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Cell Cycle Pathway Dysregulation In Human Keratinocytes During Chronic Exposure To Low Arsenite

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Abstract

Background—Arsenic is naturally prevalent in the earth's crust and widely distributed in air and water. Chronic low arsenic exposure is associated with several cancers *in vivo*, including skin cancer, and with transformation *in vitro* of cell lines including immortalized human keratinocytes (HaCaT). Arsenic also is associated with cell cycle dysregulation at different exposure levels in multiple cell lines. In this work, we analyzed gene expression in HaCaT cells to gain an understanding of gene expression changes contributing to transformation at an early time point.

Methods—HaCaT cells were exposed to 0 or 100 nM NaAsO₂ for 7 weeks. Total RNA was purified and analyzed by microarray hybridization. Differential expression with fold change ≥ 1.5 and p-value ≤ 0.05 was determined using Partek Genomic Suite™ and pathway and network analyses using MetaCore™ software (FDR ≤ 0.05). Cell cycle analysis was performed using flow cytometry.

Results—644 mRNAs were differentially expressed. Cell cycle/cell cycle regulation pathways predominated in the list of dysregulated pathways. Genes involved in replication origin licensing were enriched in the network. Cell cycle assay analysis showed an increase in G2/M compartment in arsenite-exposed cells.

Conclusions—Arsenite exposure induced differential gene expression indicating dysregulation of cell cycle control, which was confirmed by cell cycle analysis. The results suggest that cell cycle dysregulation is an early event in transformation manifested in cells unable to transit G2/M efficiently. Further study at later time points will reveal additional changes in gene expression related to transformation processes.

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Conflict of Interest

The authors declare no conflict of interest other than grants as stated in acknowledgements.

Keywords

Arsenic-induced skin cancer; mRNA; carcinogenesis; transformation; chronic exposure; cell cycle

Introduction

Arsenic is a naturally prevalent metalloid in air and water. Underground drinking water is the major source of human arsenic exposure. Globally, over 200 million people are exposed to arsenic in drinking water in levels higher than the World Health Organization recommends (10 µg/L) (WHO 2008). Most of which are concentrated in Bangladesh, Cambodia, India, Nepal and Vietnam along with countries in Latin and North America, such as Argentina, Bolivia, Chile and Mexico and the U.S.A. Several epidemiological studies in Taiwan, Bangladesh, Chile, India, Argentina and the U.S.A. demonstrated links between the arsenic exposure exceeding 10 µg/L in drinking water and increased all-cause mortality, linked not only to increased risk of lung, bladder and skin cancers, but also cardiovascular, neurological and respiratory diseases, and skin lesions (Ahsan et al. 2006; Argos et al. 2010; Argos et al. 2011; Argos et al. 2012; Chen et al. 2010; Chen and Ahsan 2004; Chen et al. 2011; Gribble et al. 2012; Haque et al. 2003; James et al. 2015; Parvez et al. 2008; Smith and Steinmaus 2009; Wasserman et al. 2014; Wright et al. 2006; Yuan et al. 2010).

Studies from arsenic-endemic regions of Taiwan showed the association of skin cancer prevalence with increased arsenic levels in drinking-water (Tseng 1977). Long-term ingestion of arsenic is associated with different types of skin cancer such as intraepidermal carcinoma (Bowen's disease, BD), squamous cell carcinoma (SCC), basal cell carcinoma (BCC), and Merkel cell carcinoma (Centeno et al. 2002; Ho et al. 2005). Immortalized human keratinocytes (HaCaT) are an *in vitro* model for normal human keratinocytes (Boukamp et al. 1988). Pi et al (2008) showed that continuous exposure of HaCaT cells for 28 weeks to 100 nM sodium arsenite malignantly transformed these cells and resulted in an aggressive SCC phenotype when inoculated into nude mice (Pi et al. 2008). The model has also been evaluated by Sun *et. al.* as an important model to study the induction of skin cancer by arsenic exposure (Sun et al. 2009). However, the early stages of the transformation process in this model have not been studied yet. Thus, within a longitudinal study using this chronic exposure model, we analyzed gene expression to gain an understanding of gene expression changes contributing to transformation at an early time point. The data reveal differential expression of a wide array of genes responsible for cell cycle regulation suggesting that cell cycle dysregulation plays a role in early events leading to transformation.

Materials and Methods

a. Cell Culture and RNA Isolation

We adopted the HaCaT model of Pi et al. (2008) for these studies. HaCaT cells were the kind gift of Dr. TaiHao Quan, University of Michigan. NaAsO₂ (CAS 7784-0698) was obtained from Fisher Scientific, Waltham, MA, USA. HaCaT cells were cultured in alpha modification of minimal essential media supplemented with fetal bovine serum (10%), penicillin (100 units/mL), streptomycin (100 µg/mL) and glutamine (2 mM). Cultures were

maintained at 37°C in a humidified 5% CO₂ atmosphere. Multiple cultures of cells (4 with and 4 without 100 nM NaAsO₂) were maintained separately for 7 weeks (Fig. 1). This NaAsO₂ concentration was selected based on blood levels observed in an epidemiological study of a population in China that used tube wells containing high concentrations of arsenic (Pi et al. 2000). The study subjects were diagnosed with chronic arsenic intoxication and arsenic-induced skin lesions and epidermal cancers (Pi et al. 2000). Cells were passaged twice a week and a million cells were plated per 100 mm dish at every passage. Total RNA was purified from the cells (quadruplicate unexposed and exposed cultures) using the mirVana™ RNA Isolation Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). RNA quality was determined using the Agilent RNA 6000 Pico Kit, Eukaryote, version 2.6 and the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). All samples used had RIN (RNA integrity number) > 9.

b. Microarray Analysis

Expression profiles of mRNA were obtained using GeneChip® PrimeView™ Human Gene Expression Affymetrix arrays (Fig. 1). Biotinylated cRNA was prepared according to the standard protocol for Affymetrix 3' IVT Express Plus Reagent Kit from 250 ng total RNA. Following fragmentation, cRNA was hybridized for 16 h at 45°C to Affymetrix Primeview Human arrays according to the Affymetrix GeneChip 3' array Hybridization User Manual. GeneChips were scanned using GeneChip Scanner 3000 7G (Affymetrix). The CEL files were imported into Partek software Version 6.6 (Partek Inc) and normalized using Robust Multi-Array (RMA) normalization. Contrasts of interest were analyzed using a 2-way ANOVA considering treatment and time. Using Partek Genomic Suite™ a list of differentially expressed mRNAs (644) (fold change |1.5|) at 7 weeks was obtained (supplementary Table 1). Data have been deposited in the GEO database, accession number GSE97306.

c. Flow cytometry cell cycle assay

HaCaT cells exposed to 0 or 100 nM NaAsO₂ for 7 weeks were seeded at density of 1×10^6 cells per 55 cm² cell culture dish with arsenite exposure maintained. After 48 hours, the cells were trypsinized, washed twice with PBS then fixed in 70% ethanol for 24 hours at 4°C. The cells were then centrifuged at 1500 g for 1 minute, suspended in PBS (400 µl), then RNaseA (10 mg/ml, 50 µl) and propidium iodide (2 mg/ml, 10 µl) were added followed by a 30 minute incubation in the dark at room temperature. Fluorescence was acquired by flow cytometry on a Becton Dickinson FACSCalibur™ (BD Biosciences) (Chowdhury et al. 2010). Cell cycle distribution was determined using FlowJo® v10.2. Student's T-Test was used for statistical analyses (p-value 0.05, was considered significant).

Results

a. Arsenite-dependent differential expression of cell cycle involved mRNAs

Analysis of the gene chip data revealed that 644 mRNAs were differentially expressed in HaCaT cells exposed to 100 nM NaAsO₂ for seven weeks. More than half of the mRNAs were suppressed with arsenite exposure and the remainder were induced (Fig. 2A). The differentially expressed genes were then loaded into Metacore™ software for pathways

analysis. The 15 pathways with FDR values ≤ 0.05 are listed in Table 1. Cell cycle and cell cycle regulation pathways are highly represented in this list (7 of 15). Other pathways that were dysregulated include epithelial-to-mesenchymal transition (EMT), cytoskeleton remodeling, apoptosis, immune response and gap junction pathways. The mRNAs populating these pathways were analyzed for potential interactions. The interacting genes are shown in a network built by Metacore™ software (Fig. 2B). Three E3 ubiquitin ligase complexes known to regulate cell cycle are present in this network (Siah1/SIP/EBI; CUL1/RBX1; SKP2/TRCP/FBXW) (Frescas and Pagano 2008; Matsuzawa and Reed 2001; Zheng et al. 2002). A subnetwork of genes involved in regulating licensing of the DNA replication origin (CDT1; MCM4/6/7 complex; MCM2) (Nishitani and Lygerou 2002) also is present.

b. Arsenite-dependent increase in G2/M compartment

The gene expression data indicated dysregulation of cell cycle was occurring in arsenite exposed cells. In order to confirm cell cycle dysregulation, cell cycle analyses were performed on the 4 exposed and 4 unexposed cultures. The results indicate that arsenite-exposed cells are accumulating in the G2/M compartment suggesting a delay in either the G2 to M phase or M to G1 phase transition (Fig. 3; Supp. Fig. 1). These results are consistent with the network results of Figure 2B.

Discussion

Arsenic is a known human carcinogen (IARC 2012). Several mechanisms of arsenic carcinogenicity including genomic instability and chromosomal abnormalities have been proposed (Chih-Hung Lee 2010). Arsenic exposure has been associated in several studies with dysregulation of DNA methylation, cell cycle, and DNA repair gene expression (Andrew et al. 2006; Ren et al. 2011; Treas et al. 2013; Zhang et al. 2007). However, there is not yet agreement on the mechanism(s) of arsenic transformation. Chronic low arsenic exposure leads to the malignant transformation of cell lines from several tissues (Achanzar et al. 2002; Barrett et al. 1989; Chang et al. 2010; Pi et al. 2008).

Transformation is likely to be driven by a series of events starting from early times of exposure and extending out to as much as thirty weeks exposure when transformation can be demonstrated to have occurred. Early events in transformation in these model systems have not yet been investigated. Thus, we have examined gene expression changes in the HaCaT model of arsenic-induced skin carcinogenesis after seven weeks' exposure to gain an understanding of the early events related to transformation.

We (unpublished data) and others (Pi et al. 2008) have observed that HaCaT cells chronically exposed to 100 nM NaAsO₂ grow more slowly than parallel cultures of unexposed cells until approximately 19 weeks exposure. Dysregulation of cell cycle control as suggested by the differential expression and pathways data is consistent with these earlier observations on cell growth kinetics. This conclusion is further supported by cell cycle assay analysis on HaCaT cells after seven weeks' exposure that showed accumulation of cells in the G2/M compartment (Figure 3). This observation is consistent with the network analysis showing induction of genes associated with licensing replication origins (CDT1, MCM2/4/6/7, Figure 2B). These observations are consistent with G2/M delays observed in

earlier studies by our laboratory (McNeely et al. 2008; Muenyi et al. 2014; States et al. 2002; Taylor et al. 2006) and by others (Ma et al. 1998; McCollum et al. 2005).

The E3 ubiquitin ligase complexes identified in the network analysis (Figure 3) are all zinc finger RING E3's that regulate cell cycle transitions other than G2 to M or M to G1. Thus, although the genes involved in replication origin licensing suggest a major impact on M to G1 transition, the induction of these E3 ubiquitin ligase genes suggests that the cells are responding to delays at other cell cycle transitions as well. Impact of arsenite exposure at multiple cell cycle transitions was demonstrated by McCollum et al in U937 cells (McCollum et al. 2005), and by S phase lengthening in MCF7 and H1299 cells (Pozo-Molina et al. 2015). Accumulation in the G0/G1 phase leading to reduction in the S phase also was observed with mouse skin fibroblast cells (m5S), mouse thymocytes and B-cell lymphoma A20 cells (Jutapon Chayapong 2017; Nohara et al. 2008). It was also reported that HL-60; derived from a patient with acute myeloid leukemia (FAB), were arrested at G1 with arsenic exposure (Zhang et al. 1998). Furthermore, Moghaddaskho et al. 2017 showed that arsenic arrested MDA-MB-231 and MDA-MB-468 cells at G2/M phase (Moghaddaskho et al. 2017). Clearly, arsenite exposure can cause cell cycle disruption in all cell cycle phases. However, delays in M phase are likely to contribute to genomic instability via induction of aneuploidy.

Conclusions

Results presented here showed that arsenite exposure induced differential gene expression indicating dysregulation of cell cycle control, consistent with slow growth of arsenite-exposed cells at early times of chronic exposure. Analysis of cell cycle distribution showed a delay at G2/M was occurring in HaCaT cells chronically exposed to 100 nM NaAsO₂ for seven weeks. This delay could be related to induction of aneuploidy, known to be caused by arsenic exposure. Further study at later time points will reveal additional changes in gene expression related to transformation processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BD	Bowen's disease
BCC	basal cell carcinoma
HaCaT cells	immortalized human keratinocytes

mRNA	messenger RNA
PBS	phosphate buffered saline
SCC	squamous cell carcinoma

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Highlights

- Arsenite induces differential expression of 644 mRNAs at early stages of exposure
- Arsenite exposure induced differential mRNA expression indicates cell cycle dysregulation
- Arsenite delays cells in G2/M compartment

Figure 1.

HaCaT cells were exposed to 0 or 100 nM NaAsO₂ for 7 weeks. RNA was purified and expression determined on Affymetrix microarrays and analyzed using Metacore software. Cell cycle analyses were performed by flow cytometry.

Figure 2.

Differential mRNA expression with arsenite exposure. A. Hierarchical clustering of mRNAs differentially expressed in HaCaT cells exposed to 0/100 nM arsenite for 7 weeks.

Microarray hybridization data from 4 exposed and 4 unexposed cultures was analyzed using Partek Genomic Suite to generate the heat map. Yellow indicates mRNAs induced by exposure, blue indicates suppression. B. Network of genes populating multiple dysregulated pathways at 7 weeks. Network of only direct interactions between genes populating multiple pathways at 7 weeks was built by MetacoreTM software.

Figure 3. HaCaT cells exposed to arsenite are increased in G2/M compartment. Four cultures each (exposed (+As)/unexposed (-As)) were analyzed by flow cytometry for cell cycle distribution. Fraction in each compartment (G1, S, G2/M) were determined and means $\pm$ SD are plotted. Student T-Test was used for statistical analyses. *p-value $\leq$ 0.05.

Table 1

Pathways populated by differentially expressed mRNAs 7 weeks.

Pathway *	FDR	# of genes in pathway	Induction ↑ Suppression ↓
Cell cycle Start of DNA replication in early S phase	4.54E-10	13	↑
Cell cycle Role of SCF complex in cell cycle regulation	7.44E-07	10	↑
Cytoskeleton remodeling Neurofilaments	0.0006747	7	↑
Cell cycle Transition and termination of DNA replication	0.0008922	7	↑
Immune response Antigen presentation by MHC class I, classical pathway	0.001548	9	↑↓
Cell cycle (generic schema)	0.001548	6	↑
Immune response IL-4-induced regulators of cell growth, survival, differentiation and metabolism	0.003904	9	↑
Development Regulation of epithelial-to-mesenchymal transition (EMT)	0.003904	9	↑
Cell cycle Regulation of G1/S transition (part 2)	0.003904	6	↑
Cell cycle Regulation of G1/S transition (part 1)	0.003928	7	↑
Apoptosis and survival Endoplasmic reticulum stress response pathway	0.007063	8	↓ Protein folding
Cell cycle ESR1 regulation of G1/S transition	0.01425	6	↑
Proteolysis Putative ubiquitin pathway	0.01568	5	↑
Transcription Ligand-dependent activation of the ESR1/SP pathway	0.04928	5	↑ cell cycle regulation
Cell adhesion-Gap junctions	0.04928	5	↑

* Pathway analysis performed by Metacore software with expression criteria |1.5| fold change, FDR 0.05.