Gene Expression of Inflammatory Molecules in Circulating Lymphocytes from Arsenic-Exposed Human Subjects

Meei-Maan Wu,[1] Hung-Yi Chiou,[2] I-Ching Ho,[1] Chien-Jen Chen,[3] and Te-Chang Lee[1,4]

[1]Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, Republic of China; [2]Institute of Public Health, Taipei Medical University, Taipei, Taiwan, Republic of China; [3]Graduate Institute of Epidemiology, National Taiwan University, Taipei, Taiwan, Republic of China; [4]Institute of Pharmaceutical Sciences, National Yang-Ming University, Taipei, Taiwan, Republic of China

Long-term arsenic exposure is associated with an increased risk of vascular diseases including ischemic heart disease, cerebrovascular disease, and carotid atherosclerosis. The pathogenic mechanisms of arsenic atherogenicity are not completely clear. A fundamental role for inflammation in atherosclerosis and its complications has become appreciated recently. To investigate molecular targets of inflammatory pathway possibly involved in arsenic-associated atherosclerosis, we conducted an exploratory study using cDNA microarray and enzyme-linked immunosorbent assay to identify genes with differential expression in arsenic-exposed yet apparently healthy individuals. As an initial experiment, array hybridization was performed with mRNA isolated from activated lymphocytes of 24 study subjects with low (0–4.32 μg/L), intermediate (4.64–9.00 μg/L), and high (9.60–46.5 μg/L) levels of blood arsenic, with each group comprising eight age-, sex-, and smoking frequency-matched individuals. A total of 708 transcripts of known human genes were analyzed, and 62 transcripts (8.8%) showed significant differences in the intermediate or high-arsenic groups compared with the low-level arsenic group. Among the significantly altered genes, several cytokines and growth factors involving inflammation, including interleukin-1 beta, interleukin-6, chemokine C-C motif ligand 2/monocyte chemotactic protein-1 (CCL2/MCP1), chemokine C-X-C motif ligand 1/growth-related oncogene alpha, chemokine C-X-C motif ligand 2/growth-related oncongene beta, CD14 antigen, and matrix metalloproteinase 1 (interstitial collagnase) were upregulated in persons with increased arsenic exposure. Multivariate analyses on 64 study subjects of varying arsenic exposure levels showed that the association of CCL2/MCP1 plasma protein level with blood arsenic remained significant after adjustment for other risk factors of cardiovascular diseases. The results of this gene expression study indicate that the expression of inflammatory molecules may be increased in human subjects after prolonged exposure to arsenic, which might be a contributory factor to the high risk of atherosclerosis in arseniasis-endemic areas in Taiwan. Further multidisciplinary studies, including molecular epidemiologic investigations, are needed to elucidate the role of arsenic-associated inflammation in the development of atherosclerosis and subsequent cardiovascular disease. *Key words:* **arsenic exposure, atherosclerosis, gene expression, inflammation.** *Environ Health Perspect* **111:1429–1438 (2003). doi:10.1289/txg.6396 available via** *http://dx.doi.org/* **[Online 23 July 2003]**

Arsenic is a well-known environmental toxin associated with an increased risk of cancer and cardiovascular disease in humans. This chemical is widely distributed because of its strong affinity with pyrite and high concentration in hydrous iron oxides (Nordstrom 2002). Natural arsenic is disseminated within our living environment by groundwater from wells drilled into arsenic-rich geologic strata or by ambient air during the process of mineral extraction (Thornton and Farago 1997; U.S. NRC 1999). Man-made sources of arsenic also include uses in agriculture, husbandry, and medicine (U.S. NRC 1999). However, the main route of exposure for the general population in arseniasis-endemic areas of the world is through the ingestion of arsenic-contaminated well water (U.S. NRC 1999; U.S. PHS 1989), including those in Taiwan, the India-Bangladesh border, and Latin America (Bagla and Kaiser 1996; Bates et al. 1992; Engel et al. 1994; Kumar 1997). The latest estimates indicate that more than 100 million people worldwide are exposed to groundwater contaminated by arsenic compounds (Chen et al. 1999).

Ingested arsenic has been associated with the development of blackfoot disease (BFD) subsequent to long-term exposure (Chen et al. 1988; Tseng 1977). BFD is a unique peripheral vascular disease endemic in the southwestern coast of Taiwan. Pathological studies have demonstrated that 70% of BFD patients have histologic lesions compatible with the changes of arteriosclerosis obliterans and 30% with the changes of thromboangiitis obliterans (Yeh and How 1963). The fundamental vascular change of BFD in both types is a severe generalized arteriosclerosis (Yeh and How 1963). Recent reports have also showed that long-term arsenic exposure is closely associated with an increased risk of hypertension, diabetic mellitus, ischemic heart disease, cerebral infarction, and carotid atherosclerosis (Chen et al. 1995, 1996; Chiou et al. 1997; Tseng et al. 2000; Wang et al. 2002). Arsenic is a seemingly independent risk factor for multiple cardiovascular end points in addition to traditional risk factors such as high fat intake, alcohol consumption, and cigarette smoking. However, the pathological mechanism by which arsenic induces changes leading to vascular disorders remains to be delineated. Response to injured endothelial cells and/or stimulating proliferation of a single smooth muscle cell have long been hypothesized for the pathogenesis of atherosclerosis (Libby et al. 2002; Ross 1986, 1999). Underlying this hypothesis, activation and recruitment of blood leukocytes, as well as continuing expression of proinflammatory factors in the lesion area, characterize all stages of atherogenesis. To date, however, the contribution of inflammatory mediators has not been investigated for arsenic-associated vascular disease in human population. Arsenite, trivalent arsenic, is generally considered a poor DNA-damaging agent at noncytotoxic concentrations in cell culture studies (Kitchin 2001). We hypothesize that arsenic-associated vascular disorders observed in the human population may arise from alterations in the expression of a variety of inflammatory genes that participate in the development of atherosclerotic lesions during long-term exposure.

To identify aberrant gene expression in inflammation that is possibly involved in arsenic atherogenicity, we used a human cDNA microarray to search for differentially

Address correspondence to T-C. Lee, Institute of Biomedical Sciences, Academia Sinica, 128 Academia Rd., Section 2, Nankang, Taipei 11529, Taiwan, Republic of China. Telephone: 02 2652 3055. Fax: 02 2782 9142. E-mail: bmtcl@ibms.sinica.edu.tw

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expressed genes in peripheral blood lymphocytes (PBLs) from arsenic-exposed individuals. Recent studies in microarray analysis concerning adverse health effects of arsenic have been focused mainly on its carcinogenic properties (Chen et al. 2001; Lu et al. 2001; Yih et al. 2002). Few gene expression studies have focused on the atherogenic effect of arsenic exposure. In this report, we first demonstrate the application of cDNA microarray technology to identify gene expression changes in PBLs from arsenic-exposed individuals and show that blood arsenic is significantly associated with changes in transcription levels of several inflammatory mediator genes that have been implicated in the atherosclerotic process. PBLs do not represent all the cells involved in progression of atheroma formation but are the only collectable cell samples from apparently healthy humans in a population study, which may reflect the inflammatory response to an environmental injury. The enhanced expression of inflammatory molecules in blood leukocytes from an arsenic-exposed population may contribute to the development of atherosclerosis associated with arsenic exposure.

Materials and Methods

Study Subjects and Tissue Samples

Sixty-four residents identified as consumers of arsenic-tainted well water in Lanyang Basin of northeastern Taiwan, Republic of China, were recruited for previous studies of arsenic toxicity (Wu et al. 2001). For the present study, frozen peripheral blood lymphocytes and plasma samples previously stored from the study subjects were analyzed. Detailed characteristics of the study area, subject recruitment and blood collection, and determination of arsenic concentration in whole blood samples have been described previously (Wu et al. 2001). Isolation, freezing, and storage of the lymphocytes in liquid nitrogen were performed according to the methods described by Venkataraman and Westerman (Venkataraman and Westerman 1986). Plasma samples were preserved at –20°C until protein assay was performed for this study. Computerized records of the serum levels of total cholesterol and triglycerides initially determined by an autoanalyzer were retrieved for the study subjects. Information on demographic or clinical characteristics, as well as lifestyle data including alcohol consumption and smoking habits of the study subjects were also obtained from previous records. All study subjects gave their consent and were free of clinical symptoms,as described in our previous study using the same population (Wu et al. 2001).

mRNA Preparation and cDNA Microarray Analysis

Because of limited samples of frozen lymphocytes, only the study subjects who had a cell number of 15–20 × 10^6 in stock were selected for the cDNA microarray hybridization analysis as an initial experiment. A total of 24 study subjects whose cells were available from the archives were further separated into groups on the basis of blood arsenic levels [low (0.00–4.32 μg/L), intermediate (4.64–9.00 μg/L) and high (9.60–46.5 μg/L)], with each group comprising eight similar age-, sex-, and smoking-frequency–matched individuals. Lymphocyte samples were thawed and cultured in RPMI-1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 20% heat-inactivated fetal bovine serum (Hyclone Laboratory, Logan, UT, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 for 68 hr. Using TRI reagent (Molecular Research Center, Cincinnati, OH, USA), we extracted a total of 30–50 μg cellular RNA from the harvested cells for each study subject, which was further pooled into groups of low, intermediate, or high arsenic levels for subsequent isolation of mRNA. mRNA was extracted using Oligotex-dT resin (Qiagen, Hilden, Germany) and was used to prepare targets for cDNA microarray hybridization and first-strand cDNA for quantitative real-time polymerase chain reaction (PCR) assay.

Seven hundred eight cDNA elements used as probes, including 662 known genes of potential significance in arsenic toxicity, 16 housekeeping genes, and 22 expressed-sequence tags (ESTs), were prepared by PCR amplification of IMAGE consortium cDNA clones and arrayed on a 5 × 8 mm nylon membrane, using methods described previously (Chen et al. 1998). Also included in the membrane chip were eight plant genes, whose hybridization results served as negative controls. The cDNA microarray hybridization experiment was performed with this 708 cDNA probes array using a colorimetric detection method described previously (Yih et al. 2002). Briefly, biotin-labeled cDNA targets were prepared from 2 μg mRNA by reverse transcriptase (Superscript II; GIBCO BRL, Gaithersburg, MD, USA) incorporation of biotin-16–2'-deoxyuridine-5'-triphosphate (Roche Diagnostic, Mannheim, Germany). After precipitation, the labeled targets were dissolved in hybridization buffer and incubated with the prehybridization-treated probes array at 65°C overnight. The hybridized arrays were then washed at room temperature twice in 2 × SSC (0.15 M NaCl/0.015 M Na citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS) for 5 min, and 3 times at 65°C in 0.1 × SSC, 0.1% SDS for 15 min. After thorough washing, the arrays were blocked and incubated with streptavidin-β-galactosidase conjugate reagent for chromagen development. After a wash to remove any unbound conjugates, an X-gal substrate solution was added to the array and incubated at 37°C for 30 min with occasional shaking. Color development was terminated by addition of phosphate-buffered saline. The signal intensity of spots on arrays was acquired using a flatbet scanner at appropriate optical resolution. Quantitative results were analyzed using GenePix Pro (version 3.0; Axon Instruments, Union, CA, USA) and Microsoft Excel 2000 software (version 9; Microsoft Corp., Taipei, Taiwan).

To allow for better comparison between hybridization experiments, a series of four array probes was prepared for each membrane using known concentrations of 10-fold serial dilutions of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) clone. A standard curve plotting the signal intensity versus the concentration of four serial-diluted *GAPDH* clones was generated for each set of gene spots to be tested on one array. By comparing the signal intensity of the tested spot to this standard curve, the relative intensity of the spot was normalized against *GAPDH* intensity. After standardization, ratios of relative intensity were calculated between arsenic groups for all gene spots. Gene-specific signal ratio was considered significant if the logarithm of the ratio differed by ≥ 3 SD from the mean $\log_2$ of the ratio for the housekeeping genes set. To date, arsenic has not been shown to have appreciable effects on the expression of these housekeeping genes.

Quantitative Reverse-Transcriptase–Polymerase Chain Reaction Analysis

The quantitation of mRNA level was carried out using a real-time SYBER Green I fluorescence detection method as described previously (Morrison et al. 1998; Wittwer et al. 1997). In brief, 1 μg mRNA was first reverse-transcribed into cDNA using random primers (Roche Diagnostic) and purified by a 30-min incubation at 37°C with RNase H (Invitrogen, Carlsbad, CA, USA) followed by ethanol precipitation. The specific cDNA of interest and a reference cDNA, *GAPDH*, were PCR-amplified separately in optical tubes and caps using the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster, CA, USA). Primer design and PCR reaction were performed according to commercial

instructions provided by Applied Biosystems. Dissociation curve analysis was performed after PCR amplification (ABI PRISM 7700; Applied Biosystems) to ensure no fluorescence contamination from nonspecific dsDNA product. Results of the derivative dissociation curve profile exhibited no nonspecific products in PCR reaction solution. All PCR reactions were performed in duplicate.

Initial template concentration of a specific gene was derived from the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of the PCR reaction. For comparison of mRNA levels between groups, relative gene expression level was first determined by subtracting from the respective cycle number of *GAPDH* gene for each group. Values were then used to calculate for relative folds normalized to the relative amounts of the same gene in the low-level arsenic group.

Enzyme-linked immunosorbent assay. Selected inflammatory molecules, including interleukin-1 beta (IL1β), interleukin-6 (IL6), chemokine C-C motif ligand 2/monocyte chemotactic protein-1 (CCL2/MCP1), and chemokine C-X-C motif ligand 1/growth-related oncogene alpha (CXCL1/GRO1) protein levels in plasma, were measured for the 64 study subjects by enzyme-linked immunosorbent assay (ELISA; Biotrak, Piscataway, NJ, USA) according to the manufacturer's instructions. Lower limits of detection of assays for IL1β, IL6, CCL2/MCP1, and CXCL1/GRO1 were 0.31, 0.31, 20.5, and 15.6 pg/mL, respectively.

Statistical Methods

For comparison of more than two groups, one-way analysis of variance (ANOVA) or chi-square test was applied where appropriate. Spearman correlation coefficient was used to determine statistical association between study variables. We performed multiple linear regression analysis to examine the effect of arsenic concentration on the protein expression level in plasma after controlling for confounding factors. Statistical significance was accepted at a level of $p < 0.05$.

Results

Differentially Expressed Genes in Lymphocytes of Arsenic-Exposed Individuals

To identify genes potentially associated with arsenic atherogenicity, we compared the gene expression profile of peripheral blood lymphocytes from 24 selected individuals of low-, intermediate-, or high-level arsenic exposure groups (Figure 1; detailed information on the 708 cDNA clones spotted on membrane chip, as well as the resultant signal intensity for each study gene, are accessible at http://www.ibms.sinica.edu.tw/~bmtcl/As-chip-TCL01-PBL.xls). Hybridization intensities of the four serially diluted *GAPDH* clones are shown on the eighth line from the top. The *GAPDH* transcription levels showed a logarithmic relation with signal intensity, and a standard curve for linear transformation was generated as described in 'Materials and Methods." Table 1 includes the relative intensities of nine housekeeping genes among groups of varying arsenic exposure; the other seven housekeeping genes were either duplicates or had an expression level

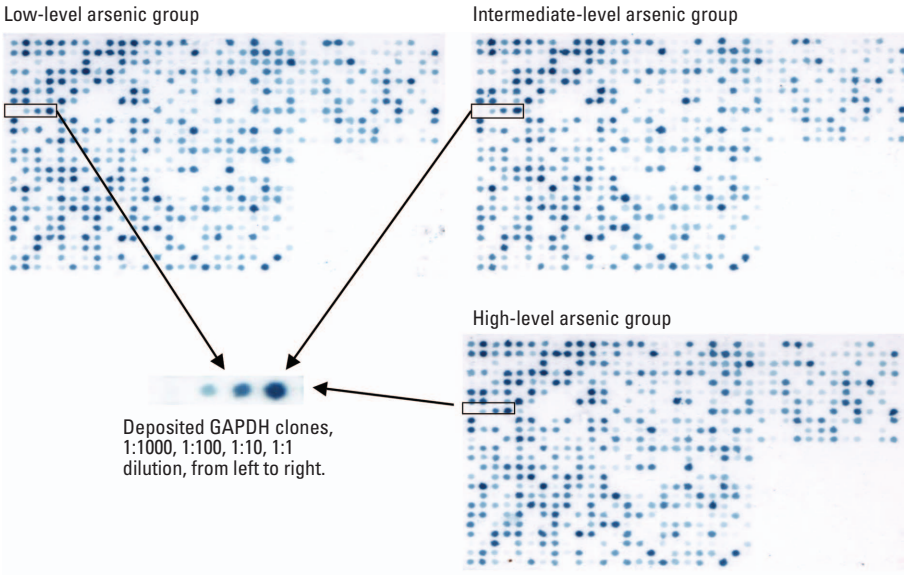

Figure 1. Section of colorimetric cDNA microarray hybridized to mRNA extracted from peripheral blood lymphocytes of various arsenic-exposed groups. mRNA was extracted from pooled total RNA samples obtained from eight individuals representative of each arsenic group. The eight individuals were age, sex, and smoking frequency-matched among groups. The array contained 708 cDNA elements, each representing an individual gene. One membrane is shown for each arsenic group. The serial-diluted *GAPDH* spots in one membrane, as shown in the box, were used to calibrate the relative intensity for each individual image in the same membrane.

Table 1. Relative intensity of mRNA levels of nine housekeeping genes in peripheral blood lymphocytes from arsenic-exposed study subjects, Lanyang Basin, Taiwan.[a,b]

Accession number[c]	Description[c]	Low (0.00–4.32)	Intermediate (4.64–9.00)	High (9.60–46.5)	Arsenic concentration in blood (μg/L) $\log_2$ (Intermediate/low)	$\log_2$ (High/low)	$\log_2$ (High/intermediate)
AA186639	Ribosomal protein S27	1313.14	1414.26	1085.65	0.107	–0.274	–0.381
AA126291	H3 histone, family 3B	1314.83	1103.04	1204.01	–0.253	–0.127	0.126
AA053244	Basic transcription factor 3	152.35	85.07	106.88	–0.841	–0.511	0.329
AA065001	Ribosomal protein S3	1510.32	1327.67	1172.98	–0.186	–0.365	–0.179
AA147674	Ribosomal protein S20	936.38	921.45	890.70	–0.023	–0.072	–0.049
AA064618	Ribosomal protein L28	204.48	246.29	161.65	0.268	–0.339	–0.607
AA131097	Ribosomal protein S5	407.75	320.50	426.96	–0.347	–0.066	0.414
M33197	GAPDH, 1:10 dilution	155.35	148.98	174.85	–0.060	0.171	0.231
H66115	Glucose phosphate isomerase	693.60	533.72	624.58	–0.378	–0.151	0.227

[a]mRNA was extracted from pooled total RNA samples obtained from 8 individuals representative of each arsenic group. The eight individuals were age-, sex,- and smoking-frequency–matched among groups. [b]Quantification of each individual gene in one group was standardized to a calibration curve established from serial dilutions of GAPDH gene of the same group. [c]Information from the UniGene database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). [d]The means of the logarithm base 2 of signal ratio (± SD) for the housekeeping genes were –0.190 (± 0.322), –0.178 (± 0.217), and 0.012 (± 0.344) for intermediate versus low, high versus low, and high versus intermediate, respectively.

below threshold. As demonstrated in Table 1, housekeeping genes showed relatively constant expression levels among groups; the means of the logarithm base 2 of signal ratio (± SD) were –0.190 (± 0.322), –0.178 (± 0.217), and 0.012 (± 0.344) for intermediate versus low, high versus low, and high versus intermediate, respectively. On the basis of the expression variation with 3 × SD from the mean log for the housekeeping genes, we identified 26 cDNA clones with an increased expression signal in intermediate- or high-level arsenic groups, and 36 cDNA clones with reduced expression in intermediate- or high-level arsenic groups compared with the low-level arsenic group. Except for five clones of EST or clones withdrawn from the Unigene database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene), the remaining 57 genes of known function included those involving growth factor or cytokine related, signaling transduction pathway, transcription regulatory components, cell-cycle control, DNA replication/repair activity, redox homeostasis, and matrix-degrading enzymes (Table 2).

Of particular interest, genes of cytokine-related or growth factors involving inflammation were significantly elevated in the high-level arsenic exposure groups (Table 2). These inflammatory molecules have recently been implicated in the atherosclerotic process for a variety of vascular diseases. A number of these genes detected by the microarray as significantly induced in lymphocytes, such as *IL1β*, *IL6*, *CCL2/MCP1*, *CXCL1/GRO1*, chemokine C-X-C motif ligand 2/growth-related oncogene beta (*CXCL2/GRO2*), CD14 antigen (*CD14*), and interstitial collagenase matrix metalloproteinase 1 (*MMP1*), were selected for a confirmation test using a real-time reverse-transcriptase–polymerase chain reaction method. As indicated in Figure 2, we reconfirmed the change profile in gene expression of these genes in parallel with the arsenic exposure group. Comparison of the colorimetric cDNA microarray method with SYBR Green I real-time PCR assay (Applied Biosystems) showed consistent fold changes in expression for these six genes.

Protein Levels of Inflammatory Molecules in Plasma of Arsenic-Exposed Individuals

Four genes detected by the microarray as significantly induced in PBL of the higher-level arsenic groups, including IL1β, IL6, CCL2/MCP1, and CXCL1/GRO1, were studied by ELISA assay to quantitatively evaluate protein expression level in plasma samples of 64 study subjects. Demographic and clinical characteristics of the study subjects by blood arsenic concentration are summarized in Table 3. As shown in this table, the three groups of varying arsenic exposure did not differ with respect to age, percentage of male gender, current smoker, serum cholesterol, or triglyceride but differed in regard to body mass index. Study subjects of high-level arsenic group were significantly underweight as compared with the other two groups ($p = 0.021$).

Table 4 shows the results of ELISA assay for IL1β, IL6, CCL2/MCP1, and CXCL1/GRO1 protein expression level in plasma of the study subjects. Although there was considerable variation within each arsenic group, a positive correlation was observed between arsenic exposures and plasma protein levels of CCL2/MCP1. Because the distribution of plasma protein levels in these study subjects was wide and skewed to the left, individual measurements of protein level were logarithmically transformed in the next regression analysis for CCL2/MCP1 to reduce the influence of extreme values on the estimates of parameters. As summarized in Table 5, we found no significant association of plasma CCL2/MCP1 protein level with body mass index, cholesterol, triglyceride, or smoking status. However, blood arsenic concentration was significantly associated with the CCL2/MCP1 protein level after adjustment for age and gender through multivariate regression analysis.

Discussion

Arsenic is an environmental contaminant that warrants high concern for human health. Long-term arsenic exposure is closely associated with adverse health effects, including several vascular disorders (Chen et al. 1996; Chiou et al. 1997; Engel et al. 1994; Tseng et al. 1995, 1996; Wang et al. 2002). The possibility that arsenic induces atherosclerosis through its actions on the change of inflammatory-related gene expression needs to be elucidated. By using cDNA microarray analysis on circulating lymphocytes from healthy arsenic-exposed individuals, we found that alteration in expression level of several genes involved in inflammation showed a positive correlation with arsenic concentration in the whole blood of study subjects. In some of study genes, a dose–response relationship between transcription level and arsenic exposure was not observed; in this case, there might be other risk factors interfering with gene expression, thus confounding the dose-dependent pattern under study in this population. As individual RNA samples were not available, the influence of a potential confounding effect was not examined. However, further studies of plasma protein level by ELISA exhibited a significant correlation with CCL2/MCP1 that remained significant after adjustment for other risk factors of cardiovascular disease. In contrast, we found no significant correlation of plasma protein levels for IL1β, IL6, and CXCL1/GRO1 with blood arsenic as observed in the gene expression studies. It is probable that because of posttranscriptional regulation, changes in mRNA expression would not show corresponding changes in protein levels. In addition, the number of study subjects for these genes may not be large enough to draw a definite conclusion on the association between plasma protein level and arsenic exposure gradient. Taken together, the enhanced expression of the inflammatory molecules observed in blood lymphocytes of arsenic-exposed study subjects may contribute to the atherosclerotic process caused by arsenic, although other gene factors cannot be excluded.

The role of inflammatory cytokines or growth factors with inflammatory reactivity has gained increasing attention in the pathogenesis of atherosclerotic lesions (Libby et al. 2002; Ross 1999). The main contributors to the risk for atherosclerosis include lipoprotein, homocysteine, hypertension, diabetes, infectious agents, and oxidant stress (Libby et al. 2002). Arsenic is widely accepted as a prooxidant stimulus. In humans, prolonged exposure to arsenic that accompanies persistent oxidative stress in the vasculature system might trigger inflammation and thereafter lead to atheroma formation. Although directed migration of mononuclear leukocytes, including T lymphocytes, into the tunica intima by chemokines produced by endothelial and smooth muscle cells characterizes the initiation of the artherosclerotic lesions, the activated leukocytes in arterial intima also secrete proinflammatory cytokines that amplify inflammatory response in the lesion (Libby 2002). How the induction of inflammatory mediators in activated T lymphocytes residing in blood circulation or in arterial intima of arsenic-exposed humans might lead to atherosclerosis requires further study. In the present study, gene expression of *IL1β* and *IL6* was elevated in association with arsenic exposure in the study subjects. *IL1β* contributes to vascular smooth muscle cell (VSMC) proliferation and lesion progression in atherosclerosis (Nathe et al. 2002). *IL6* plays a role in atherosclerosis as a mediator in chemotactic activity or in cell proliferation after stressful stimuli (Klouche et al. 2000; Verma et al. 2002). *CCL2/MCP1* is a key mediator of leukocyte transmigration

Table 2. Relative intensity of mRNA levels of differentially expressed genes in peripheral blood lymphocytes from arsenic-exposed study subjects, Lanyang Basin, Taiwan.[a,b]

Accession number[c]	Description; symbol[c]	Arsenic concentration in blood (μg/L) Low (0.00–4.32)	Intermediate (4.64–9.00)	High (9.60–46.5)	$\log_2$ (Intermediate/low)	$\log_2$ (High/low)	$\log_2$ (High/intermediate)
Growth factor or cytokine-related genes							
AA150507	Interleukin-1, beta; *IL1β*	65.12	86.88	137.87	0.416	1.082[d]	0.666
N98591	Interleukin-6 (interferon, beta 2); *IL6*	50.95	48.54	131.15	–0.070	1.364[d]	1.434[d]
H96871	Chemokine (C-C motif) ligand 2; *CCL2*	36.09	113.89	105.92	1.658[d]	1.553[d]	–0.105
W42723	Chemokine (C-X-C motif) ligand 1; *CXCL1*	19.43	22.22	56.43	0.194	1.538[d]	1.344[d]
AA487453	Chemokine (C-X-C motif) ligand 2; *CXCL2*	80.61	85.57	202.63	0.086	1.330[d]	1.244[d]
R94179	Hepatoma-derived growth factor (high-mobility group protein 1-like); *HDGF*	76.53	63.92	115.21	–0.260	0.590[d]	0.850
H11719	CD14 antigen; *CD14*	6.76	9.70	14.48	0.522	1.100[d]	0.577
H57126	Colony-stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog; *CSF1R*	*3.29*	*3.80*	*4.83*	*0.207*	*0.553*[d]	*0.346*
H87426	Interferon gamma receptor 1; *IFNGR1*	114.37	47.00	75.19	–1.283[e]	–0.605	0.678
R45384	Activin A receptor, type 1; *ACVR1*	46.86	18.22	31.19	–1.363[e]	–0.587	0.776
R39862	Activated leukocyte cell adhesion molecule; *ALCAM*	28.74	11.96	17.80	–1.265[e]	–0.691	0.574
Signal transduction pathway genes							
H11455	*RAB5A,* member *RAS* oncogene family; *RAB5A*	81.97	34.13	50.71	–1.264[e]	–0.693	0.571
R20666	Endothelial differentiation, sphingolipid G-protein-coupled receptor, 1*; EDG1*	208.10	92.21	134.84	–1.174[e]	–0.626	0.548
R43007	Annexin A7*; ANXA7*	336.47	246.85	183.48	–0.447	–0.875[e]	–0.428
R84980	Inositol 1,3,4-triphosphate 5/6 kinase*;* ITPK1	22.22	22.92	34.76	0.045	0.646[d]	0.601
N62226	Phosphatidylinositol 4-kinase, catalytic*,* alpha polypeptide; *PIK4CA*	42.87	46.04	74.82	0.103	0.804[d]	0.701
R39925	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha); *PIK3R1*	35.74	18.15	17.53	–0.978	–1.027[e]	–0.049
R42845	Myotubular myopathy 1; *MTM1*	19.61	9.95	10.39	–0.979	–0.916[e]	0.064
H07920	Mitogen-activated protein kinase kinase 6	11.58	7.49	4.69	–0.630	–1.304[e]	–0.674
T89100	Mitogen-activated protein kinase 6; *MAPK6*	196.18	62.01	88.79	–1.662[e]	–1.144[e]	0.518
T57875	Protein kinase C, iota; *PRKCI*	58.04	24.14	42.34	–1.266[e]	–0.455	0.811
R43147	Protein kinase, cAMP-dependent, regulatory, type1, alpha (tissue-specific extinguisher 1); *PRKAR1A*	357.84	156.01	187.91	–1.198[e]	–0.929[e]	0.268
AA018676	Protein kinase, AMP-activated, gamma 1 noncatalytic subunit; *PRKAG1*	260.15	152.28	142.57	–0.773	–0.868[e]	–0.095
Transcription regulatory genes							
R08560	Spleen focus forming virus (SFFV) proviral integration oncogene spi1; *SPI1*	27.18	32.21	40.65	0.245	0.581[d]	0.336
R15253	V-fos FBJ murine osteosarcoma viral oncogene homolog; *FOS*	136.24	126.03	63.85	–0.112	–1.093[e]	–0.981
H24055	Heat-shock transcription factor 2; *HSF2*	575.88	357.86	301.82	–0.686	–0.932[e]	–0.246
H07034	B-cell CLL/lymphoma 6 (zinc finger protein 51); *BCL6*	20.58	10.42	10.06	–0.983	–1.033[e]	–0.050
R39273	MAD, mothers against decapentaplegic homolog 4 (*Drosophila); MADH4*	94.47	38.76	59.69	–1.285[e]	–0.662	0.623
H18451	Transcription factor A, mitochondrial; *TFAM*	100.56	41.70	56.65	–1.270[e]	–0.828	0.442
H09636	DEK oncogene (DNA binding); *DEK*	59.54	30.49	30.07	–0.965	–0.986[e]	–0.020
H23978	General transcription factor IIB; *GTF2B*	137.75	72.43	69.72	–0.927	–0.982[e]	–0.055
R91548	Topoisomerase (DNA) I; *TOP1*	194.36	85.65	159.14	–1.182[e]	–0.288	0.894
T65211	SFRS protein kinase 2; *SRPK2*	50.82	19.55	27.73	–1.378[e]	–0.874[e]	0.504
R55052	PRP4 pre-mRNA processing factor 4 homolog B (yeast); *PRPF4B*	156.81	89.06	84.36	–0.816	–0.894[e]	–0.078
Cell-cycle control genes							
N21348	Menage a trois 1 (CAK assembly factor); *MNAT1*	3.29	2.90	6.16	–0.185	0.903[d]	1.088[d]
AA164211	Cyclin C; *CCNC*	219.44	91.80	150.92	–1.257[e]	–0.540	0.717

Continued, next page

Table 2. Continued.

Accession number[c]	Description; symbol[c]	Low (0.00–4.32)	Intermediate (4.64–9.00)	High (9.60–46.5)	Arsenic concentration in blood (μg/L) $\log_2$ (Intermediate/low)	$\log_2$ (High/low)	$\log_2$ (High/intermediate)
DNA replication/repair genes							
AA028094	Polymerase (DNA directed), delta 2, regulatory subunit 50kDa; *POLD2*	144.11	141.44	229.63	–0.027	0.672[d]	0.699
H14431	Polymerase (DNA directed), beta; *POLB*	32.88	16.48	18.06	–0.997	–0.864[e]	0.132
AA035596	Excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence); *ERCC1*	34.26	22.21	49.96	–0.625	0.544[d]	1.170[d]
AA013051	Topoisomerase (DNA) II binding protein; *TOPBP1*	193.73	78.51	142.76	–1.303[e]	–0.440	0.863
Redox homeostasis genes							
R81700	Glutathione peroxidase 4 (phospholipid hydroperoxidase); *GPX4*	24.12	16.35	34.27	–0.561	0.507[d]	1.068[d]
NM_002133	Heme oxygenase (decycling) 1; *HMOX1*	19.83	27.96	39.38	0.496	0.990[d]	0.494
R45064	Serine/threonine kinase 38; *STK38*	70.88	30.52	43.83	–1.216[e]	–0.693	0.522
T77613	Aldehyde dehydrogenase 3 family, member A2; *ALDH3A2*	23.39	10.83	12.83	–1.111	–0.866[e]	0.244
R49679	COX11 homolog, cytochrome *c* oxidase assembly protein (yeast); *COX11*	23.79	14.02	11.75	–0.763	–1.018[e]	–0.255
Matrix-degrading enzyme genes							
AA081006	Matrix metalloproteinase 1 (interstitial collagenase); *MMP1*	3.29	3.68	6.47	0.161	0.973[d]	0.812
R63637	Matrix metalloproteinase 12 (macrophage elastase); *MMP12*	4.37	5.65	6.14	0.371	0.490[d]	0.119
N33214	Matrix metalloproteinase 14 (membrane-inserted); *MMP14*	8.91	12.10	18.38	0.442	1.044[d]	0.603
R55625	Matrix metalloproteinase 19; *MMP19*	3.29	3.65	4.74	0.150	0.525[d]	0.375
Miscellaneous genes							
AA134959	Interferon-induced protein with tetratricopeptide repeats 4; *IFIT4*	25.97	28.95	37.84	0.157	0.543[d]	0.386
R32850	Major histocompatibility complex, class I, E; *HLA-E*	13.74	13.08	20.42	-0.071	0.572[d]	0.643
AA031807	Feline sarcoma oncogene; *FES*	9.40	6.98	14.28	–0.430	0.602[d]	1.032
AA031530	Brain protein I3; *BRI3*	3.64	3.53	5.28	–0.044	0.537[d]	0.581
R94976	PTD009 protein; *PTD009*	4.06	3.35	5.85	–0.275	0.529[d]	0.804
AA515390	Lamin B receptor; *LBR*	208.98	88.26	172.33	–1.244[e]	–0.278	0.965
R41478	COP9 homolog; *COP9*	75.55	40.58	41.11	–0.897	–0.878[e]	0.018
H15248	Lipase A, lysosomal acid, cholesterol esterase (Wolman disease); *LIPA*	113.71	54.16	41.92	–1.070	–1.439[e]	–0.369

[a]mRNA was extracted from pooled total RNA samples obtained from eight individuals representative of each arsenic group. The eight individuals were age-, sex,- and smoking-frequency–matched among groups. [b]Quantification of each individual gene in one group was standardized to a calibration curve established from serial dilutions of GAPDH gene of the same group. [c]Information from the UniGene database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). [d]Significantly upregulated, defined as the $\log_2$ of signal ratio (intermediate- to low- level arsenic group, high- to low-level arsenic group, or high to intermediate-level arsenic group) differs by ≥ 3 SD from the corresponding mean $\log_2$ of the ratio for the nine housekeeping genes shown in the table. [e]Significantly downregulated, defined as the $\log_2$ of signal ratio (intermediate to low-level arsenic group, high- to low-level arsenic group, or high- to intermediate-level arsenic group) differs by ≤ 3 SD from the corresponding mean $\log_2$ of the ratio for the nine housekeeping genes as shown in the table.

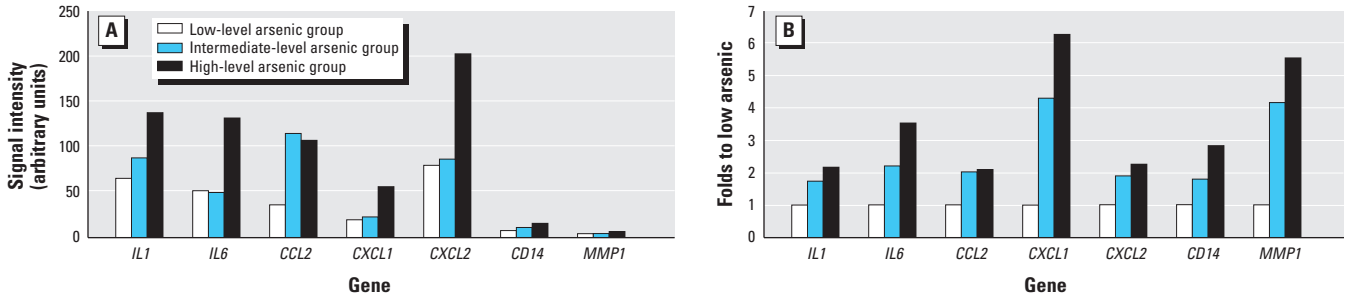

Figure 2. Relative abundance of mRNA level of *IL1β*, *IL6, CCL2/MCP1, CXCL1/GRO1, CXCL2/GRO2, CD14,* and *MMP1* in peripheral blood lymphocytes of study subjects by arsenic exposure group. mRNA was extracted from pooled total RNA samples obtained from eight individuals representative of each arsenic group. The eight individuals were age, sex, and smoking frequency-matched among groups. (*A*) Genes obtained using a colorimetric cDNA microarray method. (*B*) Genes obtained by SYBR Green I real-time PCR assay. Sequences of primers used for PCR analysis were as follows. IL1β: (F)-AGCAGAAAACATGCCCGTCTT, (R)-CCACATTCAGCACAGGACTCTCT. IL6: (F)-TGGCTGCAGGACATGACAA, (R)-TGAGGTGCCCATGCTACATTT. CCL2/MCP1: (F)-CCCAAGAATCTGCAGCTAACTTATT, (R)-AAGGCATAATGTTTCACATCAACAA. CXCL1/GRO1: (F)-TTTCTGAGGAGCCTGCAACA, (R)-ATTCCCCTGCCTTCACAATG. CXCL2/GRO2: (F)-AGAGGCTGAGGAATC-CAAGAAA, (R)-TGAGAAATGTTGACCACACACTGT. CD14: (F)-AAGGACTGCCAGCCAAGCT, (R)-TTCCCGTCCAGTGTCAGGTT. MMP1: (F)- GCAGCTTCAGTGACAAA-CATATCC, (R)- GGTGACACCAGTGACTGCACAT. GAPDH: (F)-GGAGTCCCTGCCACACTCA, (R)-GCCCCTCCCCTCTTCAAG.

to sites of inflammation and thus plays an important role in the development of artherosclerosis (Rosenfeld 2002). Enhanced *CCL2/MCP1* transcription level was also detected in lymphocytes from high arsenic level group in this study. Growth-stimulating gene expression, such as *CXCL1/GRO1* and *CXCL2/GRO2*, was upregulated in the high-level arsenic exposure group. In experimental animals, CXCL1/GRO1 protein also triggers monocyte arrest on early atherosclerotic endothelium (Huo et al. 2001). *CXCL2/GRO2* is a potent chemotactic agent for polymorphonuclear leukocytes as well (Wolpe et al. 1989). Hepatoma-derived growth factor (*HDGF*) was activated in study subjects of the high-level arsenic exposure group. Recent studies provide evidence for HDGF stimulation of DNA synthesis in VSMCs (Everett et al. 2001). CD14 molecules interact with apoptotic cells, triggering phagocytosis of the cells and also acting as a receptor that binds bacterial lipopolysaccharide, triggering inflammatory responses (Devitt et al. 1998). Colony-stimulating factor 1 receptor (*CSF1R*) encodes the receptor for macrophage colony-stimulating factor, potentially involved in promoting transforming activity (Hampe et al. 1989). Enhanced gene expression of both these genes was observed in subjects from the high-level arsenic exposure group in the present study. In contrast, mRNA levels of interferon gamma receptor 1 (*IFNGR1*), activin A receptor, type 1 (*ACVR1*), and activated leukocyte cell adhesion molecule (*ALCAM*) all exhibited downregulation in study subjects of the high-level arsenic exposure group. Repression of *IFNGR1* was unexpected, as major histocompatibility complex, class I, E (*HLA-E*) was activated in association with high arsenic levels in the study subjects. Enhanced expression of both immune-related genes should have increased the overall inflammatory response. Downregulation of *ACVR1* for activin may result in loss of induction for smooth muscle cell differentiation, and thus is involved in plaque destabilization (Engelse et al. 1999). *ALCAM* is a CD6 ligand expressed by activated leukocytes and involved in dynamic growth and/or migration (Swart 2002).

Aberrant expression of inflammatory cytokines or growth factors has been consistently noted in both *in vitro* or *in vivo* arsenic studies, although patterns of production vary between cell systems (Chen et al. 2001; Germolec et al. 1997, 1998; Lu et al. 2001; Yih et al. 2002). In cultured human keratinocytes or the Tg.Ac transgenic mice model, sodium arsenite induced a dose-dependent increase in the expression of growth factors, including granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-alpha, or tumor growth factor-alpha, but not in the expression of inflammatory cytokines such as *IL1β*, *IL6*, or *CCL2/MCP1* (Germolec et al. 1997, 1998). Altered expression in these growth factors is associated with the development of skin neoplasia (Germolec et al. 1997, 1998). Expression of *IL6*, *CCL2/MCP1*, *CXCL1/GRO1,* and *CXCL2/GRO2* is decreased in human fibroblast cells after treatment with 5 μM arsenite for 0–24 hr (Yih et al. 2002). Results of another study, however, showed an enhanced expression of inflammatory cytokines or cytokine-related components, such as *IL1* receptor and *IL6* receptor, in arsenic-transformed cells associated with malignant transformation (Chen et al. 2001). In arsenic-exposed human livers, expression of hepatocyte growth factors *IL1β*, and *IL6* receptor is also increased (Lu et al. 2001). In our study, increased gene expression of *IL1β*, *IL6*, *CCL2/MCP1*, *CXCL1/GRO1*, *CXCL2/GRO2,* and *HDGF* as detected by cDNA microarray was observed in association with blood arsenic in activated lymphocytes of study subjects who had ingested arsenic-tainted well water for an extended period of time. The specific profile change of inflammatory molecules in leukocytes of the vasculature system identified in this study may differ from that found in previous studies using different cell systems; these studies usually focused on tumor development or high-dose treatments of arsenite. Recently, in cultured VSMCs we also found elevated expression of *IL6* and *CCL2/MCP1* genes in a dose-dependent manner after 0–5 μM arsenite treatment (Lee PC and Lee TC. Unpublished data). Atherosclerotic lesions have shown proliferation of smooth muscle cells involving activation and proliferation of macrophages and T lymphocytes, cytokine production, and oxidized low-density lipoprotein accumulation (Ross 1999). Studies have indicated that cholesterol and lipid uptake are unimportant factors for ischemic heart disease or peripheral vascular disease in arseniasis-hyperendemic areas in Taiwan (Chen et al. 1996; Hsueh et al. 1998; Tseng et al. 1997). Arsenic-induced inflammatory reaction has a potential contribution to the artherogenic effect of arsenic, possibly derived from a coordinated involvement of leukocyte recruitment and smooth muscle cell proliferation.

Alteration of gene expression involving signal transduction pathways or transcription regulatory components related to arsenic exposure was also observed in this study. Most of these genes were repressed in study subjects of the high-level arsenic

Table 3. Demographic and clinical characteristics of the study subjects as determined by arsenic concentration in whole blood samples, Lanyang Basin, Taiwan.[a]

	Arsenic concentration in blood (μg/L)		
Characteristics	Low (0.00–4.32)	Intermediate (4.64–9.00)	High (9.60–46.5)
Total subjects	21	22	21
Age (years)	56.4 ± 6.7	58.7 ± 6.7	56.5 ± 9.4
Gender (% male)	33.3	54.6	33.3
Body mass index (kg/m^2)*	25.8 ± 3.8	25.4 ± 3.8	22.9 ± 3.2
Current smoker (%)	23.8	36.4	33.3
Serum cholesterol (mmol/L)	207.3 ± 33.4	219.0 ± 31.9	203.7 ± 37.8
Serum triglyceride (mmol/L)	135.2 ± 108.7	144.7 ± 87.3	117.0 ± 59.3

[a]Values are shown as means ± SD for continuous variables and percentages for dichotomous variables. *$p < 0.05$, derived from an ANOVA *F* test for the hypothesis that there was no difference among groups.

Table 4. Plasma protein level of four study genes in 64 study subjects as a function of blood arsenic exposure, Lanyang Basin, Taiwan.[a]

		Arsenic concentration in blood (μg/L)			Correlation coefficient for individual measurements	
Protein[b]	Total subjects[c]	Low 0.00–4.32 (number)	Intermediate 4.64–9.00 (number)	High 9.60–46.5 (number)	ϒ	*p*-Value
IL1b	53	0.65 ± 0.51 (18)	0.85 ± 0.53 (18)	0.74 ± 0.37 (17)	0.02	0.902
IL6	51	1.7 ± 1.8 (18)	2.4 ± 3.1 (20)	1.4 ± 0.9 (13)	–0.19	0.190
CCL2/MCP1	64	498 ± 153 (21)	530 ± 183 (22)	611 ± 254 (21)	0.24	0.060
CXCL1/GRO1	32	42.2 ± 19.4 (12)	43.8 ± 18.4 (11)	47.9 ± 30.4 (9)	–0.05	0.766

[a]Protein levels in plasma (pg/mL) are shown as mean ± SD. [b]Information from the UniGene database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene). [c]Subjects with plasma protein level below detection limit by ELISA assay were treated as having missing data.

exposure groups. Several studies employing cell lines have defined the three mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinase, stress-activated c-Jun N-terminal kinase, and p38/CSBP (CSAID-binding protein) protein kinase, that are involved in the response to lethal levels of arsenite (Cavigelli et al. 1996; Dong 2002; Liu et al. 1996; Ludwig et al. 1998; Theodosiou and Ashworth 2002). In this study, no enhanced activation of MAP kinase pathways was observed in association with arsenic exposure. Relatively low levels of arsenic may have different modes of action, as proposed by Barchowsky (Barchowsky et al. 1999). MAP kinase pathways may not be activated in the study subjects with relatively low-level arsenic exposure, such as those derived from drinking water. In contrast, the transcription factor *SPI1* (spleen focus forming virus proviral integration oncogene), which is essential for the development of hematopoietic system (DeKoter and Singh 2000), is significantly upregulated in lymphocytes from study subjects with high levels of blood arsenic. Deregulation in transcription levels can also be found for genes involved in cell cycle control and DNA replication/repair processes, including induction of menage a trois 1 (*MNAT1*), polymerase delta 2 (POLD2), and excision repair cross-complementing rodent repair deficiency, complementation group 1 (*ERCC1*) gene expression, and reduction of cyclin C (*CCNC*) and polymerase beta (*POLB*) gene expression in intermediate- or high-level arsenic groups compared with the low-level arsenic groups. In contrast to the marked induction of DNA damage-related proteins noted in previous studies of the cell culture system (Chen et al. 2001; Lu et al. 2001; Yih et al. 2002), we did not observe substantial changes of DNA damage–inducible transcripts gene expression associated with arsenic exposure in these study subjects. Perhaps the increased expression of genes regulating DNA damage response is associated mainly with overt carcinogenic events. In the present study, evidence for DNA-damaging activity in lymphocytes from arsenic-exposed study subjects was not supported. However, results of this study showed that arsenic exposure induced expression of cellular defense proteins, such as heme oxygenase 1 (*HMOX1*) and glutathione peroxidase 4 (*GPX4*). In many mammalian systems of cell culture, elevation of HMOX1 is a hallmark of increased oxidative stress induced by xenobiotic challenge, including arsenical compounds (Elbirt and Bonkovsky 1999). GPX4 is a component of the glutathione redox system that protects cells against oxidative damage induced by arsenic (Chouchane and Snow 2001; Lee and Ho 1994). Oxidative stress has been proposed as an important mechanism underlying arsenic-induced tissue damage that leads to cell death or gene expression changes (Bernstam and Nriagu 2000; Li et al. 2002; Nakagawa et al. 2002; Snow 1992). In our previous study, enhanced plasma oxidative stress levels associated with arsenic exposure were also observed for these study subjects (Wu et al. 2001). Among the genes of the MMPs family spotted on our array, *MMP1*, *MMP12* (macrophage elastase), *MMP14*, and *MMP-19* had enhanced expression in subjects from the high-level arsenic exposure group. It has long been known that increased MMP activity is important in atheroma formation (Bendeck 2002). In addition, increased production of MMPs in activated leukocytes has unfavorable effects for plaque stabilization (Libby 2002; Schonbeck et al. 1997). In arseniasis-endemic area in Taiwan, we observed an increased risk of cerebrovascular disease after long-term arsenic exposure to drinking well water (Chen et al. 1996; Chiou et al. 1997; Wang et al. 2002). In addition to formation of atheroma, arsenic-induced MMP activity leading to plaque rupture and hemorrhage might play a role in cases of advanced atherosclerosis observed in the study area.

Many inflammatory molecules including CCL2/MCP1 are regulated by nuclear factor kappa-B (NF-κB), which is mediated by oxidative stress (Kokura et al. 2002; Libermann and Baltimore 1990; Shin et al. 2002). Arsenite has been shown to induce oxygen free radicals and thereby increase NF-κB activity in cell culture studies (Barchowsky et al. 1996; Roussel and Barchowsky 2000). Enhanced plasma oxidative stress level associated with arsenic exposure was also observed for the present study subjects (Wu et al. 2001). Arsenic exposure may contribute to atherosclerosis through induction of oxidative stress and redox-sensitive inflammatory gene expression in the vasculature of exposed humans. A promoter analysis for NF-κB binding sites on those upregulated genes identified in this study may provide implicative information on gene regulation by arsenic exposure. Arsenic may alter gene expression as well by influencing promoter activity such as DNA methylation status or sequence variants. Long-term arsenic exposure in experimental animals alters DNA methylation status (Zhao et al. 1997). Whether arsenic exposure causes gene expression induction by a mechanism of demethylation or sequence variants in promoter region of all the affected genes in these study subjects needs additional experimental study.

Several issues need to be addressed. First, the cDNA microarray chip we used in this study was designed to include known genes of potential significance in arsenic toxicity; however, only a defined subset of genes was spotted in the cDNA chip because of difficulty for clone maintenance. It is possible that other gene products also play a role in arsenic-induced atherosclerosis. Second, the decision to pool the total cellular RNA from blood lymphocytes of eight individuals into one group was made to guarantee sufficient mRNA for gene expression profiling as an initial experiment. Because the extent of variability among individuals within one group was not available in this study, reproducibility of comparison between groups for RNA levels may be questioned. However, because the 24 individuals were grouped into various levels of the arsenic dose group with similar age, male/female ratio, and smoker percentage among groups, comparability of the expression profiles obtained as such should be enhanced. This matching strategy should increase the reliability of the microarray

Table 5. Linear regression analyses on the logarithmic plasma CCL2/MCP1 protein levels for 64 arsenic-exposed residents, Lanyang Basin, Taiwan.[a]

Variable	Coefficient (× 100)	SE[b] (× 100)	*p*-Value[c]
Univariate analysis model			
Age (1-year increment)	0.37	0.27	0.172
Gender (male vs female)	2.51	4.14	0.547
Blood arsenic (1-μg/L increment)	0.39	0.20	0.055
Body mass index, kg/m^2 (1 unit increment)	–0.86	0.53	0.112
Current smoker (yes vs no)	4.27	4.37	0.332
Serum cholesterol (one mmol/L increment)	0.03	0.06	0.666
Serum triglyceride (one mmol/L increment)	0.02	0.02	0.357
Multivariate analysis model			
Age (1-year increment)	0.33	0.26	0.211
Gender (male vs female)	2.97	4.10	0.472
Blood arsenic (1-μg/L increment)	0.41	0.20	0.048

[a]Plasma CCL2/MCP1 protein level (pg/mL) in logarithm scale was detected by ELISA assay. [b]SE: standard error of the coefficient. [c]Probability derived from a Wald's chi-square test for the hypothesis that coefficient = 0.

data. In addition, an alternate measure of gene expression, ELISA assay, was used to confirm the initial gene array analysis genes, which adds substantially to the reproducibility of this study. Third, as only one chip was spotted for each dose level in this study and the variability across chips was thus not obtainable, a standard curve using serial-diluted *GAPDH* clones was generated to control the variation between hybridization experiments, including variability from chip to chip. Furthermore, the variance in expression of the housekeeping genes was used to measure the significance of gene expression changes for study genes. As the variability in the expression of housekeeping genes probably overestimated the experimental variability in measuring differential expression, the resulting comparison under study should have been underestimated. Finally, the number of study subjects may not be large enough for most of the genes under study to draw a definitive conclusion on the association between expression level and arsenic exposure gradient. A larger sample size will be needed, especially for studies using diverse human population and gene markers of great experimental variability, to evaluate the effect of environmental factors on the gene expression profile.

In conclusion, this exploratory study demonstrates the potential of cDNA microarray as a method to identify candidate genes associated with arsenic exposure, an atherogenic stimulus, and provides novel investigational targets including genes involved in inflammation and immune response. Although PBL is not representative of all inflammatory cells in atherosclerotic lesion areas, the result of a dose-dependent elevation of plasma CCL2/MCP1 protein levels in the study subjects may yield insight into the response to atherogenic stimulus after long-term arsenic exposure. Further research that extends the sample size of this study as well as exploration of gene expression profile of other inflammatory cytokines and growth factors in arsenic-exposed population are needed to define the dose–response relationship between the exposure and inflammatory mediators at the population level. Multidisciplinary studies such as molecular epidemiologic investigations are also needed to elucidate the role of arsenic-associated inflammation in the induction of atherosclerosis.

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