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Effect of plasma homocysteine level and urinary monomethylarsonic acid on the risk of arsenic-associated carotid atherosclerosis

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Abstract

Arsenic-contaminated well water has been shown to increase the risk of atherosclerosis. Because of involving *S*-adenosylmethionine, homocysteine may modify the risk by interfering with the biomethylation of ingested arsenic. In this study, we assessed the effect of plasma homocysteine level and urinary monomethylarsonic acid (MMA^V) on the risk of atherosclerosis associated with arsenic. In total, 163 patients with carotid atherosclerosis and 163 controls were studied. Lifetime cumulative arsenic exposure from well water for study subjects was measured as index of arsenic exposure. Homocysteine level was determined by high-performance liquid chromatography (HPLC). Proportion of MMA^V (MMA %) was calculated by dividing with total arsenic species in urine, including arsenite, arsenate, MMA^V, and dimethylarsinic acid (DMA^V). Results of multiple linear regression analysis show a positive correlation of plasma homocysteine levels to the cumulative arsenic exposure after controlling for atherosclerosis status and nutritional factors ($P < 0.05$). This correlation, however, did not change substantially the effect of arsenic exposure on the risk of atherosclerosis as analyzed in a subsequent logistic regression model. Logistic regression analyses also show that elevated plasma homocysteine levels did not confer an independent risk for developing atherosclerosis in the study population. However, the risk of having atherosclerosis was increased to 5.4-fold (95% CI, 2.0–15.0) for the study subjects with high MMA% (≥16.5%) and high homocysteine levels (≥12.7 μmol/l) as compared to those with low MMA% (<9.9%) and low homocysteine levels (<12.7 μmol/l). Elevated homocysteinemia may exacerbate the formation of atherosclerosis related to arsenic exposure in individuals with high levels of MMA% in urine.
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Keywords: Atherosclerosis; Arsenic; Homocysteine; Biomethylation; Risk factors

Introduction

Arsenic is a metalloid element and widely distributed on earth because of its strong affinity with pyrite and high concentration in hydrous iron oxides (Nordstrom, 2002). Humans are exposed to arsenic in the environment mainly through groundwater supplies of drinking water (WHO, 1981; U.S.PHS, 1989). Epidemiological studies in Taiwan have shown that inorganic arsenic from groundwater is associated with an increased risk of peripheral arterial disease (Tseng et al., 1996), ischemic heart disease (Chen et al., 1996), and cerebral infarction (Chiou et al., 1997b). A recent report also indicated a close association of long-term arsenic exposure with the progression of carotid atherosclerosis (Wang et al., 2002), an indication of vessel narrowing in carotid artery. The arsenic-associated vascular manifestation was also observed among the residents in Chile, Mexico, Poland, and the United States, as well as vineyard workers in Germany (Engel and Smith, 1994; Lewis et al., 1999). Arsenic may act as an independent risk factor for atherosclerotic vascular diseases in humans aside from the classic risk factors of cigarette smoking, diabetes, hypertension, and hyperlipidemia. The mechanisms by which arsenic induces atherogenesis

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Table 1
Multiple linear regression analyses on plasma homocysteine levels[a] in relation to homocysteine metabolism factors and cumulative arsenic exposure

Variable	Coefficient (×100)	Standard error (×100)	*P* value
Age (year)	1.12	0.26	<0.001
Gender (male vs. female)	34.4	4.32	<0.001
Folic acid (nmol/l)	0.91	0.46	0.052
Vitamin B_{12} (pmol/l)	−0.04	0.01	0.006
Atherosclerosis status (yes vs. no)	0.26	4.48	0.954
Cumulative arsenic exposure (μg/l-year)	0.46	0.19	0.016

[a] Values are log-transformed.

are not fully understood. Generation of reactive oxidants has been related to arsenic toxicity in studies of cell culture and humans (Wang and Huang, 1994; Barchowsky et al., 1996; Wu et al., 2001, 2003). However, the sources of formation of the reactive oxygen species are not completely elucidated. Recent studies on the metabolism of arsenic involving biomethylation process conclude that it may result in the generation of reactive oxygen species and free radicals during the process (Yamanaka and Okada, 1994; Del Razo et al., 2001; Kitchin and Ahmad, 2003), suggesting a source of oxidative stress.

Metabolism of inorganic arsenic in human bodies includes sequential biomethylation processes by alternating reduction of pentavalent arsenic to trivalent and an addition of a methyl group to its trivalent form (Cullen et al., 1984). *S*-adenosylmethionine (SAM) acts as the methyl donor in the arsenic biomethylation and is subsequently demethylated to *S*-adenosylhomocysteine (SAH). Historically, methylation of arsenic has been regarded as a detoxification pathway because the arsenic metabolites, monomethylarsonic acid (MMA^V) and dimethylarsinic acid (DMA^V), are less toxic than inorganic (arsenite and arsenate) (Gebel, 2002). However, recent experimental studies have shown that the reactive intermediate metabolites, monomethylarsonous acid (MMA^{III}) and dimethylarsinous acid (DMA^{III}), are more toxic than their parent arsenite in a variety of mammalian cells (Petrick et al., 2000; Styblo et al., 2000; Mass et al., 2001; Ahmad et al., 2002). In contrast, studies on arsenic-exposed humans concluded that individuals with a lower capacity to biomethylate arsenic have a higher risk of developing arsenic-associated diseases (Del Razo et al., 1997; Hsueh et al., 1997; Yu et al., 2000; Chen et al., 2003; Tseng et al., 2005). Interestingly, all their data also indicated that study subjects with higher urinary MMA^V percentage (MMA%) or lower DMA^V percentage (DMA%) suffered from a higher risk of the reported diseases, including peripheral artery disease related to atherosclerosis (Tseng et al., 2005). Whether this risk is related to the presence of trivalent methylated products in tissues remains to be eluzcidated. Additionally, the two sequential stages of methylation efficiency involving different methylated products in individuals may likely have distinct features of health effects.

Moderate elevation of homocysteine level in plasma has recently been proposed as a significant predictor of atherosclerosis and its related complications (Hackam and Anand, 2003). Mechanism studies have demonstrated that homocysteine may induce vascular damage by promoting platelet activation, oxidative stress, endothelial dysfunction, hypercoagulability, vascular smooth muscle cell proliferation, and endoplasmic reticulum stress (Lawrence de Koning et al., 2003). As homocysteine is produced from the hydrolysis of SAH (Finkelstein et al., 1971), arsenic may contribute to the increase of homocysteine levels by consuming the SAM pool and therefore enhance the subsequent cardiovascular risk. However, this speculation requires careful examination. Plasma

Table 2
Traditional risk factors and carotid atherosclerosis

Characteristics	Patients *n* (%)	Controls *n* (%)	Unadjusted OR (95% CI)	Age–gender-adjusted OR (95% CI)
Age (years)				
<60	29 (17.8)	71 (43.6)	1.0	1.0
60.0–69.9	79 (48.5)	59 (36.2)	3.3 (1.9–5.7)[a]	3.2 (1.8–5.5)[a]
≥70	55 (33.7)	33 (20.2)	4.1 (2.2–7.5)[a]	4.0 (2.2–7.3)[a]
Gender				
Female	79 (48.5)	94 (57.7)	1.0	1.0
Male	84 (51.5)	69 (42.3)	1.4 (0.9–2.2)	1.3 (0.8–2.1)
Body mass index (kg/m²)				
<27	140 (85.9)	139 (86.3)	1.0	1.0
≥27	23 (14.1)	22 (13.7)	1.0 (0.6–1.9)	1.2 (0.6–2.3)
Current smoking				
No	95 (58.3)	119 (73.0)	1.0	1.0
Yes	68 (41.7)	44 (27.0)	1.9 (1.2–3.1)[b]	2.1 (1.0–4.4)[c]
Total cholesterol (mg/dl)				
<200	73 (45.1)	88 (54.3)	1.0	1.0
≥200	89 (54.9)	74 (45.7)	1.5 (0.9–2.2)	1.6 (1.0–2.5)[c]
HDL cholesterol (mg/dl)				
<45	19 (14.0)	13 (8.8)	1.0	1.0
≥45	117 (86.0)	134 (91.2)	0.6 (0.3–1.3)	0.8 (0.4–1.8)
LDL cholesterol (mg/dl)				
<130	61 (44.8)	80 (54.4)	1.0	1.0
≥130	75 (55.2)	67 (45.6)	1.5 (0.9–2.3)	1.6 (1.0–2.7)[c]
Triglycerides (mg/dl)				
<130	104 (64.2)	113 (69.8)	1.0	1.0
≥130	58 (35.8)	49 (30.3)	1.3 (0.8–2.0)	1.4 (0.8–2.2)
Hypertension				
No	100 (61.4)	117 (72.2)	1.0	1.0
Yes	63 (38.7)	45 (27.8)	1.6 (1.0–2.6)[c]	1.5 (0.9–2.4)
Diabetes mellitus				
No	144 (88.9)	145 (89.5)	1.0	1.0
Yes	18 (11.1)	17 (10.5)	1.0 (0.5–2.2)	1.0 (0.5–2.0)
Homocysteine (μmol/l)				
<12.7	56 (34.4)	82 (50.3)	1.0	1.0
≥12.7	107 (65.6)	81 (49.7)	1.9 (1.2–3.0)[b]	1.4 (0.9–2.4)

OR, odds ratio; CI, confidence interval; LDL, low-density lipoprotein; HDL, high-density lipoprotein.
Differences from the total number of 163 cases and controls are due to missing values.
[a] $P < 0.001$.
[b] $0.001 < P < 0.01$.
[c] $0.01 < P < 0.05$.

homocysteine concentrations have been examined in Taiwanese (Chao et al., 1999; Lin et al., 2002) and in else ethnic population for the risk of cardiovascular diseases (Hackam and Anand, 2003); this factor however has not been examined for a disease risk when combined with arsenic exposure. We attempted to investigate the additional effect of plasma homocysteine level on the risk of atherosclerosis related to arsenic exposure. MMA% or DMA% in urine involved in the two-stage methylation process is also taken into account while evaluating the atherogenic effect from plasma homocysteine level added on the risk estimates for arsenic exposure.

Materials and methods

Study subjects. Study subjects were recruited from the Lanyang Basin of Ilan County in northeastern Taiwan. Research on arseniasis in this area was begun in the early 1990s (Chiou et al., 1997b). Characteristics of the study area, recruitment of the study cohort, baseline data obtained from questionnaire interviews, and determination of arsenic concentrations in well water were described in detail previously (Chiou et al., 1997b, 2001). In brief, the Lanyang Basin is one of two areas, in which well water with high arsenic level is clustered. However, the variation of arsenic concentration in well water from the Basin area is much more striking, ranging from undetectable (<0.15 μg/l) to 3.59 mg/l. During the years of 1991 to 1994, a total of 8088 residents aged ≥40 years from 18 villages in four townships were interviewed and included as the study cohort (Chiou et al., 1997b, 2001; Chen et al., 2004). Well water samples were also collected for the determination of arsenic content at that time. In 1997–1998, an initial health examination was carried out for a subsample of 1318 residents (687 and 631 for year 1997 and 1998, respectively) from the cohort, including ultrasonographic assessment of the extracranial carotid artery (ECCA) being conducted. These examinee cohort are younger and more females than the original cohort as we expected for a study of community-based vascular disease. Before the health examination, a follow-up questionnaire was also given to update the information on lifestyle characteristics such as cigarette smoking and alcohol and tea consumption, as well as a detailed history of well water use since the previous interview in 1994. Urine sample and fasting blood were collected and stored at an appropriate temperature until use. A total of 605 examinees (88%) out of the 687 gave their consent to participate the current research project.

To assess the extent of carotid atherosclerosis for study subjects, a Hewlett-Packard SONO 1000 ultrasound system, equipped with a 7.5-MHz real-time B-mode scanner and a 5.6-MHz pulsed-Doppler mode scanner was used. The duplex scanning and operation on the participants were described in a previous study (Wang et al., 2002). For future and subsequent off-line analysis, all scans were recorded on super-VHS videotape. Indications of carotid atherosclerosis were evaluated mainly based on 2 indices: the maximal ECCA intimal–medial thickness (IMT) and the presence of ECCA plaque. The maximal IMT was measured in the far side of the common carotid artery (CCA) at the most stenotic location between 0 and 2 cm proximal to the carotid bifurcation. The ECCA plaque was assessed for 5 carotid artery segments, including the proximal CCA (0 to 1 cm proximal to the bifurcation), distal CCA (1 to 2 cm to the bifurcation), bulb, internal carotid artery, and external carotid artery. The presence of ECCA plaque was defined as irregular surface, lumen encroachment, wall thickening ≥50% of the adjacent IMT, as well as structure heterogeneity such as acoustic shadow. All the measurements were bilateral, and mean of the measurements was presented for each artery segment for both indices. Patient subjects were diagnosed according to a maximal ECCA IMT of ≥ 1.0 mm or the presence of observable plaque in any of the 5 carotid artery segments. In the initial-stage screening, two hundred and seventy nine subjects with an indication of carotid atherosclerosis were identified (46.1%). This prevalence is slightly higher than that of a previous report in a Taiwanese population (39%) (Wang et al., 2002). This difference in frequency might be due to factors in lifestyle or the high prevalence of stroke in the study area (Chiou et al., 1997b) as yet to be investigated. For the present study, a random sample of 163 patient subjects was selected. These study subjects were not substantially different from the original 279 patients in the distributions of demographic characteristics. Age (±5 years)- and sex-matched controls (n = 163) with no indication of carotid atherosclerosis were chosen from the same cohort who had undergone the ultrasonographic assessment. The diagnoses of atherosclerosis for all the 326 study subjects were reexamined and confirmed by two of our investigators (C.-L. Su and C.-T. Hong, neurologists).

Index for arsenic exposure. Well water samples were collected from each household, and the arsenic content in well water was determined during 1991–1994, by a method of hydride-generation atomic absorption spectrometry (Chiou et al., 1997b). To reflect the overall exposure to ingested arsenic for each study subject, cumulative arsenic exposure from drinking well water was applied in addition to the arsenic concentration in well water of the household. The cumulative arsenic exposure was calculated as the sum of the products derived by multiplying the arsenic concentration in well water by the years of drinking well water during the periods of living in one's household throughout the subject's life. Information on the history of well water consumption as well as a detailed residential history were obtained from the baseline questionnaire data and updated from the follow-up questionnaire.

Biochemical variables and homocysteine metabolism assay. Biochemical variables, including total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides, were assessed in 1997. All laboratory analyses were performed using a standard automatic analyzer. Height, weight, systolic blood pressure, and diastolic blood pressure were measured according to standard protocols. Hypertension was defined as (1) an average systolic blood pressure of ≥140 mm Hg, (2) an average diastolic blood pressure of ≥90 mm Hg, or (3) a history of being diagnosed as hypertensive or having taken antihypertensive medication. Subjects were considered to have diabetes, if they had ever been diagnosed by a physician or had a fasting blood sugar level of ≥126 mg/dl. For measures of total homocysteine level, plasma samples collected in 1997 were thawed and assayed by a method of high-performance liquid chromatography (HPLC) (Durand et al., 1998). Plasma folate and cobalamin levels were quantified using SimulTRAC-SNB Radioassay Kit according to commercial instructions (ICN Pharmaceuticals, Burlingame, CA).

Arsenic species in urine. 5-ml urine samples from each study subject were examined for arsenic speciation, including arsenite, arsenate, MMA^V and DMA^V, by a method of HPLC combined with hydride generation AAS as described previously (Chiou et al., 1997a). We calculated the proportion of MMA^V or DMA^V of total arsenic species and their metabolites and focused on the effect of MMA^V or DMA^V percentage (MMA% or DMA%, respectively) in the risk estimates of atherosclerosis. Urinary MMA^V percentages of 9.9% and 16.5% were taken as cut-points, which approximately represent the lower, middle and upper tertiary value of the distribution of control subjects. The corresponding tertiary cut-points for the DMA^V analysis are 71% and 83%, respectively.

Table 3
Arsenic exposure and risk of carotid atherosclerosis

Characteristics	Cases	Controls	Model I	Model II
	n (%)	n (%)	OR (95% CI)	OR (95% CI)
Arsenic concentration in well water (μg/l)				
≤50.00	25 (15.6)	39 (24.1)	1.0 (reference)	1.0 (reference)
50.01–100.00	46 (28.8)	49 (30.3)	1.6 (0.8–3.1)	1.9 (0.9–3.8)
≥100.01	89 (55.6)	74 (45.7)	2.1 (1.1–3.8)[a]	2.6 (1.3–5.0)[b]
Trend across tertiles			1.4 (1.1–1.9)[a]	1.6 (1.1–2.1)[b]
Cumulative arsenic exposure (μg/l-year)				
≤1.70	34 (21.3)	57 (35.2)	1.0 (reference)	1.0 (reference)
1.71–4.20	43 (26.9)	53 (32.7)	1.5 (0.8–2.7)	1.7 (0.9–3.2)
≥4.21	83 (51.9)	52 (32.1)	2.4 (1.4–4.3)[b]	2.9 (1.6–5.3)[c]
Trend across tertiles			1.6 (1.2–2.1)[b]	1.7 (1.3–2.3)[c]

OR, odds ratio; CI, confidence interval. Model I, adjusted for age and gender; model II, model I with the addition of current smoking, total cholesterol, hypertension, and plasma homocysteine level. Differences from the total number of 163 cases and controls are due to missing data.

[a] $0.01 < P < 0.05$.

[b] $0.001 < P < 0.01$.

[c] $P < 0.001$.

Group of Risk Factors	Arsenic Exposure	Homocysteine Level	MMA% Level	Adjusted OR (95% CI)	Grouped aOR (95% CI)
Reference	Low	Low	Low	1.0	1.0
I	Low	Low	High	0.5 (0.1-2.0)	
I	Low	High	Low	0.8 (0.2-2.7)	
I	High	Low	Low	1.7 (0.6-5.2)	1.1 (0.4-2.9)
II	Low	High	High	1.4 (0.4-5.2)	
II	High	Low	High	1.6 (0.5-4.7)	
II	High	High	Low	1.9 (0.7-5.4)	1.7 (0.6-4.5)
III	High	High	High	2.7 (0.9-7.7)	2.7 (1.0-7.8)

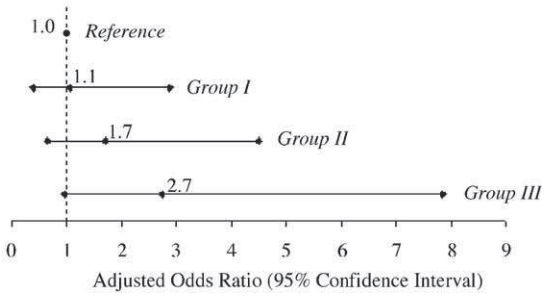

Fig. 1. Adjusted odds ratios (aOR) of atherosclerosis risk by cumulative arsenic exposure, plasma homocysteinemia level, and urinary monomethylarsonic acid percentage (MMA%). The reference group was the study subjects who were exposed to low cumulative arsenic exposure (≤1.7 μg/l-year), low plasma homocysteine level (<12.7 μmol/l), and had low MMA% (<13.4%). Data have been adjusted for age, gender, current smoking, total cholesterol, and history of hypertension. *P* for a trend test among Groups I to II: 0.006.

Statistical analysis. We first use linear regression method to analyze the relationship between plasma homocysteine level and arsenic exposure while holding constant the plasma levels of folic acid and vitamin B_{12}. These two nutrition factors are essentially involved in homocysteine metabolism (Lawrence de Koning et al., 2003). In the next atherosclerosis risk analysis, logistic regression model was used to analyze the dependence of disease risk on various risk factors in this study, including arsenic exposure, plasma homocysteine, and traditional risk factors of cardiovascular disease. The effect of a risk factor was expressed as an odds ratio (OR) and a 95% confidence interval (CI). All risk factors under study were defined as categorical variables in the regression model. To evaluate whether there was an interactive effect between plasma homocysteine level and urinary MMA% on the risk of developing carotid atherosclerosis, we estimated the risk associated with homocysteine level according to the lower, middle, or upper tertiary values of MMA% or of DMA%. The interaction of these two factors was assessed using the method, synergy index S, defined by Schlesselman and Stolley (1982). We further evaluated the combined effect of homocysteine level, MMA^V percentage, and arsenic exposure on the atherosclerosis risk and therefore classified the study subjects into eight groups according to their respective median values. All analyses were performed using SAS (Win8e) statistical software, and the statistical significance level was defined as $P < 0.05$.

Results

Relation of plasma homocysteine level with arsenic exposure

Linear regression coefficient estimates depending on plasma homocysteine level for arsenic exposure and other predictors are listed in Table 1. A significant positive association was observed in the aged, male gender and cumulative arsenic exposure, while the homocysteine level related negatively to vitamin B_{12}. No association of homocysteine level was found with plasma folate and the status of carotid atherosclerosis in the study subjects.

Traditional risk factors and carotid atherosclerosis

Table 2 shows the frequency distribution and the ORs with the 95% CIs for the classic risk factors in the 163 patients and 163 controls. Aging and current smoking were risk factors with the strongest effects on carotid atherosclerosis in this study population. Total cholesterol and LDL cholesterol were significantly higher in case subjects as compared with controls. In contrast, the effects of hypertension and plasma homocysteine level lost significance after adjusting for age and gender differences in the distribution between cases and controls. Other factors, including BMI, HDL cholesterol or triglycerides, and diabetes, revealed no evidence of an association with the development of carotid atherosclerosis in these study subjects.

Association of arsenic exposure with carotid atherosclerosis

To assess the risk of carotid atherosclerosis associated with levels of arsenic exposure, we first divided both indices of arsenic exposure into tertiles according to the distribution of the controls and then examined the trend of the ORs across the tertiles (Table 3). As shown in the table, the age–gender-adjusted analysis demonstrated a significantly higher risk of carotid atherosclerosis

Table 4
Interaction between plasma homocysteine levels and monomethylarsonic acid percentage (MMA%) for the risk of carotid atherosclerosis

Homocysteine level	MMA% < 9.9			9.9 ≤ MMA% <16.5			MMA% ≥16.5		
(μmol/l)	Patient n (%)	Control n (%)	Adjusted OR (95% CI)	Patient n (%)	Control n (%)	Adjusted OR (95% CI)	Patient n (%)	Control n (%)	Adjusted OR (95% CI)
<12.7	20 (38.5)	29 (52.7)	1.0	23 (43.4)	21 (40.4)	1.0	12 (21.8)	32 (58.2)	1.0
≥12.7	32 (61.5)	26 (47.3)	0.8 (0.3–2.1)	30 (56.6)	31 (59.6)	0.9 (0.4–2.3)	43 (78.2)	23 (41.8)	5.4 (2.0–15.0)[a]

OR, odds ratio; CI, confidence interval.
Model was adjusted for age, gender, current smoking, total cholesterol, hypertension, and cumulative arsenic exposure.
[a] $P < 0.05$ for the comparison between the strata of high or low homocysteine level.

in the upper tertile of arsenic concentration in well water compared with the first tertile (OR, 2.1; 95% CI, 1.1–3.8). Adjusting for current smoking, total cholesterol, hypertension, and plasma homocysteine level did not attenuate the relationship (OR, 2.6; 95% CI, 1.3–5.0). There was also a significant association between arsenic and carotid atherosclerosis using cumulative arsenic exposure as an index of the exposure level in this population (OR, 2.9; 95% CI, 1.6–5.3 after multivariate adjustment). The linear trends across the tertiles were significant for all models ($P < 0.05$).

Interaction between plasma homocysteine and urinary MMA% or DMA%

As indicated above, the distribution of high or low homocysteine levels was not statistically different between control and patient groups in the age–sex-adjusted analysis in Table 2. However, when we further perform a stratified analysis, according to urinary MMA% of study subjects, the association between homocysteine levels and atherosclerosis risk was different in strata of lower, middle and upper urinary MMA%, indicating a possible interaction in risk modification. As shown in Table 4, in subjects with urinary MMA% above the upper tertiary value of 16.5%, elevated plasma homocysteine level was significantly associated with a 5.4-fold increased risk (95% CI, 2.0–15.0) for carotid atherosclerosis. In contrast, the risk from the high plasma homocysteine level was not increased in the subjects with urinary MMA % less than 16.5% (OR, 0.8; 95% CI, 0.3–2.1, and OR, 0.9; 95% CI, 0.4–2.3, for the lower and middle tertiary group, respectively). On the other hand, no biological gradient among the strata of low, middle or high DMA^V percentages in urine samples (data not shown) is observed. The synergistic index ($S = 0.95$) did not reach statistical significance (χ^2 test, $P = 0.162$) in interaction estimates.

Combined effect of plasma homocysteine, urinary MMA%, and arsenic exposure

In a multivariate logistic regression analysis (Fig. 1), the risk of carotid atherosclerosis was estimated for each combination of arsenic exposure, plasma homocysteine, and urinary MMA%, using exposure to low arsenic, low homocysteine level, and low MMA^V percentage as the reference group. As is expected and shown in the Fig. 1, arsenic alone is a major risk factor in this study population (OR, 1.7; 95% CI, 0.6–5.2). Addition of high homocysteine level and high MMA^V percentage further increased the risk ratio to the arsenic-exposed individuals by 60% (OR, 2.7; 95% CI, 1.0–7.8). A trend test indicates that the atherosclerosis risk increases along with the accumulating number of the three risk factors (P for trend: 0.006).

Discussion

Our observation that carotid atherosclerosis is associated with ingested arsenic from well water is consistent with the results of our previous study carried out on a different arsenic-exposed population in southwestern Taiwan (Wang et al., 2002). In the current study, we further tested the hypothesis that arsenic exposure increases plasma homocysteine level and the subsequent risk for carotid atherosclerosis. We examined changes in plasma homocysteine levels of 326 arsenic-exposed study subjects and found that the homocysteine levels were positively correlated to the cumulative arsenic exposure through drinking well water. However, this correlation did not change substantially the independent effect of arsenic exposure on the risk of atherosclerosis in the study population. The adjusted OR (1.7-fold) for the effect of cumulative arsenic exposure was nonetheless statistically significant after controlling for plasma homocysteine level. There was only a slight change of OR from 1.8-fold, the corresponding value by dropping the homocysteine variable from the full fitted model II in Table 3 (data not shown). We also found that the levels of plasma homocysteine were not statistically related to the risk of carotid atherosclerosis in these study subjects. In other words, arsenic exposure might have an effect on plasma homocysteine levels in the study subjects, yet the biological significance of this correlation remains to be elucidated. Arsenic, acting as an independent risk factor after adjustment for other potential confounding factors, including plasma homocysteine level, should, at least, have exerted on a distinct causal pathway.

Several possible mechanisms of arsenic-induced atherosclerosis have been recently proposed based on experimental data and epidemiological evidence (Kitchin, 2001; Simeonova and Luster, 2004). Accumulating evidence demonstrated that arsenic could cause cellular redox alteration, impaired nitric oxide (NO) homeostasis, and enhanced coagulation activity, which are relevant to the dysfunction of endothelial cells (Simeonova and Luster, 2004). Endothelial dysfunction is thought to be an early event in atherosclerosis progression (Libby et al., 2002), resulting in inflammatory cell infiltration and platelet-thrombus formation (Simeonova and Luster, 2004). Exposure of endothelial cells to arsenite has been shown to induce NF-κB activation through reactive oxygen species (Barchowsky et al., 1996, 1999). It has been demonstrated that arsenite induces expression of genes encoding for inflammatory mediators including MCP-1, IL-6 and IL-8 (Simeonova et al., 2003; Lee et al., 2005). Promoter regions of these genes contain multiple binding sites for the NF-κB transcription factor. It has also been reported that arsenic increases cyclooxygenase-2 protein expression through peroxynitrite generation (Bunderson et al., 2002), suggesting a link between reactive nitrogen species and arsenic-induced inflammatory states. More recently, Bunderson et al. have also reported an association of arsenic-induced atherosclerosis with the increased expression of prostacyclin in experimental animals (Bunderson et al., 2004). Our and other reports based on arsenic-exposed human study subjects also support these laboratory findings (Wu et al., 2001, 2003; Pi et al., 2002). Taken together, arsenic-associated vascular disorders found in humans may likely arise from changes in expression levels of a variety of genes that participate in atherosclerosis through a mechanism of oxidative interference.

Most, though not all, observational studies have shown that moderately elevated plasma homocysteine levels are associated with an increased risk for premature atherosclerosis and thrombotic disease (Hackam and Anand, 2003; Fruchart et al., 2004). In experimental animals, homocysteine has been reported to accelerate atherosclerosis and amplify proatherogenic processes when

combined with other risk factors of cardiovascular disease, such as hyperlipidemia and hypertension (Matthias et al., 1996; Wang et al., 2003). Consistent with this finding, some studies have contended that the observed association between homocysteinemia and atherosclerotic events is not independent of conventional cardiovascular risk factors (Collaboration, 2002; Kolling et al., 2004). In the present study, we found no independent predictive role of elevated homocysteine levels in the risk of carotid atherosclerosis in this arsenic-exposed population. However, the risk estimates OR for elevated plasma homocysteine level were dose-dependent on the MMA% in urine samples, which indicated heterogeneity among the study subjects. The increased efficiency of metabolic methylation from arsenite to MMA^V or reduced efficiency from MMA^V to DMA^V in individuals might somehow be interrelated to plasma homocysteine levels in the development of atherosclerosis. Biomethylation is the major pathway for metabolism of arsenic, during which ROS and free radicals are concurrently produced (Cullen et al., 1984). Study subjects with higher levels of MMA% are supposedly at greater increased risk from oxidative injuries to the vascular system. Homocysteine concentrations at pathological or physiological levels have been shown to decrease the activity of glutathione peroxidase-1 (GPx-1) (Handy et al., 2005), an antioxidant enzyme. Accumulation of the ROS free radicals and subsequent oxidative damages contributing to atherosclerosis risk might therefore be enhanced. Whether GPx-1 enzyme activity is involved in the atherosclerosis in the study subjects with high levels of plasma homocysteine and high levels of MMA % after arsenic exposure, however, requires further examination.

On the other hand, there is no biological gradient in risk among the strata of low, middle or high DMA% in urine (data not shown). DMA^{III}-induced carcinogenesis has been described in animal models (Yamanaka and Okada, 1994). Although the trivalent methylated metabolites have been detected in urine of humans chronically exposed to arsenic, their associations with disease risk remains to be elucidated (Vahter, 2002). Several indices of methylation efficiency have been used in previous reports on exposed humans. Higher MMA% or lower DMA% is the most consistent predictors among the indices for the risk of arsenic-associated diseases (Del Razo et al., 1997; Yu et al., 2000; Chen et al., 2003; Tseng et al., 2005). It is unclear how an increased concentration of MMA^V relative to DMA^V would contribute significantly to an increased risk of arsenic-induced health effects. Although the mechanisms remain not fully elucidated, formation of MMA^{III} and the by-product SAH during the first methylation step may provide possible explanations (Buchet and Lauwerys, 1988; Thompson, 1993; Yi et al., 2000; Drobna et al., 2005). MMA^{III} is a reactive product harmful to tissues, and the SAH may inhibit the second step of methylation process; the latter of which could also account for the lower percentage of DMA^V observed in humans at higher risk in the same studies. In this study, no correlation of increased risk in parallel with lower DMA% was beyond our expectation. Alternatively, data variation because of small sample size may also explain. More population-based studies are needed to examine the contribution of each methylated metabolite to the observed risk following exposure to arsenic.

To assess the additional risk of atherosclerosis from the joint effect of plasma homocysteine and MMA%, on the top of the arsenic-exposed individuals, we calculated combined risk of the three risk factors and compare it to the group of low arsenic exposure, low plasma homocysteine, and low MMA%. Although arsenic alone could cause atherosclerosis in the carotid arteries of study subjects, a combination of high plasma homocysteine and high MMA% may further add a risk of ~60% (from 1.7- to 2.7-fold) to the arsenic-exposed individuals. Elevated plasma homocysteine level may result from low consumption of folic acid or vitamin B_{12} or of both in Western populations (Selhub et al., 1993). In our study on an oriental population, we also found a significantly inverse association between plasma levels of homocysteine and vitamin B_{12}. However, effect of the folate on homocysteine levels was not found in this population. Perhaps inherent or acquired heterogeneity of study subjects resulting in different risk profiles in the population studied. Although a relatively small risk for vascular disease may be difficult to detect, the combined effect from elevated homocysteine level and high MMA% may still increase a significant risk for atherosclerosis. Like many population-based studies, the observed correlation of the three factors in this population may have occurred as a random event as well. Whether there is causal relatedness should be further identified by experimental animals or confirmed by human data from different populations.

There are some potential limitations of this study. First, genetic variants of homocysteine metabolism enzymes factors were not determined for subjects, which might have contributed to some of the unexplained variation in this study. The association between homocysteine levels and atherosclerosis risk may be thus underestimated. Second, as plasma collection for the assay of homocysteine level was conducted at the almost same time as the assessment of ECCA in each study subject, the induction period for the acceleration of an atherosclerotic event due to homocysteine imposition might not have been long enough. A follow-up health examination on the study subjects in the future may overcome this limitation. Third, a larger sample size is needed to adjust for the genetic and nongenetic influences of the disease while assessing the effect of plasma homocysteine levels on atherosclerosis risk. In particular, justifying a small to moderate effect of homocysteine in the presence of a strong environmental risk factor such as arsenic requires data from large-scale studies.

In conclusion, this study demonstrated that long-term exposure to arsenic from well water is significantly associated with an increased risk of developing carotid atherosclerosis, and that the coexistence of high homocysteinemia level and high urinary MMA% may exacerbate atherosclerosis formation caused by arsenic in the carotid artery in humans. Factors involved in arsenic methylation, particularly the genetic make-up of the methyltransferase or reducing enzymes in the formation of MMA^{III}, as well as the genetic or nongenetic factors in the homocysteine metabolism likely act as risk modifiers in the development of atherosclerosis associated with arsenic. The proposition that GPx-1 enzyme may be interfered in association with homocysteine level needs to be further tested in human subjects. More studies on exposed humans or experimental animals are warranted to confirm the observed correlation of the combination of arsenic exposure homocysteinemia and high MMA% levels in this study.

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