± 2.3	6.4 ± 2.3	0.21

sBP, systolic blood pressure; dBP, diastolic blood pressure
TC, total cholesterol; TG, triglycerides; HDL-C, HDL-cholesterol
FPG, fasting plasma glucose; HOMA-R, homeostasis model assessment of insulin resistance

Table 3. Changes in metabolic parameters in subjects with less than 8000 steps per day (n=22)

	Before	After	*P* value
Body mass index, kg/m^2	24.4 ± 3.5	24.2 ± 3.5	0.17
Percent body fat, %	23.3 ± 6.1	24.2 ± 6.5	0.24
Waist, cm	85.8 ± 8.2	85.5 ± 9.0	0.47
sBP, mmHg	119 ± 11	119 ± 8.7	0.86
dBP, mmHg	77 ± 7.8	77 ± 9.2	0.95
TC, mg/dL	191 ± 27	191 ± 24	0.73
TG, mg/dL	114 ± 86	99 ± 44	0.22
HDL-C, mg/dL	59 ± 17	60 ± 15	0.47
FPG, mg/dL	96 ± 13	96 ± 13	0.64
Glycoalbumin, %	14.4 ± 1.1	14.3 ± 1.3	0.37
HOMA-R	1.97 ± 1.34	1.89 ± 1.48	0.76
TNF-α, pg/mL	1.7 ± 0.6	1.6 ± 0.6	0.12
Adiponectin, μg/mL	7.0 ± 2.4	6.9 ± 2.4	0.77

sBP, systolic blood pressure; dBP, diastolic blood pressure
TC, total cholesterol; TG, triglycerides; HDL-C, HDL-cholesterol
FPG, fasting plasma glucose; HOMA-R, homeostasis model assessment of insulin resistance

Table 4. Correlations of waist circumference, BMI and % fat (PBF) versus several metabolic parameters at baseline

	Waist		BMI		% fat	
Variables	r	p	r	p	r	p
Age	0.203	0.187	0.111	0.472	0.214	0.163
BMI	0.878	<0.0001	–	–	0.901	<0.0001
PBF	0.893	<0.0001	0.901	<0.0001	–	–
Waist	–	–	0.878	<0.0001	0.893	<0.0001
sBP	0.315	0.0373	0.306	0.0433	0.299	0.049
dBP	0.409	0.0058	0.287	0.0588	0.434	0.0033
TC	−0.047	0.760	−0.031	0.841	−0.017	0.914
TG*	0.165	0.285	0.085	0.583	0.201	0.192
HDL-C	−0.336	0.0256	−0.384	0.0101	−0.324	0.032
FPG	0.400	0.0071	0.374	0.0124	0.434	0.0032
HOMA-R*	0.662	<0.0001	0.596	<0.0001	0.667	<0.0001
Adiponectin*	−0.417	0.0049	−0.300	0.0477	−0.384	0.0102

*These values were logarithmically transformed before correlation analysis.
BMI, body mass index; PBF, percent body fat; sBP, systolic blood pressure; dBP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; HDL-C, HDL-cholesterol; FPG, fasting plasma glucose; HOMA-R, homeostasis model assessment of insulin resistance

Correlation of BMI, Percent Body Fat (PBF) and Waist Circumference with Metabolic Parameters at the Baseline (Table 4)

Significant associations were noted between BMI, PBF and waist circumference versus serum APN levels, among which the association between waist circumference and serum adiponectin levels was most pronounced. Similarly, there were considerable associations between BMI, PBF and waist circumference, and HOMA-R.

Correlation between the Number of Steps Per Day with % Changes in Metabolic Parameters During the Study Period

Among the metabolic parameters investigated, % changes in sBP showed a significant correlation ($r=-0.401$, $p=0.0069$) and BMI tended to show a correlation ($r=-0.276$, $p=0.068$) with the number of steps per day, whereas no other metabolic parameters showed a significant correlation.