CLINICAL STUDY

Relation of plasma homocysteine levels to atherosclerotic vascular disease and inflammation markers in type 2 diabetic patients

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Abstract

Objective: Both hyperhomocysteinemia and increased inflammatory activity are shown to be associated with atherosclerosis. The relation of inflammatory activity to homocysteine (Hcy) levels is not well established. In the present study, we aimed to evaluate the relation of plasma Hcy levels to atherosclerotic vascular disease and inflammatory activity in type 2 diabetic patients.

Design and methods: In total 90 type 2 diabetic patients were included in the study. Of these patients, 41 had established atherosclerotic vascular disease and 49 had no evidence of atherosclerotic vascular disease. Hcy levels and markers of inflammation, namely C-reactive protein, fibrinogen, erythrocyte sedimentation rate, interleukin-6, and tumor necrosis factor α (TNF α), were measured. Glucose regulation, C-peptide, lipid parameters, and renal functions were also studied.

Results: Both Hcy levels and inflammation markers were all significantly elevated in patients with atherosclerotic vascular disease when compared with patients without vascular disease. Reduced renal functions were responsible for the majority of Hcy elevation in patients with vascular disease. Also, renal functions were significantly associated with both Hcy levels and inflammatory markers. There was no correlation between Hcy levels and inflammation markers except for TNF α .

Conclusions: Inflammatory activity and Hcy levels are increased in type 2 diabetic patients with atherosclerotic vascular disease. Impairment of renal functions is the key factor that affects both Hcy levels and inflammation markers. Inflammation is not involved in the process by which Hcy leads atherosclerosis in type 2 diabetes.

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Introduction

Diabetes is an important risk factor for atherosclerosis, and atherosclerotic vascular disease is a major cause of morbidity and mortality among patients with diabetes mellitus. Numerous factors including hyperglycemia, hyperlipidemia, hypertension, smoking, endothelial dysfunction, and platelet and coagulation abnormalities contribute to the process of accelerated atherosclerosis in diabetes (1–3).

Atherosclerosis is considered to be an inflammatory process triggered by response to injury. The increase in the plasma inflammation markers were shown to be related with the risk of vascular disease in non-diabetic individuals (4–13). There is evidence suggesting increased inflammatory activity in type 2 diabetic patients (14–16). But the factors that promote this inflammatory process are not apparently clear. C-reactive protein (CRP), fibrinogen, and erythrocyte sedimentation rate (ESR) are well known to be increased in inflammatory conditions (6–8, 11). Additionally, interleukin-6 (IL-6) and tumor necrosis

factor α (TNF α) are the main cytokines reflecting the existence of an ongoing inflammation (13, 17).

Homocysteine (Hcy) is known to take part in the development of atherosclerosis and vascular injury and it has been suggested to contribute to the atherosclerotic process of diabetes mellitus. Several studies have demonstrated that plasma Hcy levels are elevated in diabetic patients (18–24). But the relation of inflammatory activity to Hcy levels is not well established. The present study was undertaken to compare plasma inflammation markers and Hcy levels in type 2 diabetic patients with and without atherosclerotic vascular disease and to investigate the possible relationship between inflammatory activity and Hcy levels in these patients.

Subjects and methods

Totally, 90 type 2 diabetic patients were included in the study. Of these patients, 41 had established atherosclerotic vascular disease and 49 had no evidence of

atherosclerotic vascular disease. Past history of myocardial infarction, coronary artery by-pass surgery, peripheral arterial disease, stroke or angiographically proven coronary artery disease in a patient with electrocardiography (ECG) findings or angina pectoris were considered to be evidence for atherosclerotic vascular disease. Diagnosis of myocardial infarction, peripheral arterial disease, angina pectoris, and stroke were confirmed with appropriate tests by the related sections and were registered in their patient files. The cause of the vascular disease was myocardial infarction or by-pass surgery in 14 patients, stroke in 9 patients, peripheral arterial disease in 7 patients, and in 11 of the patients coronary artery disease was established by angiography. The group without vascular disease was selected from diabetics that have no history of vascular disease, normal ECG on exercise test, and normal peripheral artery Doppler ultrasound. Patients who have experienced myocardial infarction, stroke, diabetic foot infection, and diabetic ketoacidosis in the past 3 months or patients who have infectious diseases were excluded for those conditions could affect inflammatory markers. Age, diabetes duration, and treatment types of the patients were recorded and body mass indices (BMI) were calculated. Only the patients treated with insulin or sulfonylureas were included in the study. None of the patients were taking glitazones, metformin, or insulin plus sulfonylureas. As an index of glomerular filtration rate (GFR), creatinine clearance (Ccr) was calculated using body surface area adjusted Cockcroft–Gault formula. In order to exclude possible confounding effects of uremia, patients with creatinine levels above 1.6 mg/dl were not included in the study. Urinary albumin excretion rates (AER) were measured from 24-h urine collections with an immunoturbidometric assay. Vitamin B₁₂ and folate levels were studied and patients having B₁₂ or folate deficiencies were excluded. Also, thyroid functions were checked and hypo- or hyperthyroid patients were not included in the study. Fasting morning serum and plasma samples were obtained from each patient and hemoglobin A1c (A1c), C-peptide, total cholesterol, triglyceride, high density lipoprotein cholesterol (HDL-C), apolipoprotein A (ApoA), ApoB, lipoprotein a (Lp(a)), CRP, fibrinogen, ESRs were studied. CRP levels were measured nephelometrically (Beckman Image, Beckman Coulter, Fullerton, CA, USA). Fibrinogen levels were measured coagulometrically with Stago Compact analyzer. A1c, HDL-C, triglyceride, total cholesterol, ApoA, ApoB, and Lp(a) levels were studied with RD-Hitachi automatic analyzer using Roche Diagnostic tool kits. Low density lipoprotein cholesterol (LDL-C) was calculated using the Friedwald formula. C-peptide levels were measured by chemiluminescence immunoassay using DPC Immulite autoanalyzer (Diagnostic Products Corporation, Los Angeles, CA, USA). Fasting serum samples were frozen at -70°C without delay and serum IL-6 and TNF α levels were measured by use of commercially

available ELISA kits (Bender Systems, Vienna, Austria). Plasma Hcy levels were measured using ELISA kits (Diazyme, San Diego, CA, USA).

This study was approved by the Ethical Committee of Eskisehir Osmangazi University Medical Faculty.

Student's *t*-test and Pearson's correlation analysis were performed in statistical evaluation. Multiple regression analysis was used for multivariate analysis. A *P* value <0.05 was accepted as indicating statistical significance. All statistical analyses were performed using SPSS version 10. Results are represented as mean $\pm$ s.d.

Results

Basic characteristics and clinical variables of the study population are given in Table 1. CRP, ESR, fibrinogen, IL-6, and TNF α levels were significantly higher in patients with atherosclerotic vascular disease than in those without vascular disease. Plasma Hcy levels were also significantly elevated in diabetic patients with atherosclerotic vascular disease. After adjustment for age, diabetes duration, Ccr, AER, and folate levels, Hcy levels persisted to be significantly different between groups except for Ccr and folate.

Parameters found to be significantly correlated with Hcy and inflammation markers are shown in Table 2. The correlation coefficients between the variables were not significantly different among patients with vascular disease and in those without vascular disease when analyzed separately. CRP levels were in negative correlation with Ccr ($r = -0.263$, $P = 0.012$) and in positive correlation with AER ($r = 0.212$, $P < 0.001$). We found a significant positive correlation between CRP and triglyceride levels ($r = 0.282$, $P = 0.007$) and a negative correlation with HDL-C levels ($r = -0.400$, $P < 0.001$).

Fibrinogen levels also correlated negatively with Ccr ($r = -0.273$, $P = 0.009$) and positively with AER ($r = 0.317$, $P = 0.002$). ESR levels also correlated negatively with Ccr ($r = -0.286$, $P = 0.006$) and positively with AER ($r = 0.292$, $P = 0.005$). We found a negative correlation between ESR levels and HDL-C levels ($r = -0.262$, $P = 0.013$). Both TNF α and IL-6 levels correlated negatively with HDL-C ($r = -0.295$, $P = 0.005$; $r = -0.215$, $P = 0.044$) and ApoA levels ($r = -0.288$, $P = 0.007$; $r = -0.265$, $P = 0.013$ respectively). There was a significant negative correlation between plasma Hcy levels and Ccr and ApoA levels ($r = -0.489$, $P < 0.001$; $r = -0.260$, $P = 0.014$).

It was found by univariate analysis that, among inflammation parameters, there was no correlation between inflammation markers and Hcy levels. Plasma Hcy levels correlated only with TNF α levels significantly ($r = 0.302$, $P = 0.004$; Table 3). However, by multiple regression analysis with Hcy as the dependent variable, the strongest predictors of Hcy were Ccr (negative) and TNF α independent of age, sex, BMI, IL-6, ESR, CRP, fibrinogen, Lp(a), LDL-C, AER, ApoA, hypertension, folate, and B₁₂ (Table 4).

Table 1 Clinical characteristics and laboratory data of the patients with and without atherosclerotic vascular disease.

	Patients without vascular disease <i>n</i> =49	Patients with vascular disease <i>n</i> =41	Statistical significance
Age (years)	56.24 ± 1.49	63.75 ± 1.26	<i>P</i> < 0.001 [‡]
Diabetes duration (years)	11.00 ± 0.98	16.87 ± 1.12	<i>P</i> < 0.001 [‡]
A1 _c (%)	9.80 ± 0.47	9.55 ± 0.34	
Ccr (ml/min × 1.73 m ²)	79.37 ± 3.13	62.74 ± 3.31	<i>P</i> < 0.001 [‡]
AER (mg/day)	53.91 ± 25.11	533 ± 224.77	<i>P</i> = 0.04*
Cholesterol (mg/dl)	205.42 ± 7.03	205.97 ± 6.97	
Triglyceride (mg/dl)	172.26 ± 14.36	178.24 ± 14.67	
HDL-C (mg/dl)	46.67 ± 1.69	42.63 ± 2.48	
LDL-C (mg/dl)	127.24 ± 6.24	127.97 ± 6.14	
ApoA (mg/dl)	132.36 ± 3.86	119.48 ± 4.19	<i>P</i> = 0.027*
ApoB (mg/dl)	92.31 ± 3.73	100.12 ± 4.30	
Lp(a) (mg/dl)	32.44 ± 4.04	44.93 ± 6.52	
C-peptide (ng/ml)	2.96 ± 0.24	2.76 ± 0.27	
BMI (kg/m ²)	29.49 ± 0.69	28.98 ± 0.84	
CRP (mg/dl)	0.513 ± 0.11	1.310 ± 0.36	<i>P</i> = 0.04*
Fibrinogen (mg/dl)	386.122 ± 12.06	457.585 ± 18.27	<i>P</i> = 0.002 [‡]
ESR (ml/h)	17.755 ± 1.88	32.02 ± 3.86	<i>P</i> = 0.002 [‡]
TNFα (pg/ml)	9.992 ± 1.08	18.419 ± 2.02	<i>P</i> = 0.001 [‡]
IL-6 (pg/ml)	1.428 ± 0.12	6.117 ± 1.58	<i>P</i> = 0.005 [‡]
B ₁₂ (pg/ml)	345.25 ± 132.6	478.42 ± 535.52	<i>P</i> = 0.142
Folate (ng/ml)	12.33 ± 5.23	10.02 ± 4.66	<i>P</i> = 0.035*
Homocysteine (μmol/l)	10.725 ± 0.59	13.840 ± 0.95	<i>P</i> = 0.005 ^{a†}
Patients with hypertension	18/49	26/41	<i>P</i> = 0.021*
Microalbuminuria	6/49	1/41	
Macroalbuminuria	14/41	8/41	
Treatment			
Sulfonylurea	43/49	22/41	
Insulin	6/49	19/41	<i>P</i> = 0.001 [‡]
ACEI	7/49	9/41	
Statin	8/49	4/41	
ASA	19/49	15/41	

Data are means ± s.d. Microalbuminuria: 30 ≤ AER < 300 mg/day, Macroalbuminuria: AER ≥ 300 mg/day

ACEI, angiotensin-converting enzyme inhibitor; statin, HMGCo reductase inhibitor; ASA, acetyl salicylic acid; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; Ccr, Creatinine clearance; AER, Albumin excretion rates; ApoA, Apolipoprotein; Lp(a), Lipoprotein a; BMI, Body mass indices; CRP, C-reactive protein; ESR, Erythrocyte sedimentation rate; TNFα, Tumor necrosis factor α; IL-6, Interleukin-6(IL-6).

^aModel 1, adjusted for age; *P* = 0.091, model 2, adjusted for diabetes duration; *P* = 0.012, model 3, adjusted for Ccr; *P* = 0.240, model 4, adjusted for AER; *P* = 0.009, model 5, adjusted for folate; *P* = 0.108.

**p* < 0.05, [†]*p* < 0.01, [‡]*p* < 0.001.

Discussion

Atherosclerosis is substantially more prevalent, appears earlier, and progresses more rapidly in diabetics when compared with normal population. Even if some of the established cardiovascular risk factors exist more frequently in diabetics, they cannot completely explain this increase in the macrovascular disease (1–3).

Recent investigations have suggested that atherosclerosis is an inflammatory process that is initiated as a response to injury and there is growing evidence on its relationship to inflammation parameters. The association of inflammation with vascular disease is clearly established in non-diabetic individuals (17, 25).

There is evidence suggesting increased inflammatory activity in both type 1 and type 2 diabetic patients. Although the precise reason of the increased inflammatory activity is not known, factors such as hyperglycemia, obesity, and hyperinsulinemia are all proposed to affect the inflammatory activity (11, 26). There are also reports that connect inflammatory activity to microalbuminuria (27).

In our study, markers of inflammation were all found to be significantly elevated in type 2 diabetic patients with atherosclerotic vascular disease when compared with patients without vascular disease. This finding suggests that inflammatory activity is increased in type 2 diabetics with atherosclerotic vascular disease.

Hcy is also suggested to be a causative factor for atherosclerosis. Hcy levels were found to be elevated in patients with vascular disease and were proposed to contribute to atherosclerosis in patients with coronary artery disease, stroke, and peripheral vascular disease (1, 19, 20, 22, 24, 28). Hcy-induced vascular pathologies include fragmentation of the internal elastic lamina, disruption of elastic fibers, smooth muscle hyperplasia, and arterial and venous thrombosis (28). It is clear that hyperhomocysteinemia can promote atherosclerosis, but the underlying pathophysiological mechanisms are not well understood. Endothelial cell injury, endothelial dysfunction, increased smooth muscle cell growth, increased platelet adhesiveness, enhanced LDL oxidation and deposition in the arterial wall, and direct activation of the coagulation cascade

Table 2 Correlation coefficients found to be significant by univariate analysis between inflammatory markers, homocysteine (Hcy) levels, and other cardiovascular risk factors.

	Ccr (ml/m $\times$ 1.73/m ²)	AER (mg/day)	Triglyceride (mg/dl)	HDL-C (mg/dl)	ApoA (mg/dl)
CRP (mg/dl)	$r = -0.263$ $P = 0.012$	$r = 0.212$ $P = 0.045$	$r = 0.282$ $P = 0.007$	$r = -0.4$ $P < 0.001$	
Fibrinogen (mg/dl)	$r = -0.273$ $P = 0.009$	$r = 0.313$ $P = 0.002$			
ESR (mm/h)	$r = -0.28$ $P = 0.006$	$r = 0.292$ $P = 0.005$		$r = -0.262$ $P = 0.013$	
IL-6 (pg/ml)				$r = -0.215$ $P = 0.044$	$r = -0.265$ $P = 0.013$
TNF α (pg/ml)				$r = -0.295$ $P = 0.005$	$r = -0.288$ $P = 0.007$
Hcy (μ mol/l)	$r = -0.489$ $P < 0.001$				$r = -0.260$ $P = 0.014$

HDL-C, high density lipoprotein cholesterol; Ccr, Creatinine clearance; AER, Albumin excretion rates; ApoA, Apolipoprotein; CRP, C-reactive protein; ESR, Erythrocyte sedimentation rate; TNF α , Tumor necrosis factor α ; IL-6, Interleukin-6(IL-6); HCY, Homocysteine.

may all contribute to this process (1, 18, 28). Several studies have demonstrated that Hcy levels are increased in diabetes. Elevated Hcy levels were shown to be a stronger risk factor in diabetics than in non-diabetic patients (1, 19, 20, 21, 24, 29, 30).

Plasma Hcy levels were also found to be significantly elevated in our type 2 diabetic patients with atherosclerotic vascular disease when compared with patients without vascular disease. However, after correction for variables, it is found that reduced renal functions and low folate levels were responsible for the majority of elevation of Hcy levels. Folate levels were lower in patients with vascular disease though in the normal range. It seems that plasma folate levels are important in the development of vascular disease in diabetics. Since folate levels are essential for the metabolism of Hcy, low folate levels may contribute to vascular disease either by elevating Hcy levels or through other mechanisms. The reason why folate levels were lower in patients with vascular disease is not certain, but supplementation with this nutrient may be beneficial in reducing the risk.

Inflammation markers were shown to be closely related with microalbuminuria and with renal functions that are indicators of renal endothelial injury. However, it is not clear whether inflammation is the cause of the nephropathy or inflammatory activity is elevated in response to nephropathy.

Like CRP and ESR, IL-6 and TNF α levels were also in a significant negative correlation with HDL-C levels, and in

addition they correlated negatively with ApoA levels. It is possible that these cytokines may impair the production or accelerate the catabolism of these lipoproteins that are protective against the atherosclerotic process. Increased inflammatory activity may play a mediator role in the unfavorable serum lipid changes seen in type 2 diabetes.

Plasma Hcy levels are also affected directly by the renal functions and are increased substantially with the decrease in Ccr values. The presence of nephropathy causes elevated plasma Hcy concentrations because of reduced clearance of Hcy (1, 19, 21, 29). Patients with significantly elevated creatinine levels were not included in our study. However, even among diabetic patients with relatively good renal functions, Hcy levels were significantly associated with renal functions.

In literature, both inflammation markers and Hcy levels were investigated in diabetic patients but there is not much data studying their relationship with each other. In our study, no significant relationship could be found between inflammatory parameters and plasma Hcy levels other than TNF α . But it was obvious that both inflammation and Hcy were highly related with renal functions. However, TNF α levels showed a close relationship with Hcy levels. In a study, urinary and interstitial TNF α concentrations were shown to be elevated in diabetic rats before the commencement of microalbuminuria (31). This finding suggests that TNF α may have a role in the complex pathogenesis of diabetic nephropathy. Because the other inflammation markers are not related

Table 3 Pearson correlation coefficients between plasma homocysteine levels and inflammation markers.

	CRP (mg/dl)	Fibrinogen (mg/dl)	ESR (mm/h)	IL-6 (pg/ml)	TNFα (pg/ml)
Hcy (μ mol/l)	$r = 0.146$ $P = 0.175$	$r = 0.127$ $P = 0.239$	$r = 0.160$ $P = 0.136$	$r = 0.053$ $P = 0.623$	$r = 0.302$ $P = 0.004$
Statistical significance	NS	NS	NS	NS	*

CRP, C-reactive protein; ESR, Erythrocyte sedimentation rate; TNF α , Tumor necrosis factor α ; IL-6, Interleukin-6(IL-6); HCY, Homocysteine.

* $p < 0.05$.

Table 4 Main determinants of homocysteine (Hcy) levels based on multiple regression analysis with Hcy as the dependent variable (model $r^2=0.336$; included variables: age, sex, body mass index, C-reactive protein, erythrocyte sedimentation rate, fibrinogen, LDL-C, lipoprotein a, apolipoprotein A, tumor necrosis factor α (TNF α), interleukin-6, creatinine clearance (Ccr), albumin excretion rates, hypertension, folate, B₁₂).

Independent predictors	β	P
TNF α	0.296	0.003
Ccr	-0.471	<0.001

with Hcy in our study, the relation of TNF α with Hcy levels seems to be through another factor related with atherosclerosis.

Since we did not study the levels of insulin resistance in our subjects, we could not exclude entirely that the association found between Hcy and TNF α might be due to insulin resistance. However, we could not show any relationship between these parameters and C-peptide levels and BMI of the patients that are indirect measures reflecting insulin resistance. Alternatively, insulin resistance may activate a common mechanism elevating both Hcy and TNF α levels. However, our findings are not sufficient to explain the exact nature of the association between these two variables.

In the study conducted by Aso Y and colleagues, inflammation markers IL-6, hsCRP, and fibrinogen levels were found to be significantly correlated with Hcy levels in patients with diabetic nephropathy. However, when they reevaluated the findings after adjustment for Ccr, none of the inflammation markers except IL-6 (IL-6 correlated with Hcy; $P=0.05$) remained to be significantly correlated with Hcy. Also, an association of plasma Hcy with IL-6 and C-peptide levels was shown in another study in type 2 diabetic patients, and insulin resistance was thought to be the common mechanism leading both Hcy and IL-6 elevations (32).

There are also studies investigating the relation of Hcy levels to inflammation in patients with coronary artery disease, in elderly patients, etc. with some inconsistent results suggesting a causal relationship between them (33, 34). But in all these studies, they did not take into account the GFRs of the patients. Moreover, lowering of Hcy levels by means of folic acid, pyridoxine, or cyanocobalamin did not influence the inflammatory responses (34, 35). In a study conducted in patients with chronic kidney disease starting dialysis therapy, markers of inflammation were found to be negatively correlated with Hcy levels (36).

Also, Hcy was not found to be associated with low-grade inflammation in a population with and without type 2 diabetes (37). Likewise, in another study, both Hcy and inflammation markers were found to be elevated in chronic renal failure patients. But, while there was no association between Hcy levels and oxidative stress, hemostatic activation and endothelial dysfunction, these parameters were closely associated with inflammation

parameters. These findings suggest that inflammation is not involved in the process by which Hcy leads atherosclerosis (38).

In type 2 diabetic patients, inflammatory activity can be a significant indicator of vascular disease and endothelial damage. Although Hcy elevation is also a correlate of atherosclerotic vascular disease, the connection of atherosclerosis to Hcy is not through inflammation. Impairment of renal functions is the key factor that affects both inflammation and Hcy levels. Hcy may lead to atherosclerosis by operating through alternative mechanisms other than inflammation.

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