Toxicogenomics/proteomics Report

Chronic exposure to arsenite induces S100A8 and S100A9 expression in rat RBL-2H3 mast cells

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ABSTRACT — To investigate the effects of chronic exposure to arsenite on the gene expression profiles of mast cells, microarray analysis was performed on rat basophilic leukemia RBL-2H3 cells exposed to arsenite for 28 days. Upregulated genes include calcium-binding S100 proteins such as S100A9, S100A10, S100A6, and S100A13, and granzymes B and C. Among S100 proteins, S100A9 showed the highest expression (8.62-fold of untreated cells) after 4-weeks of exposure to arsenite. As S100A8 and S100A9 comprise a heterodimer called calprotectin, and are implicated in the development of atherosclerosis and cancer, mRNA levels of both S100A8 and S100A9 were analyzed. The results demonstrated that exposure of RBL-2H3 cells to arsenite for a few weeks induces marked increases in mRNA levels of S100A8 and S100A9.

Key words: Arsenic, Mast cell, S100A8, S100A9

INTRODUCTION

Arsenic is a toxic metalloid that causes deleterious health effects on a variety of tissues. Recent geological and epidemiological studies have revealed widespread arsenic pollution in Asian groundwater. Exposure to arsenic from well water is associated with skin lesions; neurological, respiratory, hepatic, and cardiovascular dysfunctions; malignant tumors; and increased mortality (Smith *et al.*, 2000; Chen *et al.*, 2009; Argos *et al.*, 2010). On the other hand, arsenic trioxide is effective for the treatment of acute promyelocytic leukemia (APL) (Soignet *et al.*, 1998; Sanz *et al.*, 2009). However, the adverse side effects of arsenic trioxide, such as cardiotoxicity, hinder the therapeutic application of this agent (Soignet *et al.*, 2001).

In APL patients receiving arsenic trioxide, incidences of herpes simplex virus and varicella zoster virus infection have been frequently reported (Tanvetyanon and Nand, 2004; Au and Kwong, 2005; Nouri *et al.*, 2006). Infection with herpes simplex virus was also reported in a case of acute arsenic poisoning (Bartolomé *et*

al., 1999). Patients with arsenic-induced Bowen's disease showed immune dysfunctions detected by decreases in the number of Langerhans cells in the skin (Wang *et al.*, 1991) and by decreases in cutaneous and systemic CD4+ cells (Liao *et al.*, 2009). Chronic exposure of mice to arsenic from drinking water showed immunosuppressive effects as assessed by responses to influenza A infection (Kozul *et al.*, 2009a) and by the expression of genes related to immune responses (Kozul *et al.*, 2009b). Although these studies suggest that chronic exposure to arsenic affects immunological functions, limited information is available on the mechanisms underlying arsenic-induced immunomodulation.

Mast cells play important roles in immunological responses such as allergy and inflammation. Recent studies have demonstrated that mast cells are deeply involved in the development of vascular diseases such as atherosclerosis (Heib *et al.*, 2008; Bot *et al.*, 2008; Soehnlein and Weber, 2009), which is also an important outcome of arsenic poisoning (Simeonova and Luster, 2004). However, no study has ever examined the effects of arsenicals on the functions or gene expression of mast cells. In

the present study, we investigated the effects of chronic exposure to arsenite on gene expression profiles of rat basophilic leukemia RBL-2H3 cells as a model of mast cells.

MATERIALS AND METHODS

Cell culture and arsenic exposure

RBL-2H3 cells were cultured in MEM (Eagle's Minimum Essential Medium with Earle's salts) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The cells were passaged every 3-4 days. RBL-2H3 cells were chronically exposed to sodium arsenite (1 μ M) for 28 days in a 10-cm tissue culture dish. Control cells were incubated with medium alone for 28 days.

Preparation of RNAs for microarray analysis

RNA samples for microarray analysis were prepared as reported previously (Matsumoto *et al.*, 2010). Total RNA was extracted and purified from RBL-2H3 cells using the SV Total RNA Isolation System (Promega, Madison, WI, USA). Pooled total RNA (5 μ g) was applied to OpArray™ Rat V3.0 (Operon Technologies, Alameda, CA, USA), in which 28,800 genes are registered. We used the Low RNA Fluorescent Linear Amp Kit PLUS (Agilent Technologies, Santa Clara, CA, USA) to synthesize double-stranded (ds) cDNA from the total RNA and then to synthesize cRNA from the ds cDNA as a template. Total RNA was reverse-transcribed with the T7 Promoter primer. After second-strand synthesis, we used the ds cDNA as a template for Cyanine (Cy)3-/Cy5-labeled cRNA synthesis. cRNA from the control group was labeled with Cy3 and that from the arsenite-exposed group was labeled with Cy5. The OpArray slide was hybridized with 20-25 μ g of Cy3- and Cy5-labeled cRNA competitively and washed with the OpArray Hybridization Buffer Kit (Operon Technologies). A fluorescent image of the slide was then recorded with CRBIO (Hitachi Software Engineering, Tokyo, Japan). The digitized image data were processed with DNASIS Array software (Hitachi Software Engineering). The ratios of the intensity of Cy5 to that of Cy3 were calculated. Information on each gene on the slide was obtained from the National Center for Biotechnology Information (NCBI) database (Honda *et al.*, 2010).

Determination of mRNA levels by real-time reverse transcription-PCR

The same batch of RNA samples used for microarray analysis was used for the real-time reverse transcription (RT)-PCR analysis. The RT reaction was performed

in a mixture containing 50 mM Tris-HCl (pH 8.3), 70 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 1 mM dNTP, 4 units of RNase inhibitor, 2 μ g total RNA, 0.5 μ M Oligo(dT)₁₅ primer (Promega), and 5 units of reverse transcriptase in a total volume of 20 μ l. The reaction was carried out at 37°C for 1.5 hr. The RT reaction mixtures were used directly for PCR amplification. Quantitative real-time RT-PCR was performed using a TaqMan probe according to the procedure recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA). For cDNA synthesis, 450 ng of total RNA was used. The forward and reverse primers and TaqMan probes for S100A8, S100A9, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were supplied by TaqMan Assay-on-Demand Products (Applied Biosystems). PCR amplifications were always performed using universal temperature cycles: 10 min at 94°C followed by 35-45 two-temperature cycles (15 sec at 94°C and 1 min at 60°C). Fluorescence of PCR products was detected using an ABI Prism 7300/7500 Sequence Detector System (Applied Biosystems).

RESULTS AND DISCUSSION

As pharmacokinetic studies showed that plasma arsenic concentrations in leukemic patients receiving clinical doses of arsenic trioxide were less than 1 μ M (Fox *et al.*, 2008), we added 1 μ M arsenite to the medium of RBL-2H3 cells for 28 days. This duration was determined based on the length of one cycle of clinical application of arsenic trioxide.

Table 1 shows the upregulated (> 2.5) and downregulated (< 0.4) genes detected by microarray analysis. The most prominent feature of the gene expression profile shown in Table 1 is the upregulation of S100 proteins. S100A9 showed the highest (8.62-fold) expression. In addition, the expression of other S100 proteins, such as S100A10 (3.97-fold), S100A6 (3.63-fold), and S100A13 (2.83-fold), was also enhanced. S100 proteins commonly contain EF-hands for calcium-binding in their protein structure, and are involved in a variety of immunological responses (Marenholz *et al.*, 2004; Foell *et al.*, 2007). S100A9 is a 14-kDa calcium-binding protein also called myeloid-related protein (MRP) 14 or calgranulin B. It forms a heterodimer with S100A8 (MRP 8/calgranulin A). The heterodimer of S100A8/S100A9 is called calprotectin and constitutes about half of the cytosolic protein of neutrophils (Stríz *et al.*, 2004). Calprotectin plays an important role in NADPH oxidase activation in neutrophils (Kerkhoff *et al.*, 2005), and extracellular calprotectin is associated with various inflammatory diseases

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Table 1. Upregulated genes in RBL-2H3 cells treated with 1 μ M arsenite for 28 days

Name of gene	Definition	Accession No.	Ratio
Upregulated gene (> 2.5)			
S100a9	S100 calcium binding protein A9 (calgranulin B)	NM_031348	8.62
Ldhb	lactate dehydrogenase B	BC080241	6.53
Gzmb	granzyme B	AY288431	6.43
Gzmc	granzyme C	NM_001000108	4.78
Myadm	myeloid-associated differentiation marker	NM_001000454	4.52
S100a10	S100 calcium binding protein A10 (calpactin)	-	3.97
Dock1	PREDICTED: similar to Dock1 protein (LOC309081)	-	3.88
Olr1063	olfactory receptor 1063 (Olr1063_predicted)	DQ185515	3.85
S100a6	S100 calcium binding protein A6 (calcyclin)	BC088187	3.63
Tubgcp5	PREDICTED: tubulin, gamma complex associated protein 5	-	2.98
S100a13	PREDICTED: S100 calcium binding protein A13	BC085937	2.83
Giot1	gonadotropin inducible ovarian transcription factor 1	BC088284	2.82
Vnr2	vomer nasal receptor 2	XM_219292	2.81
Akt1s1	PREDICTED: AKT1 substrate 1 (proline-rich)	XM_343358	2.70
Sdccag1	PREDICTED: serologically defined colon cancer antigen 1 (LOC500664)	DW395361	2.66
Foxk2	PREDICTED: forkhead box K2	AB013732	2.65
Fbxw2	PREDICTED: F-box and WD-40 domain protein 2	XM_234725	2.65

Fig. 1. Time-dependent changes in mRNA levels of S100A8 and S100A9 in RBL-2H3 cells exposed to arsenite. RBL-2H3 cells were exposed to arsenite for 1, 2, and 4 weeks, and total RNA was extracted at each of these points for real-time RT-PCR analysis. The fluorescence of the PCR products of S100A8 and S100A9 was adjusted by that of GAPDH and expressed as a relative ratio to untreated control. Data are shown as mean $\pm$ S.E.

such as rheumatoid arthritis, atherosclerosis, and cancer (Striz *et al.*, 2004; McCormick *et al.*, 2005; Croce *et al.*, 2009; Ehrchen *et al.*, 2009; Cheng *et al.*, 2009). As calprotectin can bind zinc as well as calcium, excess production of calprotectin by genetic disorder is associated with a high concentration of serum zinc (Saito *et al.*,

2002; Sampson *et al.*, 2002; Fessatou *et al.*, 2005). However, the expression and roles of calprotectin in mast cells remain unknown.

To confirm the upregulation of both S100A9 and S100A8 in mast cells exposed to arsenite, we carried out a real-time RT-PCR analysis in RBL-2H3 cells exposed

Table 2. Downregulated genes in RBL-2H3 cells treated with 1 μ M arsenite for 28 days

Name of gene	Definition	Accession No.	Ratio
Downregulated gene (< 0.4)			
Zdhhc4	zinc finger, DHHC domain containing 4	-	0.24
Pcca	PREDICTED: propionyl-coenzyme A carboxylase, alpha polypeptide	XM_345401	0.25
Eif4g1	similar to Eif4g1 protein (LOC499581)	XM_341354	0.28
Tob2	transducer of ERBB2, 2	CF976179	0.32
Cbx1	PREDICTED: chromobox homolog 1 (Drosophila HP1 beta)	XM_340847	0.32
Sf3b1	PREDICTED: splicing factor 3b, subunit 1	XM_345318	0.36
Adck2	PREDICTED: aarF domain containing kinase 2	XM_343188	0.36
Chd7	PREDICTED: chromodomain helicase DNA binding protein 7	M93661	0.37
Hnrpa3	heterogeneous nuclear ribonucleoprotein A33 (LOC288146)		0.37
Cacna1a	calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	XM_218777	0.38
Usp42	PREDICTED: ubiquitin specific protease 42		0.39
Ankrd11	PREDICTED: ankyrin repeat domain 11	AF059530	0.39
Ankrd12	PREDICTED: ankyrin repeat domain 12	BC082085	0.39

to arsenite for 1, 2, and 4 weeks. As shown in Fig. 1, the mRNA levels of both S100A8 and S100A9 increased markedly, peaking at week 2 and then decreasing at week 4. The mRNA levels of S100A8 and S100A9 after 2-week exposure to arsenite were about 15-fold and 20-fold, respectively, of untreated control. After 4 weeks of exposure, the mRNA level of S100A8 decreased to the control level, while the mRNA level of S100A9 was still several times higher than that of untreated cells. This difference may explain why the microarray analysis carried out after 4 weeks of exposure to arsenite detected the upregulation of S100A9 but not of S100A8. Thus, the microarray and subsequent real-time RT-PCR analyses enabled us to find marked increases in mRNA levels of S100A8 and S100A9 in mast cells exposed to arsenite for 1 or 2 weeks.

In addition to S100 proteins, the expression of two types of granzymes, granzyme B (6.43-fold) and granzyme C (4.78-fold), was enhanced by arsenite exposure. As granzymes B and C are involved in cytolytic activity (Kam *et al.*, 2000), further studies on the roles of these enzymes in the manifestation of and protection against arsenic toxicity are required.

Table 2 shows the list of genes whose expression was decreased by 4 weeks of exposure to arsenite. Compared with the upregulated genes, however, there was no clear change in the expression of the genes as a group, such as S100 proteins.

In conclusion, the present study demonstrated that chronic exposure to arsenite induces expression of S100 proteins, especially S100A8 and S100A9. As the S100A8/S100A9 complex was found to promote atherosclerosis

(McCormick *et al.*, 2005; Croce *et al.*, 2009) and amplify inflammation in cancer development (Cheng *et al.*, 2008; Ehrchen *et al.*, 2009), more detailed studies are required to elucidate the roles of S100A8 and S100A9 in arsenic toxicity.

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REFERENCES

- Argos, M., Kalra, T., Rathouz, P.J., Chen, Y., Pierce, B., Parvez, F., Islam, T., Ahmed, A., Rakibuz-Zaman, M., Hasan, R., Sarwar, G., Slavkovich, V., van Geen, A., Graziano, J. and Ahsan, H. (2010): Arsenic exposure from drinking water, and all-cause and chronic-disease mortalities in Bangladesh (HEALS): a prospective cohort study. *Lancet*, **376**, 252-258.
- Au, W.Y. and Kwong, Y.L. (2005): Frequent varicella zoster reactivation associated with therapeutic use of arsenic trioxide: portents of an old scourge. *J. Am. Acad. Dermatol.*, **53**, 890-892.
- Bartolomé, B., Córdoba, S., Nieto, S., Fernández-Herrera, J. and García-Díez, A. (1999): Acute arsenic poisoning: clinical and histopathological features. *Br. J. Dermatol.*, **141**, 1106-1109.
- Bot, I., van Berkel, T.J. and Biessen, E.A. (2008): Mast cells: pivotal players in cardiovascular diseases. *Curr. Cardiol. Rev.*, **4**, 170-178.
- Chen, Y., Parvez, F., Gamble, M., Islam, T., Ahmed, A., Argos, M., Graziano, J.H. and Ahsan, H. (2009): Arsenic exposure at low-to-moderate levels and skin lesions, arsenic metabolism, neurological functions, and biomarkers for respiratory and cardiovascular diseases: review of recent findings from the Health Effects of Arsenic Longitudinal Study (HEALS) in Bangladesh. *Toxicol.*

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- Appl. Pharmacol., **239**, 184-192.
- Cheng, P., Corzo, C.A., Luetette, N., Yu, B., Nagaraj, S., Bui, M.M., Ortiz, M., Nacken, W., Sorg, C., Vogl, T., Roth, J. and Gabrilovich, D.I. (2008): Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. *J. Exp. Med.*, **205**, 2235-2249.
- Croce, K., Gao, H., Wang, Y., Mooroka, T., Sakuma, M., Shi, C., Sukhova, G.K., Packard, R.R., Hogg, N., Libby, P. and Simon, D.I. (2009): Myeloid-related protein-8/14 is critical for the biological response to vascular injury. *Circulation*, **120**, 427-436.
- Ehrchen, J.M., Sunderkötter, C., Foell, D., Vogl, T. and Roth, J. (2009): The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *J. Leukoc. Biol.*, **86**, 557-566.
- Fessatou, S., Fagerhol, M.K., Roth, J., Stamoulakatou, A., Kitra, V., Hadarean, M., Paleologos, G., Chandrinou, H., Sampson, B. and Papassotiropoulos, I. (2005): Severe anemia and neutropenia associated with hyperzincemia and hypercalprotectinemia. *J. Pediatr. Hematol. Oncol.*, **27**, 477-480.
- Foell, D., Wittkowski, H., Vogl, T. and Roth, J. (2007): S100 proteins expressed in phagocytes: a novel group of damage-associated molecular pattern molecules. *J. Leukoc. Biol.*, **81**, 28-37.
- Fox, E., Razzouk, B.I., Widemann, B.C., Xiao, S., O'Brien, M., Goodspeed, W., Reaman, G.H., Blaney, S.M., Murgo, A.J., Balis, F.M. and Adamson, P.C. (2008): Phase I trial and pharmacokinetic study of arsenic trioxide in children and adolescents with refractory or relapsed acute leukemia, including acute promyelocytic leukemia or lymphoma. *Blood*, **111**, 566-573.
- Heib, V., Becker, M., Taube, C. and Stassen, M. (2008): Advances in the understanding of mast cell function. *Br. J. Haematol.*, **142**, 683-694.
- Honda, A., Komuro, H., Nagase, H., Hozumi, I., Inuzuka, T., Hara, H., Fujiwara, Y. and Satoh, M. (2010): Microarray analysis of the liver in metallothionein-III null mice treated with cadmium. *J. Toxicol. Sci.*, **35**, 271-273.
- Kam, C.M., Hudig, D. and Powers, J.C. (2000): Granzymes (lymphocyte serine proteases): characterization with natural and synthetic substrates and inhibitors. *Biochim. Biophys. Acta*, **1477**, 307-323.
- Kerkhoff, C., Nacken, W., Benedyk, M., Dagher, M.C., Sopalla, C. and Doussiere, J. (2005): The arachidonic acid-binding protein S100A8/A9 promotes NADPH oxidase activation by interaction with p67phox and Rac-2. *FASEB J.*, **19**, 467-469.
- Kozul, C.D., Ely, K.H., Enelow, R.I. and Hamilton, J.W. (2009a): Low-dose arsenic compromises the immune response to influenza A infection *in vivo*. *Environ. Health Perspect.*, **117**, 1441-1447.
- Kozul, C.D., Hampton, T.H., Davey, J.C., Gosse, J.A., Nomikos, A.P., Eisenhauer, P.L., Weiss, D.J., Thorpe, J.E., Ihnat, M.A. and Hamilton, J.W. (2009b): Chronic exposure to arsenic in the drinking water alters the expression of immune response genes in mouse lung. *Environ. Health Perspect.*, **117**, 1108-1115.
- Liao, W.T., Yu, C.L., Lan, C.C., Lee, C.H., Chang, C.H., Chang, L.W., You, H.L. and Yu, H.S. (2009): Differential effects of arsenic on cutaneous and systemic immunity: focusing on CD4+ cell apoptosis in patients with arsenic-induced Bowen's disease. *Carcinogenesis*, **30**, 1064-1072.
- McCormick, M.M., Rahimi, F., Bobryshev, Y.V., Gaus, K., Zreiqat, H., Cai, H., Lord, R.S. and Geczy, C.L. (2005): S100A8 and S100A9 in human arterial wall. Implications for atherogenesis. *J. Biol. Chem.*, **280**, 41521-41529.
- Marenholz, I., Heizmann, C.W. and Fritz, G. (2004): S100 proteins in mouse and man: from evolution to function and pathology (including an update of the nomenclature). *Biochem. Biophys. Res. Commun.*, **322**, 1111-1122.
- Matsumoto, K., Fujishiro, H., Satoh, M. and Himeno, S. (2010): DNA microarray analysis of the liver of mice treated with cobalt chloride. *J. Toxicol. Sci.*, **35**, 935-939.
- Nouri, K., Ricotti, C.A.Jr, Bouzari, N., Chen, H., Ahn, E. and Bach, A. (2006): The incidence of recurrent herpes simplex and herpes zoster infection during treatment with arsenic trioxide. *J. Drugs Dermatol.*, **5**, 182-185.
- Saito, Y., Saito, K., Hirano, Y., Ikeya, K., Suzuki, H., Shishikura, K., Manno, S., Takakuwa, Y., Nakagawa, K., Iwasa, A., Fujikawa, S., Moriya, M., Mizoguchi, N., Golden, B.E. and Osawa, M. (2002): Hyperzincemia with systemic inflammation: a heritable disorder of calprotectin metabolism with rheumatic manifestations? *J. Pediatr.*, **140**, 267-269.
- Sampson, B., Fagerhol, M.K., Sunderkötter, C., Golden, B.E., Richmond, P., Klein, N., Kovar, I.Z., Beattie, J.H., Wolska-Kusnierz, B., Saito, Y. and Roth, J. (2002): Hyperzincaemia and hypercalprotectinaemia: a new disorder of zinc metabolism. *Lancet*, **360**, 1742-1745.
- Sanz, M.A., Grimwade, D., Tallman, M.S., Lowenberg, B., Fenaux, P., Estey, E.H., Naoe, T., Lengfelder, E., Büchner, T., Döhner, H., Burnett, A.K. and Lo-Coco, F. (2009): Management of acute promyelocytic leukemia: recommendations from an expert panel on behalf of the European LeukemiaNet. *Blood*, **113**, 1875-1891.
- Simeonova, P.P. and Luster, M.I. (2004): Arsenic and atherosclerosis. *Toxicol. Appl. Pharmacol.*, **198**, 444-449.
- Smith, A.H., Lingas, E.O. and Rahman, M. (2000): Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. *Bull. World Health Organ*, **78**, 1093-1103.
- Soehnlein, O. and Weber, C. (2009): Myeloid cells in atherosclerosis: initiators and decision shapers. *Semin. Immunopathol.*, **31**, 35-47.
- Soignet, S.L., Maslak, P., Wang, Z.G., Jhanwar, S., Calleja, E., Dardashti, L.J., Corso, D., DeBlasio, A., Gabrilove, J., Scheinberg, D.A., Pandolfi, P.P. and Warrell, R.P.Jr. (1998): Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. *N. Engl. J. Med.*, **339**, 1341-1348.
- Soignet, S.L., Frankel, S.R., Douer, D., Tallman, M.S., Kantarjian, H., Calleja, E., Stone, R.M., Kalaycio, M., Scheinberg, D.A., Steinherz, P., Sievers, E.L., Coutre, S., Dahlberg, S., Ellison, R. and Warrell, R.P.Jr. (2001): United States multicenter study of arsenic trioxide in relapsed acute promyelocytic leukemia. *J. Clin. Oncol.*, **19**, 3852-3860.
- Striz, I. and Trebichavský, I. (2004): Calprotectin - a pleiotropic molecule in acute and chronic inflammation. *Physiol. Res.*, **53**, 245-253.
- Tanvetanont, T. and Nand, S. (2004): Herpes zoster during treatment with arsenic trioxide. *Ann. Hematol.*, **83**, 198-200.
- Wang, B.J., Lee, Y.Y., Mak, C.P., Kao, H.F., Hsu, M.L. and Hsien, J.R. (1991): Quantitative and morphological changes of Langerhans cells in Bowen's disease from patients with chronic arsenicism. *J. Formos. Med. Assoc.*, **90**, 1093-1098.