Arsenic-Induced Genotoxic and Cytotoxic Effects in Human Keratinocytes, Melanocytes and Dendritic Cells

Barbara Graham-Evans¹, Hari H. P. Cohly², Hongtao Yu³ and Paul B. Tchounwou¹*

¹Molecular Toxicology Research Laboratory, NIH-Center for Environmental Health, Jackson State University, Jackson, MS 39217, USA
²Department of Surgery, University of Mississippi Medical Center, Jackson, MS 39216, USA
³Department of Chemistry, Jackson State University, Jackson, MS 39217, USA
*Correspondence to Dr. Paul Tchounwou. E-mail: paul.b.tchounwou@jsums.edu

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Abstract: Arsenical keratosis and skin cancer are among the most common health effects associated with acute and chronic exposures to arsenic. This study examines the acute and chronic dose-responses of arsenic in established human cell lines using keratinocytes (HaCaT), melanocytes (CRL1675) and dendritic cells (THP-1 + A23187). Chronic conditions were established by treating the three cell lines with at least 8 passages in 0.2 µg/mL arsenic trioxide. Cytotoxicity was assessed using the fluorescein diacetate assay after 72 hrs of exposure. Single cell gel electrophoresis (Comet assay) was used to measure DNA damage. Acute exposure to arsenic had LD₆₀ and LD₂₅ values of 0.38 µg/mL and 3.0 µg/mL for keratinocytes; 0.19 µg/mL and 0.38 µg/mL for melanocytes; and 0.38 µg/mL and 0.75 µg/mL for dendritic cells. Cytotoxicity assays for chronically exposed cells resulted in LD₆₀ and LD₂₅ values of 0.4 µg/mL and 0.8 µg/mL for keratinocytes; 0.10 µg/mL and 0.20 µg/mL for melanocytes; and 0.10 µg/mL and 1.0 µg/mL for dendritic cells. The Comet assay showed that arsenic was highly genotoxic to the three cell lines. No significant differences (p > 0.05) in DNA cleavage were observed between acute and chronic exposures. In acute exposure arsenic genotoxicity was more severe with dendritic cells while melanocytes were more sensitive to arsenic cytotoxicity. Similarly, chronically exposed dendritic cells showed the maximum genotoxic damage while melanocytes were more sensitive to arsenic cytotoxicity. In conclusion, this research shows that arsenic is dermatotoxic, showing a high degree of genotoxicity and cytotoxicity to skin cells.

Keywords: arsenic, cytotoxicity, genotoxicity, comet assay, keratinocytes, melanocytes, dendritic cells

Introduction

Arsenic exposure has been associated with skin keratosis and the development of many cancers, especially of the skin, lung and bladder, prostate, kidney and liver [1]. Low dose ingestion of arsenic does not immediate fatal consequences, however, prolonged arsenic exposure have been shown to significantly increase the risk of contracting these various forms of cancer. Because of increased risk of cancer associated with inorganic arsenic, the United States Environmental Protection Agency (U.S. EPA) has classified inorganic arsenic as a class A (known) human carcinogen [2].

Following long-term exposure to arsenic, the first changes usually observed in the skin include pigmentation changes and then hyperkeratosis. Changes in pigmentation of skin are related to alterations brought about in the components of the epidermal-melanin unit. Chronic exposure to arsenic frequently results in skin cancers [3, 4]. The skin is made up of two main layers, epidermis and dermis. The epidermis, outermost layer, provides the first barrier of protection from the invasion of foreign substances into the body. The principal epithelial cells of the epidermis are the keratinocytes. The dermis contains the melanocytes that migrate to the basal layer of the epidermis and reside there. Epidermis
and surface epithelium dendritic cells are made up of immature cells known as Langerhan cells [5]. The dendritic cells arise in the bone marrow and migrate to and seed tissues throughout the body including the epidermis. The ratio for melanocytes: basal keratinocytes is 1: 10 [6] and the ratio of dendritic cells to keratinocytes is 1:10 [7].

Arsenic has been found to be genotoxic [8]. Hei suggested that arsenic acts through a series of chemical reactions in the cell, interacting strongly with nearby molecules, and changing the structure of cellular components such as DNA [9]. Other studies have found that exposure to inorganic arsenic increases the frequency of micronuclei, chromosome aberrations and sister chromatid exchanges both in humans and experimental animals [10-11]. Arsenic has been previously shown not to affect DNA directly, but to intensify the toxic effects of other physical and chemical agents by inhibiting DNA repair, changing cell redox potential, and altering DNA methylation of cell-cycle control proteins [8]. In this study we evaluated the genotoxic and cytoxic effects of arsenic in established human cell lines represented by keratinocytes (HaCaT), melanocytes (CRL1675), and dendritic cells (THP-1+A23187) [12], following acute and chronic exposures.

Materials and Methods

Reagents and Cell Lines

Arsenic trioxide with 99.9% purity was purchased from Fisher Scientific (Suwanee, GA) and used throughout the experiments without further purifications. Reagents were purchased from Gibco (Grand Island, NY), Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT). HaCaT cell line was obtained from Dr. N. Fusenig (Division of in vitro Differentiation and Carcinogenesis, German Cancer Research Center, Germany). THP-1 and melanocytes (CRL1675) were obtained from American Type Culture Collection (Rockville, MD). The cells were cultured in a humidified atmosphere with 5% CO₂ at 37 °C.

The standard growth medium was prepared according to recommendations for specific cell lines including Dulbecco’s Minimum Essential Medium for HaCaT, RPMI 1640 for THP-1 (dendritic cells), and Vitacell medium for melanocytes (CRL 1675). Media were considered complete with the addition of 10% FBS and 1% antibiotic (penicillin and streptomycin).

Cell Treatment for Acute Cytotoxicity

Cytotoxicity assay was carried out as previously described in our laboratory [13]. Briefly, cells were counted (20,000 cells/well) and resuspended in complete medium. Aliquots of 100 µL of cell suspension were placed in wells of microtiter plates, and 100 µL of different concentrations of arsenic trioxide (0 to 200 µg/mL) were used to treat the cells. The plates were incubated for 24, 48, and 72 hours, respectively. After incubation, cells were centrifuged, washed twice with PBS, PBS discarded and aliquots of 100 µL of fluorescein diacetate (10 ng/mL) added. The plates were incubated 35 min before being read using a Fluoroskan II microplate reader (Helsinki, Finland) with an excitation wavelength of 485 nm, and an emission wavelength of 538 nm.

Cell Treatment for Chronic Cytotoxicity

In preliminary studies, several different doses of arsenic (< LD₅₀) were used to grow cells by trial and error. After 3-4 passages, we encountered very sluggish growth with eventual cell death due to arsenic toxicity. In our experience with HaCaT, we found 0.2ppm to be well tolerated. Further literature research showed that 0.5 and 1.0 µM (0.10-0.2 ppm) could be used in HaCaT for 5 months [4]. Thus, we arbitrarily chose 0.2ppm as our working dose to represent chronic exposure. The use of THP-1+A23187 to mimick dendritic cells [12] was due to the fact that dendritic cells are found in the surface epithelium along with keratinocytes and melanocytes.

Cell Treatment for Genotoxicity

Cells were counted (10,000 cells/well) and re-suspended in media with 10% FBS. Aliquots of 100 µL of the cell suspension were placed in 96 well plates, treated with arsenic trioxide concentrations at doses of LD₁₀ and LD₅₀ determined from the cytotoxicity assay data, and incubated in a 5% CO₂ at 37°C for 72 hrs. After incubation, the cells were centrifuged, washed with PBS, and re-suspended in 100 µL PBS. In a 2 mL tube, 20 µL of the cell suspension and 200 µL of melted agarose were mixed and 75 µL pipetted onto a pre-warmed slide. The slides were placed in a refrigerator at 4°C for 10-20 min and placed in chilled lysis buffer for 45 min. Slides were washed twice for 5 min with TBE and electrophoresed in a horizontal gel apparatus at 25 V for 10 min. Slides were placed in 70% ethanol for 10 min, removed, tapped to remove excess ethanol, and placed in an alkaline solution containing 99mL H₂O, 100 µL of 0.1 mM Na₂EDTA and 1g NaOH for 45 min. Slides were air dried for 2.5 hrs, stained with SYBR Green and allowed to set 4 hrs. The slides were viewed with an Olympus fluorescence microscope and analyzed using LAI’s Comet Assay Analysis System software (Loates Associates, Inc. Westminster, MD).

Statistical Analysis

Cell mortality data recorded from the cytotoxicity assay were plotted against arsenic trioxide concentrations and a linear regression analysis was performed to determine and characterize the dose-response relationship equation. This equation was then used to determine the LD₁₀ and LD₅₀ values used in subsequent genotoxicity experiments with the comet assay. For comet assay, photographs were taken to illustrate the changes in DNA morphology associated with arsenic exposure. The comet
data for DNA fragmentation and tail length were expressed as means ± SDs with n = 70, and F-statistic ANOVA was applied to determine if there were significant differences in genotoxicity with regard to arsenic treatment and cell type. Differences were considered at p value ≤ 0.05.

Results

Cytotoxicity Assay

Cytotoxicity data in terms of LD$_{10}$ and LD$_{25}$ for acutely and chronically exposed cells are shown in Table 1. In acute experiments for cytotoxicity, the LD$_{10}$ for melanocytes was lower than that of keratinocytes and dendritic cells. LD$_{25}$ dose for keratinocytes was the highest while that of dendritic cells was intermediate, and that of melanocytes was the lowest. In chronic exposure for cytotoxicity assay melanocytes and dendritic cells are more sensitive at LD$_{10}$ than keratinocytes while melanocytes are more sensitive than keratinocytes and dendritic cells at LD$_{25}$.

Table 1: Cytotoxicity (LD$_{10}$ and LD$_{25}$) of arsenic trioxide in acute and chronically-exposed skin cells.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Exposure type</th>
<th>LD$_{10}$ (ppm)</th>
<th>LD$_{25}$ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keratinocytes</td>
<td>Acute</td>
<td>0.38</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>Chronic</td>
<td>0.40</td>
<td>0.80</td>
</tr>
<tr>
<td>Melanocytes</td>
<td>Acute</td>
<td>0.19</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>Chronic</td>
<td>0.10</td>
<td>0.25</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>Acute</td>
<td>0.38</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>Chronic</td>
<td>0.10</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Comet Assay for Genotoxicity

The comet assay or single cell gel electrophoresis revealed that treatment of the cells with arsenic causes severe damage to the cells’ nuclear DNA. Figure 1 is the representative picture for all the three cell types. As shown in this figure, the nuclear DNA of untreated cells is perfectly round, but the nuclear DNA of arsenic treated cells is severely fragmented. There are several ways to measure the severity of DNA fragmentation. Here listed are the percent of DNA fragmentation (percent of DNA in the Comet tail versus total DNA), and the length of the comet tail. The higher the percent of DNA fragments, the more severe is the damage. Similarly, the longer the comet tail, the smaller is the DNA fragment, the more severe is the damage.

Figure 2 depicts the percentages of DNA fragmentation, and the lengths of comet tail in keratinocytes, melanocytes, and dendritic cells acutely (upper graphs), and chronically (lower graphs) exposed to arsenic trioxide at LD$_{10}$ and LD$_{25}$ doses. Damage to the nuclear DNA of melanocytes and dendritic cells as measured by both percent DNA damage and length comet tail of DNA fragments is similar for both LD$_{10}$ and LD$_{25}$, but significantly (p < 0.01) more severe than that of the keratinocytes (Figure 2).

Results showed when compared to controls that there was no significant difference in tail length of acutely exposed keratinocytes at LD$_{10}$, however, there was statistically significant differences (p < 0.001) for the LD$_{25}$ exposure. In chronically exposed keratinocytes, there were significant differences at all exposure levels (p < 0.001) for tail length. The percentage of DNA damage and the length of the comet tail were up in LD$_{10}$ dose but did not reach statistical significance, while DNA damage and tail length were up and reached statistical significance in LD$_{25}$ (p < 0.001) when compared to controls.

In the acutely exposed melanocytes, LD$_{10}$ tail length increased slightly more than LD$_{25}$ when compared to controls but neither reached statistical significance. In chronically exposed melanocytes, tail length for LD$_{10}$ decreased slightly while LD$_{25}$ increased significantly (p < 0.001) when compared to controls. The percentage of DNA damage in melanocytes with acute exposure was slightly increased at LD$_{10}$ and more at LD$_{25}$ when compared to controls but showed no statistical significant differences. Chronically exposed melanocytes showed %DNA damage to be significant at LD$_{25}$ only.

At acute exposures dendritic cells showed tail length to be slightly greater at LD$_{10}$ than LD$_{25}$ when compared to controls, but the difference in lengths were statistically significant at both LD$_{10}$ and LD$_{25}$ (p < 0.001). In chronically exposed dendritic cells, tail length increased at LD$_{10}$ and more at LD$_{25}$ than the controls, and reached statistical significance (p < 0.001). DNA damages in dendritic cells acutely exposed at LD$_{10}$ and LD$_{25}$ were greater than controls but did not reach statistical significance. Damages in chronically exposed dendritic cells at both LD$_{10}$ and LD$_{25}$ were greater than the controls and were significantly different (p < 0.001) (Figure 2).

Discussion

Cytotoxicity Assay

Human exposure to arsenic, a ubiquitous and toxic environmental pollutant, is associated with an increased incidence of skin cancer. It is a carcinogen that poses a significant health risk in humans. The mechanisms associated with arsenic-mediated toxicity, DNA damage and proliferation at low chronic levels of exposure remain to be examined in depth. Cell cytotoxicity measures the capacity of the intact cell to recover from the damage induced and thus forms the basis for measuring the sensitivity of the cell in question. Several studies have addressed cytotoxicity to keratinocytes using different forms of arsenic [14-16] but not arsenic trioxide.
Fig. 1: Comet assay depicting the genotoxic effect of arsenic (LD$_{10}$) to melanocytes, dendritic cells and keratinocytes acutely and chronically exposed. The bottom pictures are two control keratinocytes while the upper figures represent keratinocytes, dendritic cells and melanocytes exposed to arsenic trioxide at the dose levels of LD$_{10}$. Y-axis represents total height while X-axis gives total length of the comet.
Arsenic toxicity studies on melanocytes and dendritic cells are also lacking in the literature. Our studies found the acute LD$_{25}$ to be 3ppm for HaCaT and the chronic LD$_{25}$ to be 0.8ppm. LD$_{25}$ values of 0.38ppm and 0.25ppm in acute and chronic exposures were found for melanocytes, while values of 0.75ppm and 1.0ppm were recorded in dendritic cells, respectively (Table 1). These data indicate that arsenic is cytotoxic to the three tested skin cells, and that melanocytes appear to be more sensitive to arsenic toxicity while keratinocytes are more tolerant at both acute and chronic conditions. For the most part these data indicate that chronically-exposed cells are more sensitive to arsenic toxicity than in acute exposure with the exception of keratinocytes at LD$_{10}$ and dendritic cells at LD$_{25}$.

Comet Assay for Genotoxicity

Human activities have increased the possibility of exposure to naturally occurring metals causing a greater risk of exposure to toxic levels [17]. The exposure of metals such as arsenic constitutes a major health concern. Arsenite induces DNA damage referred to as genotoxicity in human cells within a pathologically meaningful dose range. Arsenic toxicity is cell specific; therefore, it is important that target cells be used for investigations [17]. Hamadeh et al. exposed normal human epidermal keratinocytes (NHEK) to nontoxic doses (0.005-5 µM) of arsenic (III) and that exposure simultaneously modulates DNA repair, and redox-related gene expression in NHEK [18]. Studies show that arsenic may not directly impact DNA but may inhibit some DNA repair [19]. Arsenic has been shown to induce DNA damage in human cells. Inorganic arsenic increases the frequency of micronuclei, chromosome aberrations and sister chromatid exchanges as well as inhibits DNA repair [10, 11]. Specifically, a significant increase in comet tail-length at doses 0 to 6.45 mg/kg body weight demonstrated that arsenic trioxide cause DNA damage effectively [20, 21]. Using the comet assay, we applied the alkaline treatment which aids in the unwinding and denaturation of DNA molecules, thus
allowing for the sensitive detection of single-strand damage [22].

In our studies using the three cell lines, we found for genotoxicity assay that in acute exposures there was a decrease in DNA fragmentation and tail length for keratinocytes at LD$_{25}$. This implies that probably a DNA repair mechanism may be activated thus affording protection to keratinocytes. Further, the damage to the nuclear DNA in melanocytes and dendritic cells was more severe than that of keratinocytes. In general, the DNA damage to chronically exposed keratinocytes was lower than dendritic cells and melanocytes. It can thus be conjectured that genotoxicity or cancer in the long term effect could be attributed to dendritic cells and melanocytes. When comparing dendritic cells and melanocytes one can further speculate that dendritic cells are playing a more important role as the damage to the dendritic cells is more severe than melanocytes. Thus, we have demonstrated that dendritic cells are more potent in genotoxicity implying that they may be the first trigger followed by melanocytes and then eventually affecting the key element keratinocytes in skin carcinoma.

With respect to arsenic we propose that arsenic has a multifactorial effect on the cellular elements of the epidermis (Figure 3). According to our model it is postulated that the initial change caused by arsenic is on the dendritic cell at the DNA level. The antigen presenting cell then presents arsenic very effectively to the melanocyte which causes its cell death. If the death is partial then it triggers keratinocyte to alter its division and thus lead to carcinoma of the skin. If there is extensive death then the characteristic changes in the pigmentation of the skin occur.

Our cytotoxicity data revealed that keratinocytes were more tolerant while dendritic cells were intermediate and melanocytes were most sensitive to arsenic toxicity. Pigmentation alteration due to long-term exposure of arsenic could be associated due to the direct cytotoxicity of arsenic on melanocytes. This research is the first report investigating the in vitro effects of arsenic-induced cytotoxicity and DNA damage in melanocytes, dendritic cells and keratinocytes, concomitantly in the same study. It is anticipated that data from this report will serve as a base for furthering our knowledge on arsenic modulation of cytotoxicity and DNA damage in skin cells.

Figure 3: A schematic representation of the damage caused by arsenic on the cellular elements of the skin involving keratinocytes, melanocytes and dendritic cells.
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