Toxicology and Industrial Health 2003; **19:** 157–163
www.tihjournal.com

Dose-dependent transcriptome changes by metal ores on a human acute lymphoblastic leukemia cell line

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The increased morbidity of childhood leukemia in Fallon, Nevada and Sierra Vista, Arizona has prompted great health concern. The main characteristic that these two towns share is the environmental pollution attributed to metal ore from abandoned mining operations. Consequently, we have investigated the transcriptome effects of metal ores from these endemic areas using a human T-cell acute lymphoblastic leukemia cell line (T-ALL). Metal ore from Fallon significantly increased cell growth after 24, 48 and 72 h of incubation at 1.5 μg/mL concentration, as measured by trypan-blue. Sierra Vista ore significantly increased cell growth with 0.15 and 1.5 μg/mL following 72 h of incubation. From human cDNA microarray, results indicate that in total, eight genes, mostly metallothionein (MT) genes, were up-regulated and 10 genes were down-regulated following treatment of the T-ALL cells with 0.15 and 1.5 μg/mL of metal ores at 72 h, in comparison with untreated cells. Twenty-eight metals of both ores were quantified and their presence may be associated with the cell growth rate and dose-dependent activation of transcriptomes in immature T-cells. *Toxicology and Industrial Health* 2003; **19:** 157–163.

Key words: *gene microarray; leukemia; metallothionein; metals; transcriptomes; tungsten*

Introduction

The presence of metallic ores outside its indigenous environment, which can occur through such extreme events as mineral mining or volcanic eruptions, has been known to lead to health problems for nearby residents, including higher morbidity of tumors (Masironi, 1987; Garruto, 1991). These adverse effects are especially significant and have a greater potential for harm in children (Rosen, 1995; Chattopadhyay *et al*., 2003). It has become increasingly evident that the incidence of childhood leukemia has been correlated with exposure to heavy metals. These include arsenic, chromium, cadmium, and radium (Durant *et al*., 1995; Infante-Rivard *et al*., 2001).

Two confirmed childhood leukemia clusters have emerged recently within the Fallon, Nevada (16 cases/2383 pediatric population) and Sierra Vista, Arizona (13 cases/11 287 pediatric population). Both municipalities share a history of mining and a military base. The ion plasma chromatography analyses of tree ring material from the respective areas have indicated that metallic levels had increased for tungsten from the 1980–1984 tree ring

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10.1191/0748233703th185oa

period to the 1997–2002 period in Fallon and Sierra Vista (Sheppard and Witten, 2003). There is speculation that it is the presence of such metallic pollution in the soil that has contributed to the development of the two confirmed leukemia clusters.

There are three other leukemia-associated areas in the US, located in Hoisington; Kansas, Florin, California; and Clarendon, Vermont. All three locations also share similar metal pollutions that have resulted either from byproduct of industry or due to the presence of indigenous ore.

In this study we investigated concentration-dependent effects of ore samples on transcriptome alterations and cell proliferation. The samples were gathered from Fallon and Sierra Vista. Our results demonstrated that metal ores from both areas exhibited significant increases in cell growth following 72 h of incubation, as measured by trypan-blue. Both metal ores also activated transcription of metallothioneins (MTs) genes, suggesting changes in MT genes may possibly link the proliferation ability of immature T-cells during exposure to the metal complexes collected from these areas.

Methods

Cells culture

The human CCRF-CEM cell line [(immature T-lymphocytes from acute lymphoblastic leukemia (T-ALL, Foley and Lazarus, 1967)], was obtained from American Type Culture Collection (Manassas, VA). Cells were routinely maintained as suspension cultures in RPMI-1640, supplemented with fetal bovine serum (FBS, 5%) and penicillin/streptomycin (100 IU/mL, 100 μg/mL), 2 mM L-glutamine and 20 mM HEPES buffer at 37°C in a humidified atmosphere of 5% CO_2 in air. For each experiment ($n=4$), cells were harvested at the exponential growth phase and seeded in triplicate at a density of 5×10^5 viable cells per mL in the presence of Fallon/Sierra Vista metal ore at different exposure levels (0, 0.15, and 1.5 μg/mL), as determined from the tree ring metal levels for 24, 48 or 72 h in six well-plates.

Cell viability assay

The human T-ALL cells were exposed to the metal ores at the concentrations of 0, 0.15, and 1.5 μg/mL. After incubation for 24, 48, and 72 h, viable cell numbers from each sample were counted with a conventional hemocytometer by trypan-blue exclusion.

RNA isolation

Total RNA isolation was performed as previously described (Wong *et al*., 2003). After the cells were either exposed to untreated (control) or metal ore for 72 h, 8×10^6 cells were centrifuged ($250\times g$, 10 min, 4°C). The cell pellets were lysed by the addition 1 mL of TRI reagent (Sigma, St. Louis, MO). The total RNA concentration was determined by a spectrophotometer at 260 nm and the quality of RNA was determined by running 1 μg RNA on a standard 1% denaturing formaldehyde–agarose gel. Residual DNA was digested by the addition of five units DNase I and incubated at 37°C for 45 min, followed by inactivation with 1/10 volume of the inactivation reagent. The RNA samples with purity greater than 1.8 (260/280 nm ratio) were used for microarray analysis.

cDNA microarray and data analysis

To determine the gene expression profiles of T-ALL cells, the human 5K cDNA Chips (Arizona Cancer Center Microarray Core Facility) were exposed to both ores at 0 (untreated controls), 0.15, and 1.5 μg/mL for 72 h, respectively. Preparation of the cDNA fluorescent labeling, hybridization, scanning, quantification, and subsequent analysis were performed at the University of Arizona Cancer Center Microarray Core Facility. The human 5,300 of cDNA microarray was hybridized with a mixture of Cy3-labeled cDNA, corresponding to the control cells and Cy5-labeled cDNA corresponding to the metal ore treated cells. Results were captured electronically using a GenePix 4000a laser scanner (Axon Instruments, Union City, CA) that excites the fluorescent cyanine dyes and quantifies their emissions. Raw data Cy5 (red)/Cy3 (green) ratios were normalized, filtered, background subtracted for each data set, and further analyzed by using GeneSpring® 5.0 software (Affymetrix) and the

BioRag (Bioresource for array genes at http://www.biorag.org). It has been demonstrated that genes with a fluorescent intensity ratio over 1.7, compared to untreated cells (control), were considered up-regulated, while genes with fluorescent intensity ratio less than 0.5 to the control were considered as down-regulated (Tseng *et al*., 2001). By this consideration, in order to identify genes with significantly different expressions for each gene between the control and metal ore treatment, we selected genes that had a two-fold up- or down-regulation compared to untreated cells (control).

Metal analysis

The Fallon/Sierra Vista metal ore samples were sent to the Battelle Marine Sciences Laboratory (Sequim, WA) and analyzed by ion plasma chromatography to determine their respective metal concentrations.

Statistical analysis

Data were collected and analyzed on a Macintosh computer with the Statview IV statistical program. Since the measurements are independent variables, mean changes were evaluated using post hoc linear contrasts with adjustment for multiple comparisons made using Fisher's PLSD-corrected. The statistical significance level was set at $P<0.05$.

Results

T-ALL cells were cultured in the absence (controls) or presence of Fallon/Sierra Vista metal ore at levels of 0.15, and 1.5 μg/mL for 24, 48, and 72 h. At each time point for each of the metal ore concentrations, cell numbers were measured. Figure 1 indicates that Fallon ore at 1.5 μg/mL significantly increased human T-ALL cell growth after 24, 48 and 72 h of exposure. In contrast, exposure at the concentration of 0.15 μg/mL did not show any significant change. Figure 2 shows that the Sierra Vista ore significantly increased T-ALL cell growth following 72 h incubation at both concentrations as well as following 48 h exposure at 1.5 μg/mL.

In order to study the genotoxic effects of both ores on the T-ALL cell, we conducted the next set

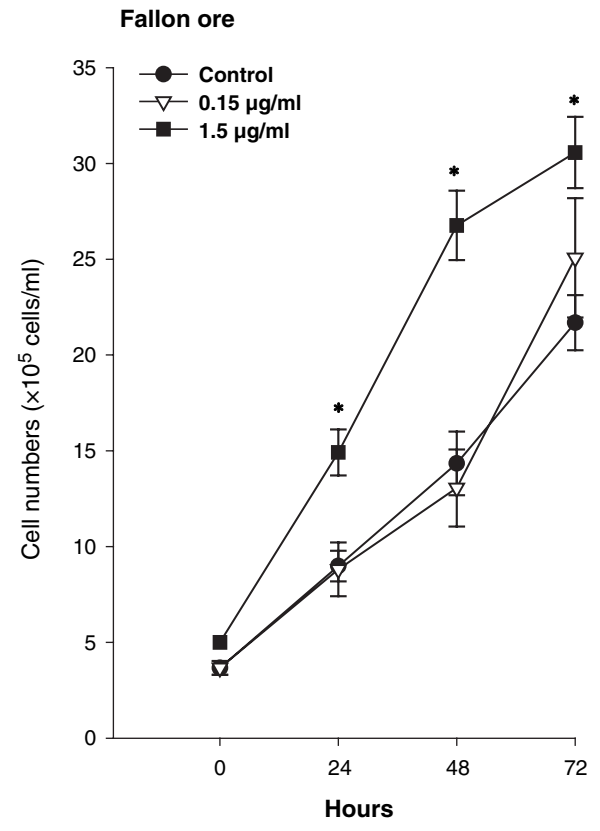

Figure 1. Cell proliferation rate after the treatment of T-ALL cells with Fallon metal ores at the concentration indicated for 24, 48, and 72 h. Viable cell numbers were determined with a conventional hemocytometer by trypan blue exclusion. Data were expressed as the mean ± SEM ($n=4$). * $P<0.05$.

of experiments by culturing T-ALL cells for 72 h in the absence (control) or presence of 0.15 and 1.5 μg/mL of Fallon or Sierra Vista ores, as both ores significantly increased cell growth after 72 h exposure. Total RNAs were extracted from the cultures and cDNAs were produced and labeled with Cy3 and Cy5 dyes. The cDNAs were mixed together and hybridized to a human cDNA microarray slide. For gene selection, we utilized $\geq$2.0-fold expression for up-regulated genes and $\leq$0.5-fold expression for down-regulated genes in comparison with untreated controls. Tables 1 and 2 show that, in total, eight genes were up-regulated and 10 genes were down-regulated following treatment of the T-ALL cells with both ores, relative to untreated cells.

Both Fallon and Sierra Vista ores at 1.5 μg/mL rapidly up-regulated eight genes (Table 1) after 72 h exposure. The first three genes were rapidly up-regulated by Fallon ore at 0.15 μg/mL and remained up-regulated at 1.5 μg/mL concentrations.

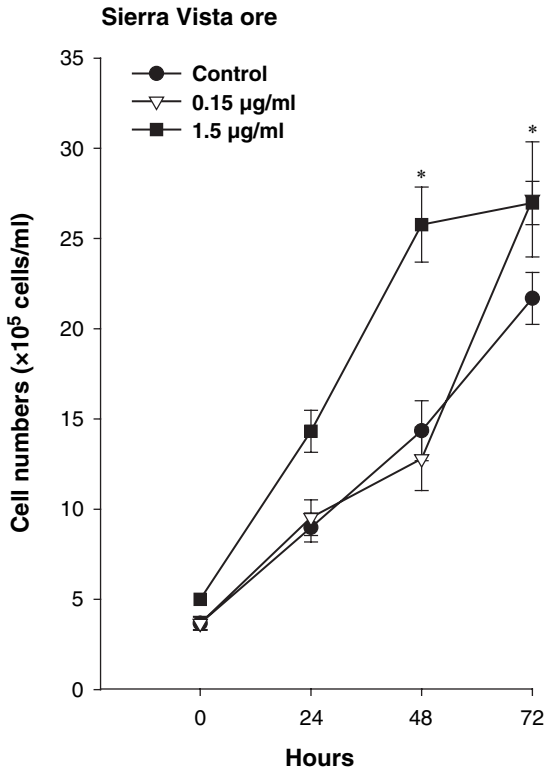

Figure 2. Cell proliferation rate after the treatment of T-ALL cells with Sierra Vista metal ores at the concentration indicated for 24, 48, and 72 h. Viable cell numbers were determined with a conventional hemocytometer by trypan blue exclusion. Data were expressed as the mean ± SEM ($n=4$). * $P<0.05$.

The responses observed with both Fallon and Sierra Vista ores were also dose-dependent. The most over-expressed genes were all members of the metallothioneins (MTs) gene family. The 10 genes that were down-regulated (Table 2) only exhibited this phenomenon at 0.15 µg/mL after the treatment of T-ALL cells by both ores. Both ores were quantified for 28 metals (Table 3).

Discussion

Lymphocytes in general and T cells in particular are important regulators of immune responses that are essential for the body's defense against environmental challenges. Metals that interfere with the normal function of the lymphocytic component of the immune system can have serious short-term and long-term health effects. Using a human T-ALL cell line, in this study, we characterized cell growth properties and the transcriptome changes induced by incubation with both metal ores. Microarray results indicate that both ores induced a dose-dependent upregulation of eight members of MT's gene family at 1.5 µg/mL following 72-h incubation. The cell culture studies also demonstrated that both ores significantly increased T-ALL cell growth by approximately 140% above control untreated cells. It is documented that human MTs are intracellular, low molecular weight, cysteine-rich proteins. MTs have unique structural characteristics to give potent metal-binding and redox capabilities that have been studied extensively since their discovery in 1957 (Margoshes and Vallee, 1957). These proteins have a major function in the homeostatic control of essential metals and the detoxification of nonessential metals (Kagi, 1991). Recently, it has been postulated that MT may have additional important functions for cell proliferation and differentiation (Studer *et al*., 1997; Cherian and Apostolova, 2000). Our results also indicate that over expression of MT genes may relate to changes in T-ALL cell growth properties. This observation is consistent with previous studies that in human T cell and breast carcinoma cell lines, suppressing MT synthesis with antisense MT oligomers not only inhibited growth of the cells, but also activated apoptosis (Abdel-Mageed and Agrawal, 1997; Tsangaris and Tzortzatou-Stathopoulou, 1998). In addition, MT expressed at a basal level in splenocytes plays an important role in the T cell mitogen-induced proliferative response (Mita *et al*., 2002). Interestingly, MT interaction with lymphocytes can motivate synergistic responses with either T- or B-cell mitogens, and can also stimulate lymphoproliferation by itself (Lynes *et al*., 1990; Borghesi *et al*., 1996). Moreover, MT may increase the number of immature T cells, but decrease their differentiation to the effectors cytotoxic T lymphocyte stage. These effects of MT on T-cell function may contribute to the immunosuppression of cell-mediated immunity that has been ascribed to inducers of MT synthesis (Youn and Lynes, 1999). This is possible because of the concentration of MT within the nucleus occurs in tumors at the proliferate edge and in tumor cells in the S phase of the cell cycles. This indicates that MT may play a role in signal transduction of growth stimulation and can be used as a marker of cell proliferation (Cherian, 1993; Tsujikawa *et al*., 1993).

Table 1. Genes up-regulated by the treatment of T-ALL cells with metal ores.

GeneBank Acc. No. #	Description	Molecular function	Fold changes			
			Fallon ore (μg/mL)		Sierra Vista ore (μg/mL)	
			0.15	1.5	0.15	1.5
H53340	Metallothionein 1G (MT1G)	Heavy metal-binding activity	2.765	2.612	1.398	4.015
H72722	Metallothionein 1B (MT1B)	Copper/cadmium binding/zinc ion binding/cytoplasm	2.427	2.517	1.226	3.383
N80129	Metallothionein 1L (MT1L)	Heavy metal-binding activity	2.161	2.325	1.305	3.378
H77766	Metallothionein 1H (MT1H)	Heavy metal-binding activity	1.858	2.749	1.061	4.091
AA872383	Metallothionein 1E (MT1E)	Heavy metal ion transporter activity	1.842	2.720	1.112	3.878
AI024402	Metallothionein 2A (MT2A)	Heavy metal-binding activity	1.835	2.312	1.245	3.194
R16596	EST-Soares fetal liver spleen	Unknown biological process	1.959	2.321	0.984	3.349
AA570216	EST-NCI_CGAP_Pr2	Unknown biological process	1.499	2.223	1.255	3.091

The results were expressed as ratios of the genes found to be up-regulated from metal ores treatment of T-ALL cell experiments, compared to untreated cells (control), by the human cDNA microarray analysis.

Table 2. Genes down-regulated by the treatment of T-ALL cells with metal ores.

GeneBank Acc. No. #	Description	Molecular function	Fold changes			
			Fallon ore (μg/mL)		Sierra Vista ore (μg/mL)	
			0.15	1.5	0.15	1.5
W86100	V-myb myeloblastosis viral oncogene (MYB)	Unknown function	0.410	0.721	0.633	1.170
T80932	Guanine nucleotide-binding protein (G protein), (GNG2)	Unknown function	0.413	N/A	0.696	N/A
R45056	Hypothetical protein MGC14433	Unknown function	0.359	N/A	0.645	N/A
H47069	Similar to RNA-binding region containing protein 2 (RNPC2)	Unknown function	0.451	N/A	0.671	N/A
N76608	ESTs, moderately similar to PRO0478 protein	Unknown function	0.471	0.919	0.735	N/A
AA455413	UDP-glucose ceramide glucosyltransferase (UGCG)	Transferase activity, transferring glycosyl groups	0.494	0.967	0.680	N/A
AA478036	B lymphoma Mo-MLV insertion region (mouse) (BMI1)	Segment specification, cell growth or maintenance	0.447	N/A	0.630	N/A
W32135	Sp3 transcription factor (SP3)	DNA-binding, transcriptional activator activity	0.431	N/A	0.570	1.002
H20138	Member RAS oncogene family (RAB6A)	GTP-binding activity, RAB small monomeric GTPase activity, protein transporter activity	0.459	N/A	0.551	N/A
R00395	HBxAg transactivated protein 2 (BAT2-iso)	Unknown function	0.524	1.21	0.496	N/A

The results were expressed as ratios of the genes found to be down-regulated from metal ores treatment of T-ALL cell experiments that compared to untreated cells (control), by the human cDNA microarray analysis.

Table 3. Metal elements of Sierra Vista and Fallon ores.

Name	Sierra Vista ore (ppm)	Fallon ore (ppm)	Earth's crust abundance* (ppm)
Eu (Europium)	0.87	0.68	1.8
Zr (Zirconium)	77	84	130
La (Lanthanum)	0.65	24.73	34
Ce (Cerium)	2.95	35.67	60
Pr (Praseodymium)	0.72	3.47	8.7
Yb (Ytterbium)	10.66	0.71	2.8
Nd (Neodymium)	4.84	12. 47	33.0
Sm (Samarium)	3.53	2.39	6.0
Gd (Gadolinium)	6.71	1.88	5.2
Tb (Terbium)	1.65	0.28	0.94
Dy (Dysprosium)	12.26	1.54	6.2
Nb (Niobium)	19.87	5.25	17.0
Ho (Holmium)	2.92	0.28	1.2
Er (Erbium)	8.97	0.77	3.0
Tm (Thulium)	1.54	0.11	0.45
Lu (Lutetium)	1.72	0.11	0.56
Pb (Lead)	58.94	276.67	10.0
Th (Thorium)	13.73	8.95	6.0
Hf (Hafnium)	3.10	2.62	3. 3
Ta (Tantalum)	3.79	0.45	1.7
U (Uranium)	4.90	5.51	1.8
W (Tungsten)	48.4	6.5	1.1
Rb (Rubidium)	717.65	74.90	60.0
Cs (Cesium)	18.37	6.53	1.9
Sr (Strontium)	15	455	360.0
Sc (Scandium)	13.0	6.2	26.0
Ba (Barium)	568	1380	340.0
Y (Yttrium)	67.08	7.34	29.0

Note: Units of ore measure are parts per million (ppm) by volume. * The abundance in the Earth's crust data reflects the average composition of continental crustal rock (http://www.webelements.com). Units are ppm by weight.

Moreover, Fallon ore down-regulated nine genes and Sierra Vista ore down-regulated one gene after treatment of T-ALL cells with 0.15 μg/mL concentration metal ores but neither down-regulate gene expression at 1.5 μg/mL, which suggests that human cells mobilize every genomic resource to overcome cytotoxicity caused by these metal mixtures. Additionally, both ores also altered a number of unknown genes (indicated by expressed sequence tags, ESTs) that may potentially contribute to the influence of those metals to the immune system. Furthermore, the explanation as to why these two ores influenced alterations in gene's expression and cell growth so differently may be attributed to the metals ore of Fallon and Sierra Vista that are very dissimilar (Table 3).

To determine whether the metal composition of ore samples is associated with changes in the T-ALL cell model, efforts were made to quantify the metal contents and to compare them to abundance content in the Earth's crust (Table 3). From data generated by using ion plasma chromatography, we speculated that these metal complexes rather than an individual metal may be involved in activation of MT genes that link cell proliferation and differentiation (Cherian and Apostolova, 2000). The reasons for this hypothesis are, first, that the metal contents of ore samples exhibit obvious differences between Fallon, Nevada and Sierra Vista, Arizona. Perhaps, differences in metal content may be attributed to variable dose-dependent effects on MT-gene transcription although both samples induced the common response pattern. Second, in this experiment, we did not measure the actual concentration of each metal in culture media but expected they may not parallel with the concentrations of samples, correspondingly, due to their different solubility. However, special consideration should be given to some well-characterized metals but not limited to Pb, U, W, and Cs that are known either to be blood toxic elements or carcinogens (Kasprzak, 1997; McCabe, Jr. *et al*., 2001; De Boeck *et al*., 2003).

Metals presence in these ores may be associated with the cell growth rate. Upregulation of MT gene transcription induced by these metal mixes may involve in several mechanisms that combine to interfere with cell proliferation/differentiation.

Acknowledgement

This study was supported by the Gerber Foundation.

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