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ORIGINAL RESEARCH

Altered Gene Expression in Human Hepatoma (HepG2) Cells Exposed to Low-Level Sodium Arsenite

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Abstract: Arsenic is present at low concentrations in nearly all aquatic environments and exposures through drinking water have been reported in many countries. Chronic exposure to high-levels of arsenic through drinking water is associated with skin, lung, kidney, liver and bladder cancer. Current evidence suggests that the liver is an important target of arsenic toxicity. cDNA microarray technology was used as a sensitive low-level monitor of the impact of arsenic in an in vitro model of exposure. In this study we examined the gene expression alteration in human hepatoma (HepG2) cells following exposure to low-levels of arsenic. HepG2 cells were exposed for 6 or 24 hours to sodium arsenite concentrations of either 0.1 µM or 10 µM. Arsenic at levels ranging from less than 0.1 µM to greater than 10 µM have been reported in drinking water sources from various parts of the world. Total RNA from treated and control cells were isolated, reverse transcribed and reciprocal labelled with Cy3 and Cy5 dyes and hybridized to a human cDNA microarray. The hybridized microarray chips were scanned, quantified and analyzed to identify genes affected by sodium arsenite exposure based on a two-fold increase or decrease in gene expression and reproducibility (affected in three or more treatments). Following filtering, normalization and hierarchical clustering initial data indicate that at least twenty-two genes were found to be commonly expressed in the treatments of sodium arsenite tested. The affected genes indicate that HepG2 cells respond to low-level sodium arsenite exposures and the cellular response is associated with alterations in expression of several genes. The affected genes were characterized as structural, immune response, regulatory and cell cycle genes. Identification of these target genes may help to highlight potential new pathways in which to further probe the effects of low-level arsenite exposures.

Keywords: arsenite, gene expression profiling, HepG2 cells, cDNA microarray

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Introduction

Arsenic (As) is a ubiquitous naturally occurring element that is present at low concentrations in nearly all aquatic and soil environments. Anthropogenic processes that include metal mining and smelting, fossil fuel combustion, and pesticide use also contribute significantly to the overall concentration of arsenic found in the environment.[1,2] Inorganic arsenic (iAs) in both pentavalent (As^{v}, arsentate) and trivalent (As^{III}, arsenite) forms is ubiquitous at varying concentrations in water, soil, air and food.[3] Many countries have documented acute and chronic arsenic toxicity in man due to the consumption of contaminated drinking water.[4,5] Arsenic is a cumulative poison and chronic exposure through contaminated drinking water has become one of the major public health problems of pandemic nature.[6]

Arsenic contamination of drinking water is a major global issue and human exposure to concentrations of greater than 50 µg/L, well above the World Health Organization (WHO) permissible limit of 10 µg/L, occur in various areas of the world.[7] Although standards have been assigned, there is considerable uncertainty about the number of drinking water systems that actually contain concentrations of arsenic equal to or below these standards. Exposure to arsenic levels that exceed the permissible limit is a common occurrence in countries world-wide and these include Countries of Chile, China, Ghana, Hungary, India, Mexico, Taiwan, Thailand and the United Kingdom.[8,9] The concern over arsenics adverse health effects originates from data, collected from several countries over the last two decades, demonstrating its toxic and carcinogenic potential in humans exposed to high levels of arsenic through drinking water for prolonged periods.[10] There have been numerous toxicological and epidemiological studies in animals and humans that have demonstrated the adverse health effects of inorganic arsenic from oral exposure through drinking water.[10] However few studies have been performed using low-level arsenic concentrations and little is known about the potential gene expression changes induced in human cells exposed to low-level arsenic concentrations.

Current knowledge about the biological effects of arsenic has primarily been derived from epidemiological and toxicological studies and the existing data suggest that arsenic has the potential to induce health effects that are multidimensional. A comprehensive assessment of published data indicate that long-term exposure to arsenic through drinking water can result in severe health disorders of which cardiovascular disease, developmental abnormalities, neurologic and neurobehavioral dysfunctions, diabetes, hearing loss, non-malignant[9] and malignant hepatic abnormalities,[11] hematologic disorders and various types of cancer; skin, bladder kidney, liver have all been reported.[10,12–16] There is no doubt that the most significant consequence of chronic arsenic exposure is cancer. Chronic exposure to high levels of arsenic has been found to be causally associated with multisite cancers in humans. Skin, lung and bladder cancer have commonly been reported. Populations with exposures to arsenic, generally at levels of 5 µM or higher, levels close to the molar concentration utilized in this study, are reported to have increased risks of cancer.[10] Although arsenic is a known carcinogen, the precise mechanisms of carcinogenesis are currently unknown.

Recent studies involving both in vitro and in vivo exposures indicate that arsenic toxicity may be mediated through its direct binding with—SH groups, or indirectly through generation of reactive oxygen species (ROS).[17] Additionally, it is thought that arsenic causes gene amplification, alters DNA methylation and causes changes in DNA repair mechanisms thereby causing uncontrolled cellular growth.[18] These findings suggest that perhaps multiple mechanisms may contribute to the toxicity of arsenic. Arsenic has been associated with various biological effects. It is likely that arsenic has multiple modes of toxicity and elucidation of the molecular mechanisms of arsenic in human cells may be critical to the understanding of arsenic. Gene expression changes are considered to be one of the earliest responses to chemical exposure and may precede overt biological outcomes by week, months or even years.[19] Changes in gene expression are important mechanisms that facilitate adaptation to chemical exposure and lead to manifestations of cellular responses to chemical exposures.[20] Analysis of gene expression changes that accompany chemical exposure are important for developing an understanding of toxicological processes initiated by particular chemical exposures.[19] Microarray technology is a tool used to determine early transcriptional responses in any given cell line[21] and could provide a mechanistic

understanding of toxicity, through the establishment of a relationship between chemical exposure and changes in genome-wide gene expression patterns.

The liver is considered a primary target for arsenic toxicity.[9,17,22] Arsenic has been shown to be cytotoxic and to induce gene expression changes in liver tissue at high levels of exposure.[23,24] The current investigation has focused on assessing the effects of low-level exposures; those that reflect concentrations generally observed in drinking water. Arsenic levels in drinking water have been reported within a range of 0.01 µM to 10 µM, in various regions of the world.[10,25,26] A concentration range of 0.1 µM to 10 µM was used in this study to represent environmental levels of arsenic in drinking water sources. These levels are not overtly cytotoxic, but have been shown to produce biological effects. Recently, Straub et al[27] has demonstrated that arsenic at lower exposure levels (250 ppb; 250 µg/l; 1.9 µM) stimulate vessel remodelling in mouse liver.[27] Therefore the purpose of this study was to assess whether exposure of Human HepG2 cells to low-levels of arsenic would affect gene expression in exposed cells.

Materials and Methods

Cell culture and chemical treatments

Human derived hepatoma (HepG2) cells were obtained from the American Type Culture Collection and cultured according to their recommendations. This cell line displays many genotypic and phenotypic characteristics of normal liver cells[28–30] and HepG2 cell cultures and cDNA microarray analyses have been established as an effective approach for characterizing the gene expression profiles of other chemicals, drugs and hepatotoxins.[31–33] The cells were grown in monolayer culture in cultured to approximately 80% confluence on 25 cm^2 cell culture flasks in Dulbecco's modified Eagles medium (DMEM) [Gibco Cat# 41500-034] supplemented with 10% fetal calf serum (Sigma), 1% 100 mM sodium Pyruvate and 1% antibiotic solution [10,000 U/ml penicillin and 10,000 µg/ml streptomycin (Gibco)] in an atmosphere of 5% CO_2 at 37 °C. The HepG2 cells were obtained from the American Type Culture Collection and were cultured according to their recommendations. HepG2 cells were grown to 80%–85% confluence, then continuously cultured in sodium arsenite (Sigma) at concentrations of 0.1 µM and 10 µM for periods of either 6 or 24 hours. Exposure dosages, sub-cytotoxic, were chosen to represent low-level arsenic concentrations found in drinking water[24,34–36] and which more than 80% cell viability was still determined by neutral red uptake.[37] Following the chemical exposure to sodium arsenite, cells were washed with phoshate-buffered saline (PBS) and immediately subjected to RNA extraction. The predominant form of arsenic in drinking water is inorganic arsenic. Arsenite [As^{3+}] is considered the most toxic form and was chosen as the chemical species for investigation in this study.

RNA isolation

The total RNA for the control and the chemically treated cells were both isolated and purified using the Trizol reagent, according to the manufacturer's protocol (Invitrogen). The concentration of the RNA was assessed by optical density readings (260 nm) using a Pharmacia GeneQuant RNA/DNA calculator. The extracted total RNA was dissolved in DEPC (diethyl pyrocarbonate)-treated water at a concentration of 3 µg/µL and 15 µg of RNA was used for microarray analysis from each sample.

Fluorescent labelling of cDNAs and hybridization

Each experiment utilized 15 µg of RNA for the microarray analysis and the experiment was performed as stated by the University Health Network (UHN) Microarray Center. A detailed description for the preparation of microarrays, labelling of cDNAs, and the hybridization reactions can be found at the UHN Microarray Centre website, http://www.cnslab.carleton.ca/~kbstorey/array/oci.pdf. Briefly, fluorescently labelled cDNAs were prepared from RNA preparations isolated from control and chemically treated cells using the protocols devised for labelling and hybridization to Human 1.7 K and 8 K-Expressed Sequence Tag (EST) microarray chips obtained from the University Health Network (UHN), Toronto Ontario. Target cDNAs from control and treated RNA samples were prepared by reverse transcription and reciprocally labelled using Cyanine 3' and Cyanine 5'-dCTP fluorescent dyes.

Scanning and quantification

The microarray chips were scanned on a scanning laser confocal microscope (Scanarray 4000 XL;

PerkinElmer, Boston, Mass). Individual 16-bit tagged image file format (TIFF) images were obtained by scanning for each of the 2 fluors. An overlay image of the 2 images was created and quantified with the Genetraffic program (PerkinElmer). Intensity values for each spot were normalized, and ratios were calculated, resulting in values for control and treated sample RNA. Individual spots had to pass a number of quality criteria to be included in the data analysis, including a minimum spot/local background intensity greater than or equal to 1, a minimum and a minimum spot intensity of 50. The fluorescent labels were detected at specific wavelengths. Cyanine 3' can be detected at 543 nm and Cyanine 5' can be detected at 633 nm. A composite image of the two images was created and quantified with the Genetraffic program (PerkinElmer). The intensity values for each spot were normalized and ratios were calculated. To be included in the data analysis each individual spot had to pass certain quality criteria, including a minimum spot/local background intensity ratio less than 1, a minimum spot/mean background intensity less than 1 and a minimum spot intensity of 50.

Data analysis: Quantification, normalization, hierarchical clustering

Data were stored in and analyzed with the Quantarray Microarray database and Analysis System (Iobion Informatics, La Jolla, Calif), as well as the Significance Analysis for Microarrays program.[38] Scanned 16-bit TIFF images representing each hybridized microarray slide and the associated quantification data files were entered into the database with a complete annotation of the experiments based on the current Minimum Information About Microarray Experiments standards for microarray experiments (http://www.mged.org).

Each hybridization data set was normalized with LOWESS (Locally Weighted Scatter Plot Smoother) subarray normalization (http://oz.berkeley.edu/tech-reports/). LOWESS normalization uses a local weighted smoother to generate an intensity-dependent normalization function. In subarray normalization, each subarray or grid is normalized individually to correct for variation in local mean signal intensities across the surface of the array. Log2-normalized data sets were compared to ascertain potential statistical differences in gene expression between control and chemically treated cells. A repeated permutation procedure was performed to ascertain potential statistical differences in gene expression between the control and chemically treated cells.[38] The median false discovery rate, based on analysis of permuted data sets, was less than 1.0%, and only genes with a minimum 2-fold change in expression were selected.

The screened data was then organized into a gene table which was further filtered by comparing spot intensity ratios between experimental and control chips. The filtered genes were subjected to cluster analysis (hierarchical clustering) to organize the gene set into meaningful subsets and groups. The hierarchical tree analysis was performed using a minimum distance value of 0.001, separation ratio of 0.5, and the standard correlation distance definition. Clustering involves computing distances between data elements; whereas the inverse of distance is similarity. In the experiment, data elements are cDNA signals representing gene expression ratios.[39] If two genes have similar expression profiles across the various treatment concentrations, the distance between them will be small. On the other hand, if two genes have dissimilar profiles across the different treatments, the distance between them will be large. Clusters are detected using agglomerative methods. Agglomerative methods start with each item in its own cluster and then successively combine the two closest clusters until all items are grouped together. The result is a tree where item are joined by very short branches if they are very similar and by increasingly longer branches as their similarity decreases Genetraffic™ user manual, Iobion Informatics.[39–41]

Genes isolated by these methods were determined by entering the corresponding IMAGE clone number into the SOURCE database (http://source.stanford.edu/cgi-bin/sourceSearch). As a result, the corresponding gene name was identified form the identification number provided by University Health Network Microarray Center (http://www.cnslab.carelton.ca/~kbstorey/array/oci.pdf).

Results

Low-level sodium arsenite-mediated gene expression changes

In the present study we tested two concentrations of low-level sodium arsenite for the detection of any corresponding changes in gene expression in HepG2

cells exposed to this chemical. HepG2 cells exposed to 0.1 μM of sodium arsenite for 6 and 24 hours and to 10 μM of sodium arsenite for six hours were collected for gene expression array measurements. With the inclusion of reciprocal hybridization, a total of 80600 genes were included in the initial microarray analysis of gene expression changes in HepG2 cells treated with sodium arsenite. For the purpose of data analysis (summarized in Fig. 1), spots of low intensity and high variability were filtered out using several parameters as outlined in the material and methods section. Following the filtration criteria outlined in Figure 1, the parameters set form normalization and selecting for genes that expressed values that were Log2 > 1 and Log2 < -1, the number of genes was reduced to 1,118. This included 224 genes that were upregulated and 964 genes that were downregulated. Subsequent to further filtering, 22 genes were grouped by hierarchical clustering for the identification of both common and unique genes expressed following the treatment with low-level sodium arsenite. In our system 19 genes were identified as common genes [two-fold change in gene expression (up or down) observed in treatments] for respective sodium arsenite treated HepG2 cells. Of the 19 common genes identified in sodium arsenite treated cells, 42% exhibited a two-fold increase in gene expression and 58% exhibited a two-fold decrease in gene expression as compared to the untreated cells. A total of 3 genes were considered unique genes [three-fold change in gene expression (up or down)] for respective sodium arsenite treated cells. Of the unique genes identified 67% were upregulated and 33% were down-regulated for the respective sodium arsenite treated cells.

The chemical treatments of sodium arsenite that induced similar changes in gene expression profiles (up or down-regulated) in HepG2 cells are summarized in Table 1.

Hierarchical clustering of data indicates that 22 genes exhibit similar changes in expression profiles across the exposure durations and sodium arsenite concentrations tested. In some cases, a dose and time dependent gene expression change (up or down) was observed in HepG2 cells treated with sodium arsenite. iThe greatest change in expression profiles, (up or down), of the 22 genes identified, were observed in HepG2 cells exposed to 0.1 μM for six hours. Decreases in spot intensity with increasing exposure time and concentration may suggest a threshold effect in gene expression change for some genes observed in our study.

As summarized in Figure 2, the greatest change in expression [up (red) or down (green)] for brain-derived neurotrophic factor, keratin 1 and fibrinogen, as compared to control, was observed in HepG2 cells exposed to 0.1 μM sodium arsenite for 6 hours.

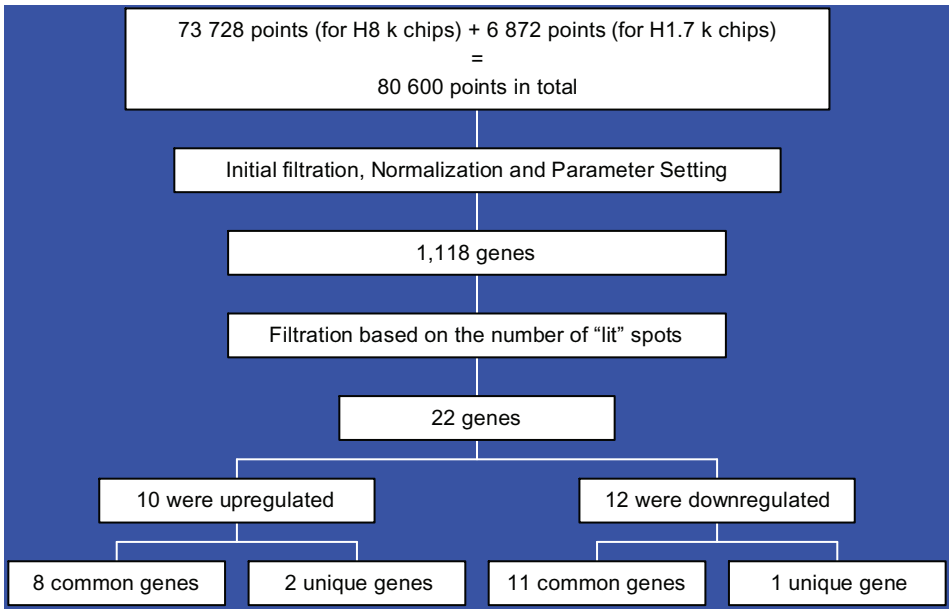

Figure 1. Schematic summary of data analysis and reduction resulting in genes with significant changes in gene expression. (Common genes: two-fold increase or decrease in gene expression. Unique genes: three-fold increase or decrease in gene expression).

Table 1. List of the 22 genes identified exhibiting similar changes in expression profiles across concentrations and durations of exposure tested.

Common upregulated genes	Common downregulated genes
Coagulation factor II (thrombin) receptor-like 2	Apoptosis Inhibitor 5
Microfibrillar-associated protein I	Alpha-fetoprotein
Insulin-like growth factor binding protein I	DC2 protein
Interleukin 1 receptor, type I precursor	Similar to 60S ribosomal protein L23a
Angiotensinase C	Reticulocaltin 2, EF-hand calcium binding domain
Osteonectin	Fork head box A3
Solute carrier family 25	v-kit Hardy Zuckerman 4 feline sarcoma viral oncogene homologue
Metallothionein 3 (growth inhibitory factor) (neurotrophic)	Ring finger protein 141
	Calcium binding protein P22
	Enolase 2 (gamma, neuronal)
	Down-regulator of transcription 1, TBP-binding negative cofactor 2
Unique upregulated genes	**Unique downregulated genes**
Brain-derived neurotrophic factor	Fibrinogen, gamma polypeptide
Keratin 1 (epidermolytic hyperkeratosis)	

Genes demonstrating altered expressions (up or down), in HepG2 cells exposed for 6 and/or 24 hours and 0.1 µM and/or 10 µM of sodium arsenite, were subsequently classified into functional groups. The genes were grouped as structural, immune, regulatory and cell cycle associated genes.

i. Structural genes: Treatment with sodium arsenite induced the up-regulation of microfibrillar-associated protein 1. The human MAGP1 (or MFAP2) genes code for the microfibril-associated glycoprotein-1 (MAGP-1), an extracellular matrix protein of microfibrillar structures[42]

ii. Immune response: Treatment with sodium arsenite induced a two-fold up-regulation of the interleukin 1 receptor. The gene encodes a protein that modulates interleukin 1 related immune and inflammatory responses.

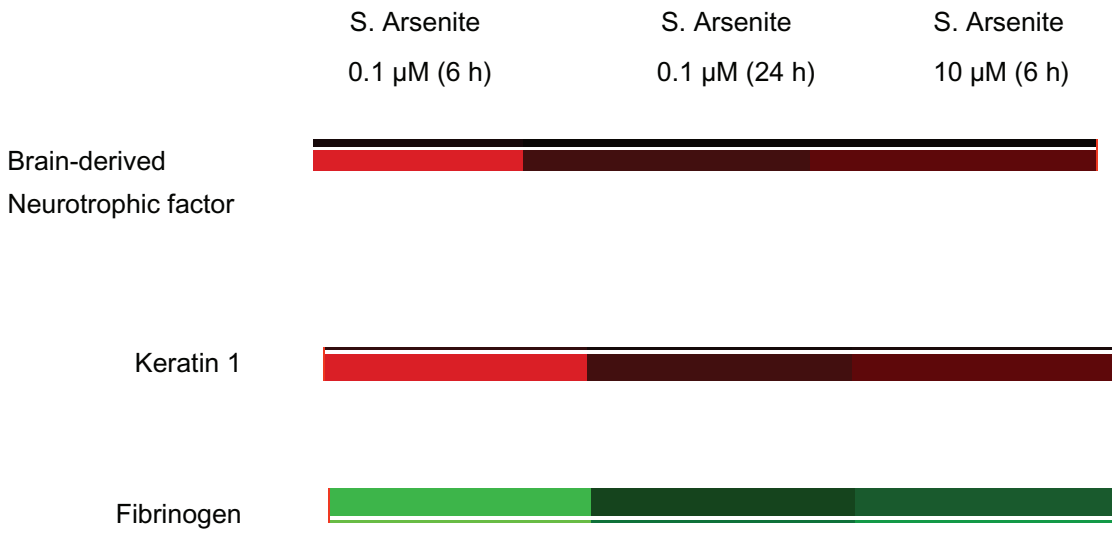

Figure 2. Expression pattern at the various concentrations tested for the unique genes.

iii. Regulation and signalling: Sodium arsenite treatment induced the up-regulation of several genes associated with regulatory and/or signalling functions. Angiotensinase c, coagulation factor II (thrombin) receptor-like 2 and metallothionein 3 were up-regulated in exposed HepG2 cells. Human Angiotensinase c (HUMPCP), also known as prolylcarboxypetidase gene, is a candidate gene for essential hypertension,[43] encodes a lysosomal enzyme and is a regulator for both renin-angiotensin system and the kallikrein-kinin system.[44] Coagulation factor II (thrombin) receptor-like 2 belongs to a clustered gene family involved in the regulation of thrombotic responses.[45] Metallothionein 3 is associated with the stress response[46] is considered a hypoxia-inducible gene[47] and is involved in growth regulation.[48] Calcium binding protein P22 and enolase 2 (gamma, neuronal) were down regulated in HepG2 cells exposed to sodium arsenite.

iv. Cell cycle control: Cells exposed to sodium nitrate resulted in the upregulation of Osteonectin was significantly up-regulated when compared to the control cells. Osteonectin is an extracellular matrix (ECM) protein which is secreted by various cell types, and regulates tissue remodeling and cell proliferation.[49] Apoptosis inhibitor 5 and alpha-fetoprotein were significantly down-regulated. Alpha fetoprotein (AFP) functions as a regulatory factor in the growth of tumor cells[50] and apoptosis inhibitor 5 functions as suppressor of E2F-dependent apoptosis[51] E2-promoter binding factor (E2F) family proteins are important regulators of cell cycle progression.

Discussion

In the present study, we have analyzed the effects of two sodium arsenite concentrations at the genomic level and have generated a gene expression profile in human hepatoma (HepG2) cells. We have shown that exposing HepG2 cells for 6 and/or 24 hours to low-level sodium arsenite concentrations induce changes in gene expression in HepG2 cells as determined by at least a two-fold difference in expression patterns between control and treated groups.

The microarray analysis indicate that at least 22 genes showed a significant change in gene expression (45% up-regulated and 54.5% down-regulated), as observed by a two or three-fold difference from control vs cells treated for 6 and/or 24 hours with sodium arsenite at concentrations of 0.1 μM and 10 μM. Hierachical clustering of microarray data demonstrated that genes have similar expression profiles across both chemical treatments and durations of exposure. The affected genes were divided into several categories, and these included: structural genes (microfibrillar-associated protein 1), immune response genes (Interleukin 1 receptor type 1), regulatory/signalling factors (metallothionein 3, angiotensinase c) and cell cycle genes (Osteonectin). Together, the genes identified in this study encompass many cellular processes. These findings suggest that the cellular response to low-level sodium arsenite exposure is complex and may be associated with alterations in the expression of many genes.

In the present study HepG2 cells, treated with sodium arsenite exhibited an up-regulation of microfibrillar-associated protein 1 (MAGP1). The human MAGP1 (or MFAP2) gene codes for the microfibrillar-associated glycoprotein-1 (MAGP-1) an extracellular matrix protein of microfibrillar structures.[42] Microfibril-associated glycoprotein is a major structural component of connective tissue microfibril and the human MAGP1 (or MFAP2) gene has been shown to possess proangiogenic activities.[52] Arsenic has been shown to demonstrate pro-angiongenic effects and to promote tumor angiongenesisl.[53] Chronic exposure of isolated B16–F10 tumor cells to low-dose arsenic was found to stimulate angiogenesis and proliferation through a (hypoxia inducible factor) HIF-dependent stimulation of angiogenesis.[54] Arsenic exposure in utero has also been shown to cause alterations in the extracellular matrix expression of airway smooth muscle in mice exposed in utero to 100 ppb (ug/l) or less of arsenic through drinking water.[55]

HepG2 cells exposed to low-levels of sodium arsenite exhibited an up-regulation of interleukin 1 receptor gene (IL-1R1). This gene along with interleukin 1 receptor, type II (IL1R2), interleukin 1 receptor-like 2 (IL1RL2), and interleukin 1 receptor-like 1 (IL1RL1) form a cytokine receptor gene cluster in a region mapped to chromosome 2q12.[56] The protein encoded by IL-1R1 is a cytokine receptor that belongs to the interleukin 1 receptor family.[57] This protein is a receptor for interleukin alpha (IL1A), interleukin beta (IL1B), and interleukin 1 receptor, type I (IL1R1/IL1RA) and

is an important mediator involved in many cytokine induced immune and inflammatory responses.[58] Members of the interleukin-1 receptor (IL-1R) family play important roles in immunity and inflammation and initiate intracellular signalling cascades leading to the activation of nuclear factor-kappaB (NF-kappaB) and other transcription factors that stimulate the expression of a variety of genes that shape an appropriate immune response.[59]

Clinical signs of inflammation in many tissues have been documented in human populations exposed to low doses of arsenic.[60] Excessive or altered inflammatory processes contribute to severe and diverse human diseases including cardiovascular disease, diabetes[61] and cancer.[62] Arsenic associated vascular disorders reported in humans are associated with the altered expression of numerous inflammatory genes.[63] Low-level nonapoptotic concentrations of sodium arsenite have been shown to activate NF-kappaB in exposed U937 human monocyte leukemia cells.[60] Activation of inflammation/NF-kappaB signaling has also been demonstrated in infants born to mothers exposed to arsenic through drinking water.[64] Although, low arsenic exposures through drinking water have been associated with inflammatory disease in humans and low-level arsenic exposures activate NF-kappaB in cell exposure models,[64] further studies are necessary to fully characterize the biological significance of the up-regulation of interleukin 1 receptor gene as demonstrated in this study.

Angiotensinase c, coagulation factor II (thrombin) receptor-like 2 and metallothionein 3 were up-regulated in exposed HepG2 cells. Human Angiotensinase c (HUMPCP), also known as prolylcarboxypeptidase gene, is a candidate gene for essential hypertension,[43] encodes a lysosomal serine carboxypeptidase that cleaves COOH-terminal amino acids linked to proline, as in angiotensin II and III and [des-Arg9] bradykinin enzyme and regulates both renin-angiotensin and the kallikrein-kinin systems.[44] Hypertension in addition to other various cardiovascular disorders have been associated in human populations chronically exposed to arsenic through drinking water.[8,65–67] The biological significance of the up-regulation of HUMPCP as seen in sodium arsenite exposed HepG2 cells requires further study, however the up-regulation of this gene may provide some insight into the potential mechanisms involved in the clinical outcome of hypertension demonstrated in populations exposed to arsenic through drinking water.

Coagulation factor II (thrombin) receptor-like 2 belongs to a clustered gene family involved in the regulation of thrombotic responses.[68] Coagulation factor II (thrombin) receptor-like 2 (F2RL2) is a member of the large family of 7-transmembrane-region receptors that couple to guanosine-nucleotide-binding proteins. F2RL2 is also a member of the protease-activated receptor family and is activated by thrombin, mediates thrombin-triggered phosphoinositide hydrolysis and is expressed in a variety of tissues. Thrombin serves as a central effector of the coagulation cascade by converting fibrinogen to fibrin and by activating platelets and other cells through protease-activated receptors (PARs).[69,70] Thrombin has also been reported to play a pivotal role in the initiation of angiogenesis by indirectly regulating and organizing a network of angiogenic molecules through distinct mechanisms involving PARs.[71]

It may be possible that arsenic induced angiogenic, apoptotic or haematological effects involve protease-activated receptors. Monomethylarsonous acid (MMA (III)) and dimethylarsinous acid (DMA(III)), highly reactive and toxic trivalent methylated metabolites of arsenic, have been found to induce procoagulant activity and apoptosis in isolated human platelets, mechanisms that may be important in arsenic-associated CVDs.[72]

MT genes belong to a superfamily with four isoforms (MT's 1,2,3 and 4) identified.[73] Metallothionein 3 was up-regulated in exposed HepG2 cells in the present study. The expression of MT3 was initially thought to be found only in the normal human brain, has been identified in kidney, breast, pancreas, intestine, bladder and prostate cancer.[74] Metallothionein 3 is has been shown to be associated with the stress response,[46] is hypoxia-inducible[47] and is involved in growth regulation.[48] MT-III upregulates VEGF production in brain endothelial cells by a HIF-1alpha-dependent mechanism.[75] The biological significance of MT3 expression in cancers has not yet been fully determined. However the up-regulation of MT3 demonstrated in our study of arsenic exposure may provide some insight to probe into the mechanisms of arsenic carcinogenicity.

Osteonectin was significantly up-regulated in HepG2 cells exposed to sodium arsenite compared to untreated cells. Osteonectin is an extracellular matrix

(ECM) protein that is secreted by various cell types, and regulates tissue remodeling and cell proliferation.[49] An increase in osteonectin gene expression has been detected in fibrotic liver[49] and its enhanced expression has been suggested to play a role in liver fibrosis.[49,76] Expression levels of osteonectin gene have been associated with heat shock,[77] differentiation[78] and culture shock.[79] Epidemiological studies have clearly indicated an association between arsenic exposure and abnormal liver function, liver fibrosis and cirrhosis.[8,46] Fibrosis of the liver is common in areas where populations are exposed to arsenic through drinking water.[80] Mechanisms involving osteonectin may contribute to arsenic induced liver fibrosis.

Apoptosis inhibitor 5 and alpha-fetoprotein were significantly down-regulated in sodium arsenite treated cells. Both are important regulators of the cell cycle. Alpha fetoprotein (AFP) functions as a regulatory factor in the growth of tumor cells[50] and apoptosis inhibitor 5 functions as a suppressor of E2F-dependent apoptosis.[51] E2-promoter binding factor (E2F) family proteins are important regulators of cell cycle progression. Arsenic has been shown to induce apoptosis in various cells lines including HepG2[81] and human hepatocytes.[82] It may be possible that the apoptotic effects of arsenic involve mechanisms that are associated with apoptosis inhibitor 5 and alpha-fetoprotein.

Alterations in gene expression have been observed in other model systems of arsenic exposure.[46,60,83] Overexpression of alpha-fetoprotein and metallothionein-1 has been observed in fetal mouse primary liver cell cultures exposed, for 72 hours, to inorganic arsenic at concentrations similar to those employed in our studies.[46] Subchronic exposure, to inorganic arsenate, through drinking water, at environmentally relevant levels, has been shown to alter expression of cyclin D1, p27, ILK, PTEN and beta-catenin in rat liver.[84] Expression changes in cyclin D1 have also been observed in the skin of K6/ODC mice exposed, through drinking water, to sodium arsenite for 4 weeks.[85] Expression changes for transcripts involved in angiogenesis, lipid metabolism, apoptosis, cell cycle control and immune responses have been observed in mouse lung isolated from mice exposed to arsenic, through drinking water, for 5 weeks, at levels commonly found in United States drinking water sources.[83] Differences in gene expression changes are reported in other models of arsenic exposure as compared to the current study. These differences may be related to the cell types, species, and tissue utilized as well as the dose, route of exposure and chemical nature of arsenic species and the design of the study (in vitro vs. in vivo). In this study, there is no direct evidence linking the gene expression alterations to specific biologically significant outcomes however, the microarray analysis does provide a global view of molecular alterations induced by sodium arsenite at relevant environmental exposure levels.

Conclusions and Future Directions

The microarray results indicate many interesting genes that are differentially expressed in response to sodium arsenite exposure. These findings suggest that sodium arsenite induces a complex cellular response in HepG2 cells and provides a global view of molecular alterations induced by sodium arsenite at relevant environmental exposure levels. The validity of the microarray data is by far very large due to the presence of duplicate spots for each gene, reciprocal labeling experiments on separate chips, and extensive normalization and filtering parameters used in our experiments. It is desirable to further validate these results by RT-PCR (Reverse Transcription-Polymerase Chain Reaction) and Northern blot analysis. In order to further explore the effect of sodium arsenite, treatment of the cells with drinking water contaminants in combination will illustrate any synergistic activity between drinking water contaminants. This is important because the chemicals are found together in water supplies and the likelihood of being exposed to drinking water contaminants together is probable. In the future a further objective is to expose other human cell types to examine the possibility of cell type heterogeneity in the response and to determine the regulatory networks by analyzing gene interactions. It is likely that changes in gene expression may be dependent on duration of exposure. Thus future studies will examine gene expression changes in cells exposed to arsenic for durations greater than 24 hours. This will be crucial to identify specific biochemical and metabolic pathways and to establishing the interplay between genes and the net physiological response of cells to drinking water contaminants such as arsenic.

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Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors report no conflicts of interest.

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