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Low folate levels may be an atherogenic factor regardless of homocysteine levels in young healthy nonsmokers

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Abstract

Low folate and high homocysteine levels are emerging as important risk factors for atherosclerosis and predictors of early coronary heart disease. We evaluated folate and homocysteine levels, compared them with endothelial function, and analyzed their association with the methylenetetrahydrofolate reductase (MTHFR) and endothelial nitric oxide synthase genotypes. We recruited 71 young healthy male nonsmokers without overt cardiovascular or renal disease. Plasma homocysteine levels were enhanced 2-fold in the subjects with the MTHFR 677T/T compared with the others (P = .0001) and also enhanced in the subjects with the endothelial nitric oxide synthase −786C allele (P = .031). Homocysteine levels were independently predicted only by the MTHFR genotype. A relationship between folate and homocysteine levels was not significant. Plasma folate levels were associated independently either with high-density lipoprotein cholesterol levels or with endothelial function in the brachial artery. These results suggest that low folate levels may be a risk factor for cardiovascular diseases regardless of homocysteine levels and that the subjects with lower folate levels should be recommended for dietary folic acid supplementation to elevate endothelial function and probably increase high-density lipoprotein cholesterol levels.

1. Introduction

A search is being carried out to identify new risk factors for atherosclerotic cardiovascular diseases because conventional risk factors including hypercholesterolemia, hypertension, smoking, and diabetes disease account for no more than 50% of all cases [1]. High plasma homocysteine [2] and low folate [3,4] levels are emerging as important and independent risk factors for atherosclerosis and predictors of early coronary heart disease. Folic acid serves as a cofactor in the synthesis of essential amino acid methionine from homocysteine and has been shown to play an essential role in the synthesis of methionine regeneration. However, the effects of plasma folate and homocysteine levels on endothelial function or on other prognostic factors for cardiovascular diseases are not well established.

We hypothesized that plasma folate and homocysteine levels differently contribute to other coronary risk factors and atherosclerosis. In the present study, we recruited healthy young male subjects before the progression of atherosclerotic lesions and evaluated their folate and homocysteine levels to compare them with conventional risk factors and with adiponectin levels or endothelial function in the brachial artery as assessed by ultrasound. We recruited only nonsmokers because current smokers have shown impaired endothelial function even if they refrained from smoking for more than 12 hours [5], and folate levels may be reduced by long-term smoking [6]. Because the plasma homocysteine levels are affected by the genotype of the methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism [7-9] and common polymorphisms of endothelial nitric oxide synthase (NOS3) [10], we also analyzed the association with the MTHFR C677T genotype and NOS3 G894T and T−786C genotypes.

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2. Methods

2.1. Study subjects

All subjects were volunteers, were free of any sign or symptoms of heart disease, and were taking no medication including antidiabetic, antihypertensive, and lipid-lowering drugs or dietary supplements of vitamins B6 and B12 and folate. They were apparently healthy with normal renal function. They consisted of 71 young male nonsmokers (mean age, 30.3 ± 4.2 years; range, 25-39 years). This study was approved by the Ethics Committee of Nagoya University, and written informed consent was obtained from all subjects.

2.2. Biochemical analyses

An overnight fasting venous blood sample was obtained from all subjects. Standard assays were used to measure serum concentrations of total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides, as well as insulin, glucose, and hemoglobin A_{1c} (HbA_{1c}) levels. Plasma folate and homocysteine levels were determined by a chemiluminescence enzyme immunoassay and high-performance liquid chromatography with fluorescence detection [11], respectively. The homeostasis model assessment of insulin resistance (HOMA-IR) was used to estimate insulin sensitivity. Plasma total adiponectin and high-molecular-weight (HMW) adiponectin concentrations were measured by sandwich enzyme-linked immunosorbent assay (Otsuka Pharmaceutical, Tokyo, Japan, and Fujirebio, Tokyo, Japan, respectively) [12].

2.3. Genotyping of the MTHFR C677T polymorphism and NOS3 G894T and T−786C polymorphisms

Genomic DNA was prepared from peripheral blood leukocytes using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). Genotypes for the MTHFR C677T polymorphism and NOS3 G894T and T−786C polymorphisms were determined by polymerase chain reaction–restriction fragment length polymorphism as previously reported [13,14].

2.4. Brachial artery physiology

Brachial artery function was measured using the noninvasive technique described by Celermajer et al [15] on the same day as the blood sampling. Using high-resolution ultrasound cardiography (SONOS 5500; Agilent Technologies, Palo Alto, CA), the end-diastolic diameter of the right brachial artery and blood flow by pulse wave Doppler ultrasound were measured. The diameter of the right brachial artery was measured from the anterior to the posterior interface between the media and adventitia, and the mean value of 3 measurements was calculated.

Measurements of flow-mediated dilation (FMD), an endothelium-dependent response, were taken at baseline, then at 1 minute after forearm hyperemia was produced by releasing a forearm cuff inflated to 250 mm Hg for 5 minutes, and finally at rest after the subject had been lying quietly for 10 minutes. After the diameter had recovered to the level of the baseline diameter, glyceryl trinitrate–induced dilation (GTN), an endothelium-independent response, was assessed 3 and 5 minutes after the sublingual application of 300 μg glyceryl trinitrate.

2.5. Statistical analysis

Results were expressed as means ± SD. Data were analyzed using the Statistical Package for the Social Sciences, version 16.0 (SPSS, Chicago, IL). Continuous variables were tested for normal distribution by the Kolmogorov-Smirnov test. Because the levels of triglycerides, fasting insulin, and HOMA-IR were not normally distributed, they were logarithmically transformed before statistical analysis. Pearson correlation coefficients (r) were applied to identify variables associated with variations in plasma folate or homocysteine levels. Genotype frequencies were compared with values predicted by Hardy-Weinberg equilibrium using the χ^2 test. The unpaired Student t test was used to calculate the statistical significance between 2 groups. A value of $P < .05$ was considered statistically significant.

3. Results

3.1. Baseline characteristics of study participants

Table 1 shows the baseline characteristics of the subjects of the study. They had never been diagnosed with diabetes or cardiovascular disease. Of the 71 subjects enrolled in the study, there were 8 with hypertension (systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure

Table 1
Clinical characteristics of healthy male nonsmokers

Variables	
Age (y)	30.3 ± 4.1
BMI (kg/m^2)	22.7 ± 2.5
Systolic blood pressure (mm Hg)	120 ± 12
Diastolic blood pressure (mm Hg)	71 ± 9
Total cholesterol (mmol/L)	4.93 ± 0.78
LDL cholesterol (mmol/L)	3.04 ± 0.67
HDL cholesterol (mmol/L)	1.52 ± 0.35
Triglycerides (mmol/L)	1.22 ± 1.17
Apolipoprotein A-I (mg/dL)	136 ± 21
Apolipoprotein B (mg/dL)	84.5 ± 19.5
Fasting glucose (mmol/L)	5.42 ± 0.60
HbA_{1c} (%)	4.71 ± 0.29
Fasting insulin (pmol/L)	66 ± 54
HOMA-IR value	2.81 ± 2.93
Total adiponectin (μg/mL)	6.93 ± 3.37
HMW adiponectin (μg/mL)	3.50 ± 2.26
Folic acid (nmol/L)	17.7 ± 8.7
Homocysteine (μmol/L)	10.5 ± 4.8
FMD	4.68 ± 2.19
GTN	16.2 ± 4.6
FMD/GTN	0.30 ± 0.13

Values are expressed as means ± SD.

≥90 mm Hg), 7 with hypercholesterolemia (total cholesterol >6.2 mmol/L and/or LDL cholesterol >4.1 mmol/L), and 13 with hypertriglyceridemia (>1.7 mmol/L). Two subjects had fasting glucose levels greater than 7.0 mmol/L. The subjects with obesity (>25 kg/m^2) numbered 10, but there was only 1 subject with hyperinsulinemia (>240 mmol/L).

The distributions of the MTHFR C677T polymorphism and the NOS3 G894T and T−786C polymorphisms were compatible with the Hardy-Weinberg equilibrium (χ^2 = 1.66, 1.86, and 0.72; P = .20, .17, and .39, respectively).

3.2. Relationship of variables with plasma folate or homocysteine levels

Plasma folate levels were more strongly correlated with HDL cholesterol and apolipoprotein A-I, and were inversely correlated with fasting insulin levels. The inverse correlation of plasma folate levels with plasma homocysteine levels did not reach statistical significance (r = −0.178, P = .14) (Table 2) and were not significant even after adjustment for HDL cholesterol levels. Notably, the folate levels significantly affected the FMD/GTN ratio in the brachial artery as the endothelial function. Furthermore, the folate levels had a tendency to be associated with the presence of the MTHFR 677T/T genotype and the NOS3 894T allele. A forward stepwise multiple regression analysis was performed including all variables given in Table 1 to examine significant contributors to the prediction of the folate levels, resulting in the selection of only 2 variables, HDL cholesterol and the FMD/GTN ratio. After adjusting for homocysteine levels, folate levels were still associated with HDL cholesterol (P = .0002) and the FMD/GTN ratio (P = .029).

The plasma homocysteine levels were much more dependent on the MTHFR C677T genotype than the folate levels were. Furthermore, the plasma homocysteine levels were associated with the presence of the NOS3 −786C allele; but there was no more significant correlation after adjustment for the presence of the MTHFR 677T/T genotype. No variables were associated with the homocysteine levels except for the two. A forward stepwise multiple regression analysis was performed to examine significant contributors to the prediction of the homocysteine levels, resulting in the selection of only the MTHFR 677T/T genotype.

3.3. Characteristics of the study population according to the MTHFR and NOS3 polymorphisms

Table 3 shows the difference in variables according to the MTHFR C677T genotype. The presence of the MTHFR 677T/T genotype significantly increased the homocysteine levels and decreased the apolipoprotein A-I, HDL cholesterol, and adiponectin levels.

In terms of NOS3 polymorphisms, the NOS3 −786C allele significantly increased the homocysteine levels; and the NOS3 894T allele had a tendency for decreased folate levels (Tables 4 and 5). The NOS3 894T allele significantly

Table 2
Correlations (r) between characteristics and plasma folate or homocysteine levels in young healthy male nonsmokers

	Folate	P value	Homocysteine	P value
Age	0.006	.96	0.078	.52
BMI	−0.042	.73	−0.095	.43
Systolic blood pressure	−0.048	.69	0.086	.48
Diastolic blood pressure	−0.181	.13	0.002	.99
Total cholesterol	0.160	.19	−0.030	.80
LDL cholesterol	−0.054	.66	−0.047	.70
HDL cholesterol	0.433	.0002	−0.082	.50
Triglycerides	−0.197	.10	0.080	.51
Apolipoprotein A-I	0.380	.0012	−0.074	.54
Apolipoprotein B	0.020	.87	0.018	.88
Fasting glucose	−0.096	.42	−0.067	.58
HbA_{1c}	−0.069	.57	−0.082	.50
Fasting insulin	−0.239	.046	0.063	.60
HOMA-IR value	−0.234	.051	0.048	.69
Total adiponectin	−0.039	.75	−0.092	.45
HMW adiponectin	0.007	.96	−0.094	.44
Folate			−0.178	.14
Homocysteine	−0.178	.14		
FMD	0.183	.13	−0.092	.44
GTN	−0.112	.35	0.131	.28
FMD/GTN	0.252	.036	−0.132	.27
MTHFR T/T genotype	−0.224	.063	0.437	.0001
NOS3 894T allele	−0.233	.052	−0.011	.92
NOS3 −786C allele	−0.165	.17	0.257	.031

Triglycerides, fasting insulin, and HOMA-IR were logarithmically transformed before statistical analysis. The absence and presence of genotypes are denoted 1 and 2, respectively.

Table 3
Physical and biochemical characteristics of subjects assigned by the presence of the MTHFR 677C allele

MTHFR C677T	C/C (n = 28) or T/C (n = 37)	T/T (n = 6)	P value
Age (y)	30.4 ± 4.0	30.3 ± 4.9	.96
BMI (kg/m^2)	22.8 ± 2.6	21.7 ± 1.6	.32
Systolic blood pressure (mm Hg)	120.1 ± 12.4	124.3 ± 12.0	.43
Diastolic blood pressure (mm Hg)	71 ± 9	75 ± 10	.35
Total cholesterol (mmol/L)	4.93 ± 0.78	4.27 ± 0.78	.030
LDL cholesterol (mmol/L)	3.04 ± 0.7	2.64 ± 0.68	.14
HDL cholesterol (mmol/L)	1.52 ± 0.34	1.29 ± 0.28	.094
Triglycerides (mmol/L)	1.22 ± 1.17	1.09 ± 0.45	.95
Apolipoprotein A-I (mg/dL)	138.1 ± 21.0	117.8 ± 18.5	.026
Apolipoprotein B (mg/dL)	85.3 ± 19.1	76.2 ± 23.3	.28
Fasting glucose (mmol/L)	5.42 ± 0.60	5.14 ± 0.31	.22
HbA_{1c} (%)	4.70 ± 0.29	4.85 ± 0.20	.23
Fasting insulin (pmol/L)	66 ± 55	86 ± 71	.28
HOMA-IR value	2.77 ± 2.98	3.23 ± 2.52	.42
Total adiponectin (μg/mL)	7.21 ± 3.36	4.02 ± 1.76	.026
HMW adiponectin (μg/mL)	3.70 ± 2.26	1.50 ± 0.78	.022
Folic acid (nmol/L)	17.7 ± 8.7	11.4 ± 1.1	.063
Homocysteine (μmol/L)	9.9 ± 4.1	17.3 ± 6.4	.0001
FMD	4.71 ± 2.13	4.35 ± 2.98	.70
GTN	16.0 ± 4.4	18.2 ± 6.3	.26
FMD/GTN	0.31 ± 0.13	0.25 ± 0.17	.37

Values are expressed as means ± SD. Triglycerides, fasting insulin, and HOMA-IR were logarithmically transformed before statistical analysis.

Table 4
Physical and biochemical characteristics of subjects assigned by the presence of the NOS3 894T allele

NOS3 G894T	G/G (n = 53)	G/T (n = 15) or T/T (n = 3)	*P* value
Age (y)	30.2 ± 4.1	30.9 ± 3.9	.57
BMI (kg/m^2)	22.5 ± 2.4	23.3 ± 2.9	.24
Systolic blood pressure (mm Hg)	121 ± 13	119 ± 10	.68
Diastolic blood pressure (mm Hg)	70 ± 10	74 ± 7	.19
Total cholesterol (mmol/L)	4.91 ± 0.74	4.97 ± 0.90	.80
LDL cholesterol (mmol/L)	2.97 ± 0.60	3.22 ± 0.85	.18
HDL cholesterol (mmol/L)	1.57 ± 0.35	1.37 ± 0.29	.027
Triglycerides (mmol/L)	1.17 ± 1.27	1.36 ± 0.84	.13
Apolipoprotein A-I (mg/dL)	140.0 ± 20.7	125.7 ± 20.4	.014
Apolipoprotein B (mg/dL)	83.4 ± 17.8	87.7 ± 24.0	.42
Fasting glucose (mmol/L)	5.38 ± 0.56	5.55 ± 0.71	.30
HbA_{1c} (%)	4.68 ± 0.30	4.82 ± 0.22	.081
Fasting insulin (pmol/L)	62 ± 53	80 ± 58	.093
HOMA-IR value	2.4 ± 2.5	3.4 ± 2.9	.041
Total adiponectin (μg/mL)	7.0 ± 3.1	6.8 ± 4.1	.81
HMW adiponectin (μg/mL)	3.5 ± 1.9	3.6 ± 3.2	.90
Folic acid (nmol/L)	18.9 ± 9.6	14.3 ± 3.0	.052
Homocysteine (μmol/L)	10.6 ± 4.8	10.4 ± 4.8	.88
FMD	4.78 ± 2.06	4.40 ± 2.58	.53
GTN	16.0 ± 4.7	16.7 ± 4.3	.56
FMD/GTN	0.31 ± 0.13	0.26 ± 0.13	.16

Values are expressed as means ± SD. Triglycerides, fasting insulin, and HOMA-IR were logarithmically transformed before statistical analysis.

Table 5
Physical and biochemical characteristics of subjects assigned by the presence of the NOS3 −786C allele

NOS3 T−786C	T/T (n = 58)	T/C (n = 13) or C/C (n = 0)	*P* value
Age (y)	30.6 ± 4.2	29.7 ± 3.5	.49
BMI (kg/m^2)	22.9 ± 2.6	21.8 ± 2.3	.15
Systolic blood pressure (mm Hg)	119 ± 12	127 ± 14	.024
Diastolic blood pressure (mm Hg)	71 ± 9	73 ± 12	.31
Total cholesterol (mmol/L)	4.96 ± 0.76	4.81 ± 0.86	.53
HDL cholesterol (mmol/L)	1.52 ± 0.34	1.47 ± 0.24	.40
LDL cholesterol (mmol/L)	3.04 ± 0.65	3.01 ± 0.79	.87
Triglycerides (mmol/L)	1.21 ± 1.22	1.25 ± 0.95	.81
LDL particle size (nm)	26.3 ± 1.0	26.0 ± 1.2	.38
MDA-LDL (U/L)	111.3 ± 41.3	84.8 ± 45.9	.036
Apolipoprotein A-I (mg/dL)	137.4 ± 22.0	131.8 ± 19.0	.40
Apolipoprotein B (mg/dL)	84.9 ± 18.7	83.0 ± 23.4	.76
Fasting glucose (mmol/L)	5.19 ± 0.28	5.47 ± 0.64	.050
HbA_{1c} (%)	4.70 ± 0.30	4.67 ± 0.24	.62
Fasting insulin (pmol/L)	69 ± 59	52 ± 23	.37
HOMA-IR value	3.0 ± 3.2	2.0 ± 1.0	.29
Total adiponectin (μg/mL)	7.1 ± 3.4	6.1 ± 3.0	.35
HMW adiponectin (μg/mL)	3.7 ± 2.4	2.7 ± 1.6	.16
Folic acid (nmol/L)	18.4 ± 9.2	14.7 ± 4.9	.17
Homocysteine (μmol/L)	9.9 ± 4.2	13.1 ± 6.4	.031
FMD	4.84 ± 2.29	3.96 ± 1.55	.19
GTN	15.8 ± 4.5	17.7 ± 4.8	.19
FMD/GTN	0.31 ± 0.13	0.24 ± 0.12	.10

Values are expressed as means ± SD. Triglycerides, fasting insulin, and HOMA-IR were logarithmically transformed before statistical analysis. MDA indicates malondialdehyde.

decreased HDL cholesterol and apolipoprotein A-I levels, whereas the NOS3 −786C significantly increased systolic blood pressure.

4. Discussion

In the present study, we showed that the folate levels were associated with HDL cholesterol and the endothelial function in the brachial artery, whereas the homocysteine levels were independently predicted only by the MTHFR genotype in young healthy nonsmokers before the progression of atherosclerotic lesions occurred because of genetic and environmental contributions. The inverse correlation between the plasma folate and homocysteine levels was not statistically significant in the recruited subjects in this study.

It is well known that the determination of plasma homocysteine levels is partially dependent on MTHFR genotypes. We previously showed in patients with coronary heart disease that the MTHFR 677T/T genotype had the effect of elevating plasma homocysteine levels by 14% [13]. In the present study, we found a strong difference in homocysteine levels between groups defined on the basis of the MTHFR genotype. This difference occurred among recruited subjects characterized as young healthy men exhibiting no renal failure in this study. It should be noted that considerable hyperhomocysteinemia can be caused by renal disease [16,17]. In MTHFR deficient mice, elevated homocysteine levels have been reported to reduce apolipoprotein A-I [18], which was consistent with our findings. The plasma adiponectin levels were also decreased in the subjects with the MTHFR 677T/T genotype, but its significance remains to be elucidated.

Folic acid is an important cofactor in the transfer and utilization of 1-carbon moieties and plays a key role in the synthesis of nucleic acids and, in particular, in methionine regeneration from homocysteine. Folic acid also possesses antioxidant potential and inhibits NOS3 uncoupling, resulting in the maintenance of NO synthesis in endothelial cells [19]. On the other hand, the common excess metabolite homocysteine has been shown to induce premature atherosclerosis and fatal thrombosis such as myocardial infarction and stroke [20]. Recently, it has been shown that homocysteine disrupts the growth and survival of endothelium cells by altering promoter DNA methylation as a pathway associated with homocysteine [21]. Hyperhomocysteinemia raised by oral methionine loading has been reported to impair endothelial function [22], and folic acid supplementation in patients with hyperhomocysteinemia can improve endothelial function [23,24]. Our results indicate that homocysteine levels were independently predicted only by the MTHFR genotype. By contrast, folate levels were associated not only with HDL cholesterol and apolipoprotein A-I levels, but also with the endothelial function. Therefore, low folate levels appear to affect endothelial function regardless of homocysteine levels. Although folic acid

supplementation increased HDL cholesterol levels in healthy postmenopausal women [25], few studies have reported a correlation between folic acid and HDL cholesterol. A low HDL cholesterol concentration is an independent and powerful risk factor for coronary heart disease; therefore, the close relationship between folate levels and HDL cholesterol concentrations could be one of the mechanisms by which atherosclerosis develops in subjects with lower folate levels. The previous report found that the NOS3 894T/T genotype was a risk factor for hyperhomocysteinemia [10], but our studies involved only 4 cases of the 894T/T genotype and found no association with the homocysteine levels (10.5 ± 1.6 μmol/L). By contrast, our results showed that the homocysteine levels were affected by the eNOS T−786C genotype rather than by the G894T genotype.

Previous studies have shown that high-dose folic acid supplementation improves endothelial function [23,26,27]. Therefore, dietary supplements of folic acid are suggested to retard the development of atherosclerosis, leading to lower morbidity and mortality in patients with cardiovascular diseases. For the past decade, several long-term mass studies have been performed using dietary supplements of folic acid including vitamins B12 and B6 [28-31]. Lonn et al [28] have reported that supplements combining folic acid and vitamins lowered homocysteine levels by 2.2 μmol/L, but did not reduce the risk of major cardiovascular events in patients with vascular disease. Other recent studies also failed in reducing morbidity and mortality in atherosclerotic patients [29,31,32]. Plasma homocysteine levels have been reported to have little or no influence on the carotid intimal-medial thickness compared with the conventional atherosclerotic risk factors [33]. These results suggested that intervention with folic acid could not be recommended at present.

The reason that the long-term treatment with folic acid has no beneficial effects on reducing cardiovascular events may be that the increased plasma folate levels are not directly associated with the improvement of endothelial function, although studies involving a small number of subjects have shown that several-week supplementation of folic acid has beneficial effects on endothelial function [22,25]. The long-term beneficial effect of folic acid supplementation on endothelial function has not yet been reported, and these clinical trials have not recruited only the subjects with low folate levels. However, a clinical study using homocysteine-lowering therapy with folic acid proved to decrease the incidence of major adverse events after percutaneous coronary intervention [30]. Limitations of this study include the small number of subjects and the lack of a trial of folic acid supplementation to evaluate changes in the homocysteine levels and endothelial function. If we evaluate carotid intimal-medial thickness or pulse wave velocity in addition to endothelial function, the results would be more convincing.

In conclusion, we demonstrated that plasma folate levels were associated independently either with HDL cholesterol levels or with endothelial function in the brachial artery, whereas plasma homocysteine levels were dependent on the MTHFR genotype. Therefore, low folate levels may be a risk factor for cardiovascular diseases regardless of homocysteine levels; and the subjects with lower folate levels should be recommended for dietary folic acid supplementation to elevate endothelial function and probably increase HDL cholesterol levels.

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