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Association of plasma homocysteine with serum interleukin-6 and C-peptide levels in patients with type 2 diabetes

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Abstract

Hyperhomocysteinemia is an independent risk factor for atherosclerotic disease. Because serum markers of inflammation and the metabolic syndrome are also associated with atherosclerotic disease and insulin resistance, we investigated whether plasma homocysteine (Hcy) levels were associated with serum markers of inflammation and factors of metabolic syndrome in 223 elderly patients with type 2 diabetes mellitus. The levels of plasma Hcy and serum interleukin-6 (IL-6), high-sensitivity C-reactive protein, and C-peptide were measured. The C677T mutation of methylenetetrahydrofolate reductase (MTHFR) gene was detected using the polymerase chain reaction–restriction fragment length polymorphism method. The number of abnormal metabolic factors (presence of diabetes, blood pressure $\geq$130/85 mm Hg, triglycerides $\geq$150 mg/dL, high-density lipoprotein cholesterol <35 mg/dL (men) or <39 mg/dL (women), or body mass index >25 kg/m^2) was assessed. Elevated plasma Hcy levels correlated significantly with serum IL-6 (r = 0.25, P < .001), C-peptide (r = 0.22, P < .01), and the number of abnormal metabolic factors (r = 0.20, P < .01), but not with C-reactive protein. Multiple linear regression analysis revealed that log-transformed IL-6, serum C-peptide, vitamin B_{12}, and creatinine were significant determinants of plasma Hcy levels. The correlation between Hcy and IL-6 levels was strongest in those with TT genotype of C677T MTHFR among 3 genotypes. The association between plasma Hcy and serum IL-6 levels supports the hypothesis that the activation of innate immunity is involved in the pathogenesis of arteriosclerosis in patients with diabetes mellitus who are homozygous for the TT genotype of C677T MTHFR.

1. Introduction

A moderate increase in plasma homocysteine (Hcy) concentration is known to be a new risk factor for arteriosclerotic disease [1-3]. We have shown previously that elevated plasma Hcy is associated significantly with the presence of both symptomatic and asymptomatic macrovascular disease in diabetic patients [4,5]. Hcy may have several actions on vascular cells: impairment of endothelial function, enhancement of low-density lipoprotein oxidation, and promotion of smooth muscle cell proliferation as a result of increased free radical generation [6]. Hcy can also activate the coagulation system, thereby promoting arteriosclerosis and thrombosis [6]. However, the exact molecular mechanisms of action remain poorly understood.

It is, however, well established that inflammation has a role in the pathogenesis of atherosclerosis, diabetes, and insulin resistance [7]. Increased levels of inflammatory markers, such as the high-sensitivity C-reactive protein (CRP) and interleukin-6 (IL-6), can predict increased risk of cardiovascular disease [8,9]. Serum IL-6 is also a predictive marker for the development of type 2 diabetes [10], as it is involved in the pathogenesis of insulin resistance [11,12], and it stimulates insulin secretion [13]. The proinflammatory cytokines (IL-6 and tumor necrosis factor α [TNF-α]) released by the activation of innate immunity also favor the progression of atherosclerosis in diabetes mellitus [7].

Because Hcy may act directly on monocytes and endothelial cells to stimulate IL-6 production in in vitro studies [14,15], we postulated that there may be an association between plasma Hcy and serum IL-6 levels in patients with type 2 diabetes. It has been reported that plasma Hcy levels are associated with hyperinsulinemia [16,17]. It is possible that the relationship between high Hcy and increased IL-6 levels originates through a common mechanism such as insulin resistance. Few studies have demonstrated a clear relation between Hcy and inflammatory cytokines in clinical studies [18,19]. Therefore, this study investigated whether

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levels of plasma Hcy were independently associated with serum IL-6 in elderly patients with type 2 diabetes mellitus.

2. Subjects and methods

2.1. Subjects

We recruited 223 outpatients older than 65 years with type 2 diabetes from 463 participants in the Quality-of-Life Study in the Elderly Diabetes, which began in 1997 [20]. Participants were registered consecutively at the Department of Endocrinology diabetes clinic in the Tokyo Metropolitan Geriatric Hospital. Patients were excluded if they had severe dementia or aphasia. Twenty-nine subjects were excluded because they had either chronic renal failure with a serum creatinine level of >1.3 mg/dL, were taking vitamin supplements or hormone replacement therapy to prevent or slow osteoporosis, or had another endocrine disease. Using these criteria, 223 patients (78 men and 145 women) were enrolled in this study. They had a mean age of 74 ± 5 years, a diabetes duration of 13.9 ± 8.2 years, and a mean body mass index (BMI) of 23.0 ± 3.4 kg/m^2. Although the participants had a higher activities of daily living score (11.6 ± 2.4 vs 10.6 ± 3.2, $P < .001$) than nonparticipants, there were no significant differences in age (74.9 ± 5.3 vs 75.7 ± 6.5 years) and duration of diabetes (13.9 ± 8.2 vs 14.6 ± 9.0 years) between the 2 groups.

The definition of diabetic complications and ischemic heart disease (IHD) has been previously reported [20]. The prevalence of retinopathy, microalbuminuria, persistent proteinuria, and neuropathy was 37%, 28%, 24%, and 60%, respectively. IHD was considered to be present when diabetic patients had one of the following: (1) a history of myocardial infarction characterized by typical clinical symptoms, typical electrocardiogram changes, and enzymatic changes in creatinine phosphokinase and creatinine phosphokinase–myocardial band (MB), or (2) a history of angina pectoris, postload electrocardiographic findings, and postload cardiac scintigram findings confirmed by coronary angiography as previously reported [20]. A diagnosis of asymptomatic and symptomatic cerebral infarction was made using the medical history and neurological deficits and was confirmed by the finding of a focal positive T2–high-intensity lesion larger than 3 mm correlating with a T1–low-intensity lesion on brain magnetic resonance images [5]. The prevalence of asymptomatic stroke, symptomatic stroke, angina, and myocardial infarction was 32%, 19%, 12%, and 4%, respectively.

2.2. Laboratory methods

Venous blood was drawn to determine blood glucose, HbA1c, and serum concentrations of total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglycerides (TGs), and creatinine according to established methods.

Fasting plasma Hcy was determined by modified high-performance liquid chromatography with fluorescence detection as previously reported [21].

Fasting serum folate and vitamin B_{12} levels were measured by chemiluminescent assays [5], whereas serum vitamin B_6 was measured by high-performance liquid chromatography [5].

The concentrations of serum IL-6 (intra-assay precision; coefficient of variation [CV] = 2.5%, n = 20) were measured using a chemiluminescent enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, Minn, USA) [22]. The immunoassay was calibrated against a highly purified *Escherichia coli*–expressed recombinant human IL-6 produced at R&D Systems. The levels of highly sensitive CRP (intra-assay precision; CV = 2.9% at the 0.1 mg/dL CRP level) were measured using a latex enhanced immunonephelometric assay on a BN II analyzer (Dade Behring, Tokyo, Japan) [23].

Fasting serum C-peptide and immunoreactive insulin levels were measured by radioimmunoassay methods using commercial kits, with C-peptide providing an index of insulin secretion and/or resistance. Insulin sensitivity was estimated using a homeostasis model assessment for insulin resistance {HOMA-IR; (fasting glucose [measured in millimoles per liter]) (fasting insulin [measured in microunits per milliliter])/22.5} [24].

The C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene was detected using the polymerase chain reaction (PCR)–restriction fragment length polymorphism method [25]. Venous blood samples were applied to genomic DNA extracting columns according to the manufacturer's protocol. The genomic DNA was amplified by the PCR method using a GeneAmp PCR kit and primers as previously reported [26]. The amplification products were treated with *Hin*fI, followed by electrophoresis in 9.6% polyacrylamide gels, and staining with ethidium bromide. This method allowed us to detect a point mutation at nucleotide 677, which produces an amino acid substitution from alanine to valine in the MTHFR gene. Three genotypes were identified, which included the CC, CT, and TT genotypes. The homozygous TT genotype is a thermolabile form and has reduced MTHFR activity.

2.3. Assessment of metabolic syndrome

The presence of the metabolic syndrome was defined as 3 or more of the following metabolic risk factors: presence of diabetes, blood pressure ≥130/ 85 mm Hg, TG ≥150 mg/dL, HDL-C <35 mg/dL (men) or <39 mg/dL (women), or BMI >25 kg/m^2. The number of abnormal metabolic factors (ie, positively occurring metabolic factors) was also assessed between patients. Then, the association of the presence of the metabolic syndrome and number of abnormal metabolic factors with plasma Hcy was examined.

2.4. Statistical analysis

The correlation between variables was assessed by calculating Spearman rank correlation coefficients. The patients were divided into 3 groups based on Hcy levels.

The differences among the 3 groups were analyzed using analysis of variance with Dunnett multiple comparison test. Because of a skewed distribution, serum IL-6 was log-transformed and designated as log (IL-6). To examine whether the potential association between Hcy and IL-6 level was independent, we performed stepwise multiple linear regression analyses using the following 3 models: (1) age, sex, BMI, systolic blood pressure (SBP), HDL-C, HbA1c, smoking (yes/no), log (IL-6), folate, vitamin B_{12}, vitamin B_6, serum creatinine, and Hcy; (2) age, sex, C-peptide, smoking, HbA1c, log (IL-6), folate, vitamin B_{12}, vitamin B_6, creatinine, MTHFR TT genotype (yes/no), and Hcy; and (3) age, sex, number of metabolic factors, smoking, HbA1c, log (IL-6), folate, vitamin B_{12}, vitamin B_6, creatinine, MTHFR TT genotype, and Hcy levels. Statistical analyses were conducted using the SPSS statistical software package for Windows (version 11.0; SPSS, Chicago, Ill, USA).

3. Results

3.1. Plasma Hcy and Serum IL-6 levels

The subjects in this study were divided into 3 groups based on plasma Hcy levels: ≤7.4, 7.5 to 9.4, and ≥9.5 nmol/mL. The highest Hcy group had significantly higher IL-6, log (IL-6), and C-peptide levels than the other 2 groups (Table 1). Serum levels of vitamin B_{12} and B_6 were lower in the highest Hcy group than in lowest Hcy group. There were no significant differences in BMI, duration of diabetes, HbA1c, and serum levels of folate and CRP among the 3 groups, although age and prevalence of men were highest in the highest Hcy group.

Table 1
Clinical characteristics of diabetic patients grouped according to plasma Hcy levels

	Lowest Hcy group (n = 72)	Middle Hcy group (n = 77)	Highest Hcy group (n = 74)
Range of Hcy (nmol/mL)	4.3-7.4	7.5-9.4	9.5-32.6
Age (y)	73.7 ± 5.1	75.4 ± 5.2	75.8 ± 5.5*
Sex (men, %)	20.8	33.8	50.0*
BMI (kg/m^2)	22.5 ± 3.5	22.7 ± 3.2	23.8 ± 3.3
Duration of diabetes (y)	13.9 ± 8.2	15.3 ± 8.4	12.5 ± 7.6
HbA1c (%)	7.5 ± 1.3	7.8 ± 1.4	7.2 ± 1.3
Treatment of diabetes (diet/oral drugs/insulin, %)	33:53:14	26:64:10	31:58:11
Serum folate (ng/mL)	10.1 ± 4.1	8.9 ± 3.3	8.8 ± 4.7
Serum vitamin B_{12} (pg/mL)	902 ± 435	785 ± 337	639 ± 352**
Serum vitamin B_6 (ng/mL)	16.9 ±17.2	17.2 ± 28.2	11.7 ± 9.7*
MTHFR types (CC/CT/TT, %)	34:51:14	25:51:24	44:41:15
Serum IL-6 (pg/mL)	0.85 ± 1.81	1.02 ± 1.80	2.10 ± 5.61*
Log (IL-6)	−0.75 ± 0.99	−0.30 ± 0.97*	−0.16 ± 1.1**
CRP (g/dL)	105 ± 128	145 ± 161	91 ± 86
Serum C peptide (ng/mL)	1.9 ± 0.9	2.2 ± 0.9	2.4 ± 1.1*

DBP indicates diastolic blood pressure.
* $P < .05$ vs the lowest Hcy group.
** $P < .001$ vs the lowest Hcy group.

Table 2
Spearman rank correlation coefficients between plasma Hcy or serum IL-6, and metabolic factors among diabetic patients

	Plasma Hcy	Serum IL-6
Serum IL-6	0.25*	–
CRP	0.05	0.34*
BMI	0.14**	0.15**
HbA1c	−0.10	0.18***
SBP	0.20***	0.02
DBP	0.20***	−0.01
TGs	0.02	0.05
HDL-C	−0.16**	−0.14**
Presence of metabolic syndrome	0.21***	0.07
Number of metabolic factors	0.20***	0.09
Serum C-peptide	0.20***	0.12
Serum insulin****	0.09	0.20**
HOMA-IR****	0.06	0.17**

* $P < .001$.
** $P < .05$.
*** $P < .01$.
**** Spearman rank correlation coefficients were calculated after excluding patients receiving insulin therapy.

Among the diabetic patients, Spearman rank correlation coefficients between Hcy and IL-6, or metabolic factors were assessed (Table 2). Plasma Hcy levels were significantly correlated with serum IL-6 but not with CRP. As expected, serum IL-6 correlated significantly with CRP levels. Plasma Hcy levels were associated significantly with BMI, blood pressure, HDL-C, fasting serum C-peptide, the number of abnormal metabolic factors, and presence of the metabolic syndrome (defined as 3 or more metabolic factors). Serum IL-6 levels were correlated with BMI, HbA1c, HDL-C, fasting serum insulin, and HOMA-IR but not with the number of abnormal metabolic factors.

3.2. C677T MTHFR genotypes

The frequencies of C677T MTHFR genotypes were 35% for CC genotype, 47% for CT genotype, and 18% for TT

Table 3
Effects of C677T MTHFR genotypes on plasma Hcy, serum IL-6 levels, or correlation between Hcy and IL-6

	MTHFR		
	CC genotype	CT genotype	TT genotype
Genotype frequency (%)	35	47	18
Hcy (nmol/mL)	10.1 ± 4.4	9.1 ± 4.1	9.0 ± 2.6
IL-6 (pg/mL)	1.8 ± 5.4	1.3 ± 2.4	0.6 ± 0.4
Log (IL-6)	−0.19 ± 1.13	−0.41 ± 1.0	−0.76 ± 0.98*
Spearman correlation coefficients between Hcy and IL-6	0.19	0.20*	0.51**

* $P < .05$ vs MTHFR CC genotype.
** $P < .001$ vs MTHFR CC genotype.

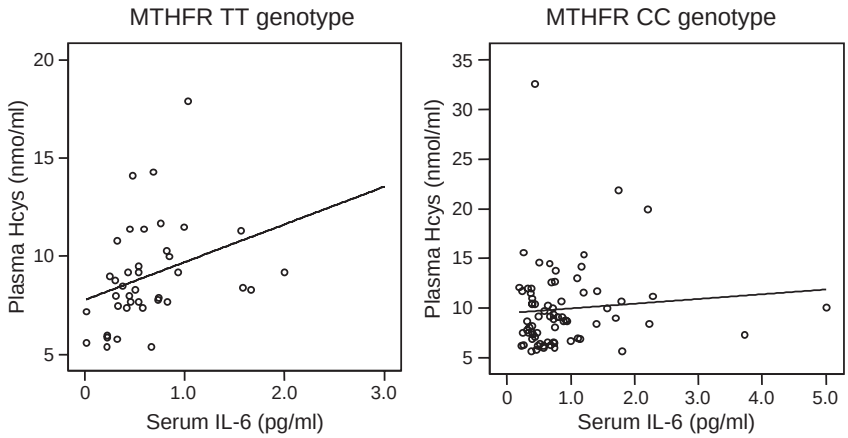

Fig. 1. Relationship between Hcy and IL-6 levels in diabetic patients with TT genotype (A) or CC genotype (B) of C677T methylenetetrahydrofolate reductase. A significant correlation between Hcy and IL-6 levels was observed in those with TT genotype (r = 0.51, P < .001), but not in those with CC genotype (r = 0.19, P = NS).

genotype (Table 3). These genotypes did not affect plasma Hcy levels or the prevalence of stroke and IHD, as previously reported [5]. Although we observed significant correlations between Hcy and IL-6, the correlation was highest in those with TT genotype (r = 0.51, P < .001) compared with those with CT genotype (r = 0.20, P < .05) or CC genotype (r = 0.19, P = NS) (Table 3 and Fig. 1). In contrast, the CC genotype of C677T MTHFR increased log (IL-6) levels compared with those with the TT genotype. Serum C-peptide levels were also higher in patients with the CC genotype than those with the TT genotype or CT genotype (2.5 ± 1.1 vs 2.0 ± 0.9 or 1.9 ± 0.8 ng/mL, both P < .05).

3.3. Multivariate analysis

To examine whether the association between Hcy and IL-6 was independent, we performed a stepwise multiple linear regression analysis using the following variables: age, sex, BMI, SBP, HDL-C, HbA1c, smoking, log (IL-6), folate, vitamin B_{12}, vitamin B_6, and serum creatinine. The multivariate analysis showed that age (standardized regression coefficient, β = .159, P = .011), BMI (β = .157, P = .014), SBP (β = .199, P = .002), low vitamin B_{12} (β = −.159, P < .001), serum creatinine (β = .269, P < .001), and log (IL-6) (β = .143, P = .027) remained significant variables associated with Hcy levels (R^2 = 0.299). Even when the number of abnormal metabolic factors was included in the model, the association of Hcy with log (IL-6) (β = .149, P = .024), the number of abnormal metabolic factors (β = .166, P = .011), vitamin B_{12} (β = −.271, P < .001), and serum creatinine (β = .252, P < .001) remained significant (R^2 = 0.252). Because serum C-peptide correlated with a number of abnormal metabolic factors (r = 0.38, P <0 .001) as well as HOMA-IR (r = 0.72, P < .001), we designated the C-peptide as an index of insulin resistance. When we entered the C-peptide into the model instead of the number of abnormal metabolic factors, significant determinants of plasma Hcy level were log (IL-6) (β = .190, P = .011), C-peptide (β = .159, P = .029), vitamin B_{12} (β = −.333, P < .001), and serum creatinine (β = .175, P = .019)(R^2 = 0.223).

As previously reported [5], there was a significant association between Hcy levels and asymptomatic and symptomatic magnetic resonance–defined cerebral infarction (10.2 ± 4.9 vs 8.5 ± 3.3 nmol/mL, P < .05; and 11.8 ± 9.2 vs 8.5 ± 3.3 nmol/mL, P < .05). Linear regression analysis showed that the presence of cerebral infarction and IHD did not affect the relation between Hcy and log (IL-6) levels (β = .192, P = .007; and β = .222, P < .001).

4. Discussion

In this study, we showed an independent association between elevated Hcy and IL-6 levels, but not CRP, in patients with type 2 diabetes. Previous studies on associations between plasma Hcy levels and serum inflammatory markers were controversial. Mojiminiyi et al [18] investigated the correlations between CRP or IL-6 and plasma Hcy levels in diabetic patients with or without coronary heart disease (CHD). Although they found a significant correlation of CRP with Hcy in patients with CHD, but not in patients without CHD, serum IL-6 was not associated with plasma Hcy. Erren et al [19] reported that plasma Hcy had no correlation with IL-6 and CRP in patients with CHD or peripheral artery disease. These discrepancies may be explained by the differences in ages, races, and prevalence of macrovascular complications and Hcy-related gene mutations in the subjects studied.

In addition, we found that the association between Hcy and IL-6 levels was highest in those with TT genotype of C677T MTHFR gene polymorphism. The high prevalence of TT genotype in our studies, compared with that of whites in other studies (approximately 12%) [27,28], might explain the significant association between Hcy and IL-6 in the present study. The C677T MTHFR TT variant has been associated with immune activation such as acute rejection of kidney transplantation [29]. The C677T MTHFR mutation might be

involved in the modulation of Hcy-induced activation of the immune system, leading to the pathogenesis of atherosclerosis and insulin resistance in diabetes mellitus [13].

In contrast, the C677T MTHFR CC genotype, which did not have a Hcy-elevating effect, increased both serum IL-6 and C-peptide levels. Because both serum IL-6 and C-peptide levels reflect the degree of insulin resistance [9-12], it appears that the C677T MTHFR polymorphism may have a complex modulating effect on the secretion and/or sensitivity of insulin, independent of Hcy levels [30].

It can be speculated that the association between Hcy and IL-6 levels may be caused by the actions of Hcy on the activation of innate immunity. Elevated Hcy may stimulate IL-6 secretion in monocytes [14] and vascular endothelial cells [15]. Hcy may also induce other proinflammatory cytokines such as monocyte chemoattractant protein 1 and IL-8 in human monocytes and smooth muscle cells, IL-1β production in human monocytes, and TNF-α production in monocyte-derived macrophages [31-33]. The expression of cytokines has been shown to occur through activation of nuclear factor-κB, a transcription factor involved in mediating downstream inflammatory processes [34]. In addition, there is evidence that folic acid treatment in hyperhomocysteinemia subjects inhibits the release of oxidized low-density lipoprotein–stimulated chemokines and monocyte chemoattractant protein 1 in peripheral mononuclear cells [33]. These findings, in combination with our observation of an association between Hcy and IL-6 levels, support the hypothesis that Hcy stimulates low-grade inflammation in the vascular wall. However, our finding that there is a lack of correlation between Hcy and CRP suggests that Hcy levels may not be a simple reflection of inflammation.

It is well established that IL-6 is involved in the development of insulin resistance [9-11]. Therefore, the link between Hcy and IL-6 may reflect the insulin-resistant state, which occurs in diabetes mellitus. In our study, we found a significant correlation between Hcy and C-peptide, or number of abnormal metabolic factors in multivariate analyses, whereas serum IL-6 levels were related to serum insulin levels and the HOMA-IR index. These results are in accordance with the concept that insulin resistance may lead to a common mechanism for the elevation of Hcy and IL-6 levels. In the Framingham Offspring Study, elevated Hcy was associated with features of insulin resistance syndrome [16]. Emoto et al [17] showed that insulin-sensitivity indices measured by the insulin clamp procedure were significant contributors to Hcy levels. Dicker-Brown et al [30] have also suggested that MTHFR activities may be reduced by hyperinsulinemic conditions. However, in our multivariate analyses, the association between Hcy and IL-6 levels persisted after adjustment for the features and number of abnormal metabolic factors or serum C-peptide. These results imply that factors other than insulin resistance may contribute to the association between Hcy and IL-6 levels.

However, this study does have its limitations. Because the study was a cross-sectional design, it is not possible to ascribe a cause-and-effect relationship between Hcy and IL-6 levels. A prospective intervention study is necessary in elderly patients with diabetes to determine whether reducing Hcy levels with folic acid treatment leads to a decrease in IL-6 levels.

In conclusion, this study demonstrated that the association between elevated Hcy levels and serum IL-6 or C-peptide was independent of age, sex, vitamin status, and serum creatinine. In addition, the TT point mutation of C677T MTHFR gene modulated the association between Hcy and IL-6. Therefore, serum IL-6 levels provide further evidence of the role of innate immune activation for arteriosclerosis in patients with diabetes mellitus [7].

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