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Elevated levels of plasma homocyst(e)ine and asymmetric dimethylarginine in elderly patients with stroke

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Abstract

Cerebrovascular risk factors, including hypertension, smoking, diabetes mellitus, aging, dyslipidemia, and hyperhomocyst(e)inemia are linked to endothelial dysfunction. Endothelial-derived nitric oxide (NO) has inhibitory effects on key processes in atherothrombosis. Although asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO synthase, is associated with atherosclerotic disease, there has been no report on association of ADMA with ischemic stroke. Here we investigated the relation of plasma ADMA, stroke, and homocyst(e)inemia in the elderly. Plasma ADMA and homocyst(e)ine concentration was determined using high-performance liquid chromatography and fluorescence detection. Patients with ischemic stroke had significantly higher concentrations of plasma ADMA than controls (1.85 ± 1.32 vs. 0.93 ± 0.32 μmol/l, $P = 0.0001$). After adjustment for risk factors, elevated ADMA levels, above 90th percentile of normal controls (≥ 1.43 μmol/l) was associated with stroke (OR = 6.05, 95% CI; $2.77 - 13.3$, $P = 0.02$). ADMA plasma levels were positively correlated to homocyst(e)ine levels ($r = 0.43$, $P = 0.01$). Multiple logistic regression analysis revealed that hyperhomocyst(e)inemia (plasma homocyst(e)ine concentration ≥ 15.0 μmol/l) was a significant predictor of elevated ADMA level. Altogether, findings indicate that elevated ADMA concentrations are at increased risk for ischemic stroke in the elderly, and may account for increased risk of stroke in patients with hyperhomocyst(e)inemia.

Keywords: Asymmetric dimethylarginine; Homocyst(e)ine; Nitric oxide synthase; Stroke; The elderly

1. Introduction

Normal endothelium has substantial roles in the regulation of vascular tone, control over thrombosis, and interactions of the vessel wall with circulating blood elements [1–3]. Endothelial-derived nitric oxide (NO) plays a role as a potent vasodilator [1,2], and inhibits platelet activation, leading to reduced platelet adhesion and aggregation [4,5]. NO also suppresses leukocyte adhesion [6], and vascular smooth muscle proliferation [7]. Endothelial vasodilator dysfunction has demonstrated in asymptomatic subjects with cardiovascular risk factor including hypertension, hypercholesterolemia, diabetes mellitus, smoking, aging, and hyperhomocyst(e)inmia, as well as in patients with established atherosclerosis [8–11]. In such conditions, the supplementation of L-arginine that serves as the substrate for the enzyme nitric oxide synthase, which converts arginine to citrulline and NO, restored endothelial-dependent vasodilation [12]. NO is synthesized from L-arginine by a family NO synthase, with endothelial, neuronal, and macrophage isoforms. This reaction can be selectively inhibited by methylated L-arginine derivatives, including *N*-monomethyl-L-arginine and N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA) which act as competitive antagonists at the active site of the enzyme [13]. In human plasma, concentrations of dimethylarginine are ten times greater than that of *N*-monomethyl-L-arginine [14].

It has been found that plasma ADMA concentrations are elevated in subjects with conditions accompa-

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nied by endothelial dysfunction, such as hypercholesterolemia [15–17], diabetes mellitus [18,19], and chronic renal failure [14,20], and in patients with atherosclerotic disease [21]. Aging is also associated with progressive endothelial dysfunction [22] and elevated plasma ADMA concentration [18]. Various mechanisms involved in the pathogenesis of atherothrombotic stroke include atherosclerotic processes and thrombophilic conditions, which may be linked to L-arginine-nitric oxide pathway. Reduction in NO bioavailability may be responsible for the biologic association of endothelial dysfunction with atherosclerotic vascular disease. In animal model, inhibition of endogenous NO synthesis lead to the development of atherosclerosis. Conversely, the supplementation of L-arginine slows progression of disease and may reverse the atherosclerotic process [12,23–27]. The severity of endothelial vasodilator dysfunction is related to the number of cardiovascular risk factor [9]. These considerations raise the plausibility that elevated plasma concentration of ADMA, an endogenous nitric oxide synthase inhibitor, could be in part responsible for increased risk of ischemic stroke. Therefore, we examined whether ADMA is associated with cerebral infarction in the elderly. Since hyperhomocyst(e)inemia recognized as a risk factor of cerebral infarction [28], is a common finding in the elderly [29], the relationship between plasma ADMA and homocyst(e)inemia was also investigated.

2. Materials and methods

2.1. Subjects

Three groups of subjects were included for the study. Stroke patients between ages 65 and 85 years, who attended the outpatient clinic, at Samsung Seoul Hospital (Seoul, South Korea) have consecutively been recruited from April through September of 1998. The diagnosis of cerebral infarction was made on the basis of neurological deficit accompanied by corresponding abnormal findings on brain magnetic resonance imaging (MRI), which was assessed by two neuroradiologists who had not been informed of the data. All MRI studies were performed with a GE Signa 1.5-T scanner (Milwaukee, WI). Thirty-six patients with recurrent stroke and 35 patients with first stroke consented to the study. They had survived from first or recurrent stroke during the previous year and maintained outpatient care and daily activity with or without partial aids.

Blood was drawn from antecubital vein at overnight fasting state. Multiple blood chemistry, blood count, thyroid hormone, and thyroid stimulating hormone were examined. Past medical history of the patients with stroke were obtained through investigator's interviews. Excluded from the study were 19 patients with following conditions; ischemic heart disease, intracranial hemorrhage, atrial fibrillation, cancer, renal dysfunction (serum creatinine level ≥ 132.6 μmol/l), hypothyroidism, alcoholism, and user of estrogen or multivitamin. Twenty-seven patients with first stroke and 25 patients with recurrent stroke were selected for the case group. Thirty-five healthy subjects without clinical evidence of stroke or ischemic heart disease were consecutively enrolled in outpatient clinic, with matching for sex and age within 2 years. Study subjects were all unrelated Koreans. The study was approved in Ethic Committee. Informed consents were obtained from their family members or participants.

2.2. Determination of plasma homocyst(e)ine and N^G,N^G-dimethyl-L-arginine concentration

Plasma homocyst(e)ine concentration was determined as described in the previous study [29]. Plasma ADMA concentration was determined, using high-performance liquid chromatography (HPLC) with 0-phthaldialdehyde (OPA) precolumn fluorescence derivatization, according to a modification of a previously published method [30]. N^G,N^G-dimethyl-L-arginine, OPA, 2-mercaptoethanol, tricholoracetic acid, and DL-homocysteine were obtained from Sigma (St. Louis, MO). HPLC system was as follows; Waters 510 HPLC pumps, 717 plus autosampler; Waters 474 scanning fluorescence detector.

ADMA was extracted from plasma with solid-phase extraction cartridges (Supelco, Bellefonte, PA). The column was activated with methanol, conditioned with TCA (2%) and loaded with sample. ADMA was eluted in methanol and fresh triethylamine prepared daily. The eluent was evaporated to dryness at under nitrogen and dissolved in distilled water. Derivatization with OPA was performed in the autoinjector for 1 min. With a multigradient program, the derivative of ADMA was separated on LiChrospher 100 RP-18 column (4×125 mm, 5 μm) with 100 RP-18 guard column (Darmstadt, Germany). Fluorescence detector was set at excitation 338 nm and emission 450 nm. Retention time was 45 min and intra-assay coefficient of variation was less than 2.5%. Recovery rates were 81.4 ± 4.5%. Inter-assay coefficient was less than 4.2% and detection limit was 0.02 μmol/l.

2.3. Statistical analysis

χ^2-test for categorical variable was applied for comparison between groups. Continuous values among groups were tested using ANOVA, followed by Duncan test for multiple comparison. For plasma ADMA values, non-parametric analysis was used because of its skewed distribution. Median test or Kruskal–Wallis

test was used when appropriate. Pearson correlation coefficient was calculated to examine a possible correlation in continuous variables. Multiple logistic regression analysis was used to estimate the adjusted odds ratio for cerebral infarction. Hyperhomocyst(e)inemia indicated plasma total homocysteine concentration more than 15.0 μmol/l. Hypercholesterolemia was defined as serum cholesterol level ≥ 6.2 mmol/l. Hypertension was defined in subjects who were currently taking antihypertensive medication or in subjects with blood pressure ≥ 160 mmHg systolic and/or ≥ 95 mmHg diastolic three times consecutively. Diabetes was considered in subjects who were currently using oral hypoglycemic agents or insulin, or in subjects with fasting plasma glucose ≥ 7.8 mmol/l and postprandial 2 h glucose ≥ 11.1 mmol/l. SAS (version 6.12) statistical software (Cary, NC) was used.

3. Results

3.1. Distribution of plasma ADMA concentrations, demographic and laboratory data

The clinical characteristics and laboratory data of 52 patients with stroke and 36 healthy controls are shown in Table 1. The distribution of gender and age did not differ among three groups. The proportions of diabetes mellitus were higher in patients with stroke than in normal controls ($P = 0.005$). On the other hand, the proportion of hypercholesterolemia, smoking and hypertension did not differ among patients with first or recurrent stroke and healthy controls. Hyperhomocyst(e)inemia was more frequent in patients with stroke than in controls ($P = 0.001$).

Plasma ADMA concentration was determined in 87 subjects. Mean concentrations were 1.26 μmol/l; maximum 6.68; minimum 0.52; mode 1.43. Mean plasma ADMA was significantly higher in patients with stroke than in controls (1.85 ± 1.32 vs. 0.93 ± 0.32 μmol/l, $P = 0.0001$). There was no difference of ADMA concentration between men and women (1.59 ± 1.35 vs. 1.40 ± 0.92 μmol/l, $P = 0.45$). The prevalence of upper median values of ADMA (≥ 1.26 μmol/l) was substantially higher among patients with stroke than among normal controls as shown in Table 2. ADMA levels more than 75 percentile (≥ 1.54 μmol/l) were observed in patients with stroke, with a trend of dose–response relationship ($P < 0.0001$) (Table 2). Patients with recurrent stroke had higher mean ADMA level than that of those with first stroke (2.28 ± 1.63 vs. 1.46 ± 0.77 μmol/l, $P = 0.0001$).

3.2. Association between plasma ADMA concentrations and stroke risk factors

In control group, plasma ADMA concentration was not significantly correlated to age ($r = 0.32$, $P = 0.06$), serum cholesterol ($r = -0.24$, $P = 0.19$), and creatinine level ($r = -0.12$, $P = 0.52$). Patient group also did not exhibit significant correlation between plasma ADMA levels and age ($r = 0.11$, $P = 0.43$), levels of serum cholesterol ($r = -0.10$, $P = 0.45$), and creatinine ($r = 0.05$, $P = 0.93$). ADMA concentration was positively correlated to plasma total homocyst(e)ine levels ($r = 0.43$, $P = 0.01$), as shown in Fig. 1. Multiple logistic regression analysis revealed that hyperhomocyst(e)inemia was a significant predictor of elevated ADMA level, above 90 percentile of control group (≥ 1.43 μmol/l) with odds ratio of 3.71 (95% CI: 2.16–6.36, $P = 0.02$) (Tables 3 and 4).

Table 1
Clinical characteristics and laboratory data among patients with first or recurrent cerebral infarction, and control groups[a]

Variable	Recurrent infarction ($n = 25$)	Cerebral infarction ($n = 27$)	Healthy controls ($n = 35$)
M/F (%)	11/14 (44/56)	10/17 (37/63)	17/18 (49/51)
Age (years)	74.5 ± 9.4	73.9 ± 9.8	73.3 ± 8.9
Hemoglobin (g/dl)	12.3 ± 1.4	12.4 ± 1.5	12.4 ± 1.4
Glucose (mmol/l)	6.4 ± 0.83	6.6 ± 1.02	6.2 ± 0.86
Cholesterol (mmol/l)	4.58 ± 0.93	5.15 ± 1.14	5.18 ± 0.78
Homocyst(e)ine (μmol/l)	19.3 ± 7.9[b]	18.7 ± 8.8[b]	9.9 ± 4.1
Creatinine (μmol/l)	113.2 ± 18.6	114.5 ± 17.8	116.6 ± 19.2
Folate (nmol/l)	16.7 ± 7.9	17.8 ± 7.6	21.3 ± 8.9[b]
Vitamin B_{12} (pmol/l)	476.4 ± 160.7	493.5 ± 175.2	483.8 ± 182.5
Smoker (%)	8 (36.4)	10 (32.2)	12 (34.3)
Hypertension (%)	12 (48.0)	14 (51.8)	20 (57.1)
Diabetes mellitus (%)	7 (28.0)[c]	12 (44.4)[c]	3 (8.6)
Hypercholesterolemia (%)	1(4)	4 (14.8)	3 (8.6)
Hyperhomocyst(e)inemia (%)	12 (48.0)[d]	13 (48.2)[d]	3 (8.6)

[a] Values were expressed as mean ± standard deviation.
[b] Kruskal–Wallis test, $P = 0.001$.
[c] χ^2-test, $P = 0.005$.
[d] χ^2-test, $P = 0.001$.

Table 2
Comparison of plasma ADMA level among patients with cerebral infarction and controls[a]

Variable	Recurrent infarction ($n=25$)	Cerebral infarction ($n=27$)	Healthy controls ($n=35$)	P-value
ADMA (μmol/l)	2.28 ± 1.63	1.46 ± 0.77	0.93 ± 0.32	0.0001
Upper median (%)[b]	20 (80.0)	16 (59.3)	9 (29.7)	<0.0001
Upper 75 percentile (%)[c]	13 (52.0)	10 (37.0)	0	<0.0001
Upper 90 percentile (%)[d]	6 (24.0)	3 (11.1)	0	0.01
Upper 95 percentile (%)[e]	4 (16.0)	1(3.7)	0	0.03

[a] ADMA levels were tested using Kruskal–Wallis test.
[b] Plasma ADMA concentration: (≥1.26 μmol/l),
[c] (≥1.54 μmol/l),
[d] (≥2.11 μmol),
[e] (≥4.64 μmol).

3.3. Association between upper median ADMA concentration and ischemic stroke

After adjustment for hypercholesterolemia, hypertension, smoking, diabetes, and advanced age, logistic regression analysis revealed that elevated ADMA level (≥ 1.43 μmol/l) was associated with stroke (OR = 6.05, 95% CI: 2.77–13.3, $P=0.02$). Compared with patients with first stroke, patients with recurrent stroke had higher mean level and higher proportion of upper median ADMA levels (Table 2). When multiple logistic regression analysis was conducted, upper median ADMA level of total subjects was at a higher risk for recurrent stroke (OR = 7.03, 95% CI: 3.42–14.4, $P=0.007$).

4. Discussion

Although atherothrombotic vascular occlusion is the common cause of stroke, there was no report on association of plasma ADMA, a potent inhibitor of NO synthase, with stroke. In this study, we found that elevated levels of plasma ADMA was at increased risk for cerebral infarction. This finding does not prove whether elevated ADMA levels is a cause or consequence of stroke. However, close-response relationship of ADMA to recurrent stroke suggests the role of elevated level of ADMA in pathogenesis of cerebrovascular disease. It seems that plasma ADMA concentrations presented are sufficient to inhibit NO production, then reduce endothelium dependent vasodilation [31] and increase leukocyte adherence [32]. ADMA may be elaborated in sufficient quantity to inhibit NO synthesis within NO producing cells. In a recent study [19], accumulation of ADMA in endothelial cells of diabetic rat was to sufficient to inhibit cyclic guanosine monophosphate production. In human studies, plasma ADMA levels was positively correlated to carotid intima-media thickness [18]. Reduction in NO bioavailability was demonstrated in patients with peripheral arterial occlusive disease [21]. Kielstein et al. [20] reported that plasma ADMA level in hemodialysis-treated patients exhibited a negative correlation to nitrate excretion, suggesting inhibited NO production. These biological evidences support a causal association between ADMA and cerebrovascular disease.

In previous study [29], we observed that hyperhomocyst(e)inemia is associated with cerebral infarction and the severity of cerebral atherosclerosis. The mechanisms by which homocyst(e)inemia leads to atherothrombosis are poorly understood. Tawakol et al. [10] demonstrated that hyperhomocysteinmia was associated with impaired endothelium-dependent vasodilation in elderly human. Azuma et al. [33] demonstrated that accumulation of ADMA content is associated with decreased NO production from regenerated endothelial cells from the rabbit carotid artery. Homocyst(e)inemia is associated with direct endothelial injury [34]. We found that hyperhomocysteinemia was a significant predictor of ADMA concentration, after adjustment of covariates. In individuals with hyperhomocyst(e)inemia, elevated plasma ADMA, in part, may account for vasodilatory dysfunction and risk of atherothrombosis.

ADMA is arisen from the catabolism of proteins containing methylated arginine residues. ADMA is metabolized by the enzyme dimethylarginine dimethy-

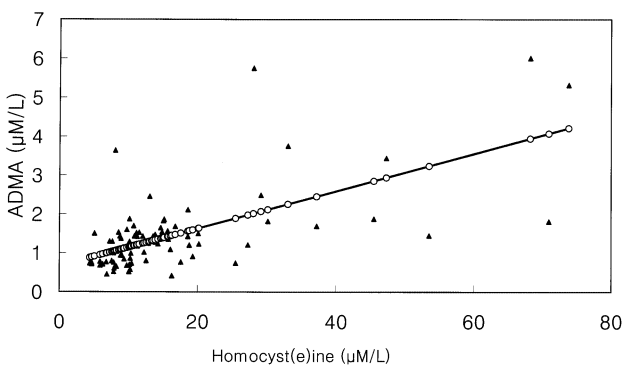

Fig. 1. A positive correlation was found between concentrations of homocyst(e)ine and ADMA in a studied elderly population ($n=87$) ($r=0.43$, $P=0.02$).

Table 3
Predictors of elevated plasma ADMA based on multiple logistic regression analysis in subjects with or without cerebral infarction

	Parameter estimate	Standard error	*P*-value
Age	2.28	0.56	0.001
Male	0.80	0.58	0.17
Hypertension	0.28	0.53	0.60
Diabetes mellitus	1.39	0.63	0.03
Hypercholesterolemia	1.14	1.07	0.29
Hyperhomocyst(e)inemia	1.31	0.54	0.02

laminohydrolase (DDAH), which hydrolyzes ADMA to L-citrulline and dimethylamine [35,36]. Recently, Ito et al. [37] showed reduced DDAH activity in hypercholesterolemic rabbits, which suggest that endothelial vasomotor dysfunction in hypercholesterolemia may be due to reduced degradation of ADMA. It remains to be established whether hyperhomocysteinemia can reduce DDAH activity. ADMA is elaborated and metabolized in vascular endothelial cell, [38] but the origin of elevated ADMA concentration in patients with vascular disease is unknown. Increased oxidative stress in the vessel wall may reduce DDAH activity, then induce accumulation of endogenous ADMA.

Endogenous dimethylarginines are excreted in urine. Vallance et al. [14] reported elevated ADMA concentrations in patients with chronic renal failure and a possible association with diminished renal excretion. In this study, subjects with elevated creatinine concentration were excluded. Regardless of renal function, relation of elevated ADMA to homocyst(e)inemia remains to be investigated. In patients with end-stage renal disease, hyperhomocysteinemia is a predictor of cardiovascular morbidity and mortality [39], which need to be evaluated, in relation to ADMA.

Table 4
Adjusted odds ratio of 90th percentile plasma ADMA of controls for cerebral infarction based on multiple logistic regression analysis

Variable	Odds ratio	95% confidence interval	*P*-value
Male	1.39	0.67–2.89	0.64
Age	7.38	3.67–14.9	0.004
Smoking	1.22	0.41–3.74	0.84
Hypertension	1.99	1.06–3.74	0.27
Diabetes mellitus	9.68	3.22–24.0	0.01
Hypercholesterolemia	5.58	2.01–15.9	0.10
90th percentile plasma ADMA	6.11	2.72–13.5	0.02
Hyperhomocyst(e)inemia	8.25	3.32–20.1	0.01

In summary, in the elderly studied, elevated plasma ADMA concentrations are associated with increased risk for cerebral infarction. In addition, ADMA plasma levels were positively correlated to homocyst(e)ine levels and hyperhomocyst(e)inemia was a significant predictor of accumulation of endogenous ADMA. These suggest that accumulation of endogenous ADMA may account for increased risk of stroke in hyperhomocyst(e)inemia. Prospective study is needed to confirm a causal association between ADMA and stroke.

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