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Inorganic arsenic inhibits the nucleotide excision repair pathway and reduces the expression of XPC

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Abstract

Chronic exposure to arsenic, most often through contaminated drinking water, has been linked to several types of cancer in humans, including skin and lung cancer. However, the mechanisms underlying its role in causing cancer are not well understood. There is evidence that exposure to arsenic can enhance the carcinogenicity of UV light in inducing skin cancers and may enhance the carcinogenicity of tobacco smoke in inducing lung cancers. The nucleotide excision repair (NER) pathway removes different types of DNA damage including those produced by UV light and components of tobacco smoke. The aim of the present study was to investigate the effect of sodium arsenite on the NER pathway in human lung fibroblasts (IMR-90 cells) and primary mouse keratinocytes. To measure NER, we employed a slot-blot assay to quantify the introduction and removal of UV light-induced 6-4 photoproducts (6-4 PP) and cyclobutane pyrimidine dimers (CPDs). We find a concentration-dependent inhibition of the removal of 6-4 PPs and CPDs in both cell types treated with arsenite. Treatment of both cell types with arsenite resulted in a significant reduction in the abundance of XPC, a protein that is critical for DNA damage recognition in NER. The abundance of RNA expressed from several key NER genes was also significantly reduced by treatment of IMR-90 cells with arsenite. Finally, treatment of IMR-90 cells with MG-132 abrogated the reduction in XPC protein, suggesting an involvement of the proteasome in the reduction of XPC protein produced by treatment of cells with arsenic. The inhibition of NER by arsenic may reflect one mechanism underlying the role of arsenic exposure in enhancing cigarette smoke-induced lung carcinogenesis and UV light-induced skin cancer, and it may provide some insights into the emergence of arsenic trioxide as a chemotherapeutic agent.

Keywords

Arsenic; UV light; Nucleotide Excision Repair; XPC; skin cancer; lung cancer

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest

1. Introduction

Inorganic arsenic is a metalloid found in moderate levels in the Earth's crust. It is one of the world's oldest known carcinogens [1] and it is classified as a class I carcinogen [2]. Humans can be exposed to arsenic by drinking contaminated water [3], inhaling certain substances in occupational settings [2], ingesting contaminated food (summarized in [4]) and using tobacco products [5]. It is estimated that approximately 200 million people in the world are exposed to drinking water contaminated with potentially harmful levels of arsenic [6]. In the US, exposure to contaminated drinking water occurs in many regions throughout the country [7]. According to the Agency for Toxic Substances and Disease Registry (ATSDR), arsenic ranks number one on the priority list of hazardous substances found at National Priority List sites (<http://www.atsdr.cdc.gov/SPL/index.html>).

Exposure to arsenic increases the risk for cancer development at multiple organ sites including the skin [8–10], lung [11, 12], liver (summarized in [13]), and bladder [14]. Arsenic has been used for centuries for a range of medicinal purposes, and topical treatment with an arsenic compound called Fowler's solution (discovered in 1786) was used to treat a host of diseases including malaria, syphilis, asthma, chorea, eczema, and psoriasis [15]. It was over one hundred years later that Hutchison proposed that Folwer's solution was a human skin carcinogen [16]. Correlations between arsenic exposure and internal cancer development were first observed in regions of the world where populations were exposed to levels of arsenic between 10–100 fold greater than the 2002 EPA standard and current World Health Organization Guideline level of 10 µg/L in drinking water [17]. In these regions of the world, such as parts of Bangladesh, India, China, Argentina, Chile, and other countries (Reviewed in [7]), high arsenic contamination in the water is a consequence of local geographical deposits of the metal and poor procedures for decontaminating water. In the US, exposure to arsenic is generally more moderate. However, it has been estimated that arsenic concentrations exceed 20 µg/L in 5% of regulated water systems [7] and arsenic exposure in the US has been tentatively linked to skin [18], bladder [19] and lung cancers [20]. Interestingly, arsenic has also been adopted as a chemotherapeutic agent, with arsenic trioxide (ATO) used with success to treat acute promyelocytic leukemia [21–23].

Arsenic exposure likely contributes to cancer development by multiple mechanisms. Evidence indicates that arsenic impacts genotoxicity, although it does not directly interact with DNA to produce DNA damage (reviewed in [24]). Rather than directly producing DNA damage, arsenic is often considered a co-carcinogen that influences the mutagenicity and carcinogenicity of other agents. Co-treatment of cells with arsenic and UV light results in a reduction in the removal of photoproducts produced by UV light [25–27] and an increase in mutation rates produced by UV light [27–30]. Co-treatment of cells with arsenic increases the levels of DNA adducts produced by benzo[a]pyrene diol-epoxide (BPDE) which is a metabolite of benzo[a]pyrene (B[a]P), a compound present in tobacco smoke [31, 32]. Consistent with this observation, arsenic reduces the removal of BPDE adducts [33, 34] and increases the frequency of mutations formed by BPDE [35]. These observations are directly relevant to the types of cancer associated with exposure to arsenic; specifically, arsenic increases non-melanoma skin cancer and lung cancer incidences in humans for which UV light and tobacco smoke exposure, respectively, are risk factors [9, 36].

Several studies have investigated how arsenic may act as a co-carcinogen using mouse models. When added to drinking water, arsenic enhances the production of DNA damage and the mutagenicity of topically applied B[a]P, but it does not produce mutations or DNA damage in the absence of B[a]P [37, 38]. Arsenic increases the multiplicity and size of skin tumors produced by dimethylbenz[a]anthracene (DMBA), a polycyclic aromatic hydrocarbon (PAH), but it does not alter skin carcinogenesis in the absence of DMBA [39, 40]. It also greatly increases the formation of UV-induced skin tumors in hairless mice [41], but it does not induce tumor formation in the absence of UV damage [42].

Many of the agents that arsenic interacts with as a co-mutagen or co-carcinogen, including UV light and tobacco smoke, produce DNA lesions that are removed by the nucleotide excision repair (NER) pathway. UV light produces cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4 PPs), both substrates for NER [43]. Tobacco smoke contains several classes of chemicals which can react with DNA, forming adducts that are removed by NER. Multiple polycyclic aromatic hydrocarbons (PAHs) from combustion of tobacco (as well as fossil fuels and other organic matter) produce bulky, DNA distorting adducts that are generally repaired by NER. PAH-induced adducts include (+)-*trans*-BPDE-*N*²-dG, the primary stereoisomeric adduct formed by B[a]P, which is clearly removed by NER (reviewed in [44]). Tobacco smoke also contains a class of aromatic amines called 4-aminobiphenyls (4-ABP) which can produce DNA adducts recognized by NER, including *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (dG-C8-ABP) [45].

The NER pathway removes helix-distorting DNA lesions which can cause mutations and drive carcinogenesis. This is clearly illustrated by decades of investigation of the disease Xeroderma Pigmentosum (XP). Patients with XP are deficient in NER and have greatly elevated levels of skin cancer and other forms of cancer [46–48]. In mammals, at least 20 different proteins participate in NER, including the XPA-G factors that are singly defective in the 7 corresponding complementation groups of XP [49–51]. The tumor suppressor factor p53 also impacts NER efficiency probably by transcriptional regulation of the *XPC* and *DDB2* genes [52–55]. NER, sometimes referred to as global genomic NER (GG-NER), can remove damage from anywhere in the genome. A subpathway of NER called transcription-coupled NER (TC-NER) selectively removes damage from the transcribed strands of expressed genes. These two pathways differ in their mechanism of DNA damage recognition. In NER, DNA damage recognition is accomplished by XPC, which is stabilized by its binding partners RAD23B and CENTRIN2 [56] and is assisted by the UV-damaged DNA binding protein DDB2 (the product of the *XPE* gene). In TC-NER, damage is recognized by the stalling of the RNA polymerase complex at the site of damage (reviewed in [57]). After DNA damage recognition, the subsequent steps are the same for NER and TC-NER. The multi-subunit complex TFIIH contains helicase activities that produce additional unwinding of DNA, which produces double-strand/single-strand DNA junctions. After DNA unwinding several NER components are recruited to the site of the lesion, including XPA, which is likely used to verify the presence of the DNA lesion and that the required NER factors are present for the subsequent steps of the pathway. Next, the endonuclease activities of the XPF/ERCC1 complex and XPG produce single-strand incisions flanking the damaged site. The original integrity of the DNA is restored after an approximately 30 nucleotide region of DNA containing the lesion is excised, and the gap is

filled by pol δ or pol ϵ , using the undamaged strand as a template, with DNA ligase III α or DNA ligase I filling in the final nick in the DNA (reviewed in [49]).

The molecular mechanisms by which arsenic may act as a co-carcinogen are under debate. One possibility is that arsenic inhibits the removal of DNA damage produced by carcinogens such as UV light and certain compounds present in tobacco smoke, thus exacerbating their mutagenic effects. NER has been suggested as a candidate pathway since it removes the DNA damage introduced by these agents [34, 58–62]. In the present study, we have examined the impact of arsenic (as the trivalent ion arsenite) on NER using an immuno-blot assay to directly measure DNA lesions specifically removed by NER. NER function was inhibited with increasing concentrations of arsenite in both human fibroblasts and mouse keratinocytes. Additionally, NER protein and RNA levels were measured in both cell types in response to arsenite treatment, and a concentration-dependent decrease in XPC protein and XPC, XPA, and DDB2 RNA levels was observed. Finally, a possible mechanism in which arsenite inhibits XPC protein expression by altering protein turnover was investigated. Our findings support the hypothesis that arsenic can promote carcinogenesis by interfering with the NER-specific repair of DNA damage introduced by other carcinogens, and provides insights into how arsenic may additionally function as an anti-cancer drug, especially in tandem with DNA damage-inducing chemotherapeutics.

2. Materials and Methods

2.1 Cell Culture

The human lung fibroblast cell line, IMR-90, was grown in minimal essential medium (Eagle) containing Earle's salts (Mediatech) supplemented with 0.1 mM non-essential amino acids (Lonza), 2 mM glutamine (Mediatech), 100 units/ml penicillin, 100 μ g/ml streptomycin and 10% fetal bovine serum (Sigma). Cultures of primary mouse keratinocyte cells were isolated as described [63]. Briefly, cells were taken from the epidermis of newborn C57BL/6J mice 1–3 days after birth and grown in keratinocyte growth media composed of an equal volume of low calcium (LoCal) media containing minimum essential medium (Eagle) with Earle's salt, without calcium, 8% chelexed FBS, 2 mM glutamine, 100 units/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL fungizone and 0.06 mM CaCl_2 and fibroblast conditioned LoCal medium. In order to condition the LoCal medium, primary mouse fibroblast cells were grown in it until confluent, and the medium was removed, filter sterilized with a 0.22 micron filter, and stored at -80°C until use. IMR-90 and mouse keratinocyte cells were maintained in a 5% CO_2 incubator at 37°C .

2.2 Treatment of Cells with Arsenite

IMR-90 and mouse keratinocyte cells were grown to confluency and then treated with arsenite for 24 h unless otherwise stated. Control (untreated) cells were treated by adding comparable volumes of water, the solvent used to dissolve sodium arsenite, to the medium. In the repair assays, sodium arsenite was added to the medium for the duration of the repair interval.

2.3 Treatment of Cells with MG-132

MG-132, a potent inhibitor of proteasome mediated proteolysis, was prepared from a 2mM stock solution from EMD Bioscience (catalog # 475791) stored at -20°C . The solution was thawed immediately prior to treatment and diluted for use. To investigate a potential involvement of the proteasome in the turnover of XPC in cells treated with arsenite, confluent IMR-90 cells were treated with a range of concentrations of arsenite for 16 h in the presence or absence of MG-132. After treatment, cells were counted and lysed, and XPC levels were measured by Western Blotting.

2.4 Analysis of Cell Viability

IMR-90 and mouse keratinocyte cells were grown and treated with arsenite as described above. Cells were trypsinized, washed with chilled PBS, centrifuged, and resuspended in 10 mL of chilled PBS. Cells were counted in triplicate for each arsenic exposure using trypan blue dye exclusion.

2.5 Measurement of Nucleotide Excision Repair

The removal of 6-4 PPs and CPDs from total genomic DNA was measured using an immunoblot assay as previously described [64, 65] with some modifications. Cells were grown and treated with a range of arsenite concentrations as described above. They were then washed twice with PBS, irradiated with UV-C light to introduce photolesions (20 J/m^2 UV-C to measure the removal of 6-4 PPs and 2 J/m^2 UV-C to measure the removal of CPDs). A lower dose of UV light was used to measure the removal of CPDs since CPDs are introduced at a much higher frequency than 6-4 PPs. Additionally, the reduced DNA damage burden of a lower UV-dose could permit the cells to remove more of the CPD lesions, and since CPD lesions are a poor substrate for NER in general, this could allow a much more significant removal of the CPD lesions by the cells. After irradiation, cells were either lysed immediately or after incubation in medium containing the same concentration of arsenite for increasing periods of time to permit repair. Whole genomic DNA was isolated using a Promega wizard DNA isolation kit. The concentration of the isolated DNA was measured using a fluorometer and Hoechst-dye. The DNA was then denatured and equal amounts from each sample were applied onto a Hybond nitrocellulose membrane (Biorad) using a slot blot apparatus (100 ng of DNA per slot for the detection of 6-4 PPs and 20 ng of DNA per slot for detection of CPDs). The membrane was baked in a vacuum oven at 80°C for 1 h, treated with 5 % nonfat dry milk (Blotto, Santa Cruz Biotechnology) in TBST (10 mM Tris pH 8.0, 150 mM NaCl and 0.1% Tween-20) for 1 h, and incubated overnight at 4°C with mouse monoclonal antibodies (1:10,000 dilution) specific for either 6-4 PPs or CPDs (CAC-NM-DND-002, CAC-NM-DND-001, Cosmo Bio USA) in 1% dry milk and TBST. The following day, the membrane was washed extensively with TBST and then incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution, Thermo Scientific) in 1% dry milk and TBST for 2 h at room temperature. After washing, chemiluminescence (ECL – Plus, GE Healthcare Bio-Sciences Corp) and fluorimaging were used to detect the photolesions. Image Quant computer software was employed to quantify the signal detected by the phosphorimager. The percent repair at each time point was calculated by dividing the signal strength of the slot-blot band obtained at each repair time

point by the signal strength of the band obtained at the zero time point. For most membranes, a DNA ladder of irradiated DNA was used as an internal control for uniformity of slotting, antibody incubation and development with chemiluminescence. Unirradiated samples of DNA were also loaded to detect any nonspecific binding of the antibodies to DNA which in general was found to be insignificant.

2.6 Western analysis

The effect of arsenic treatment on the abundance of XPC, XPA, and β -actin in IMR-90 cells and mouse keratinocyte cells was determined by Western blotting. The following antibodies were used: for XPC in both cell types, sc-74411 (Santa Cruz Biotechnology), for XPA in IMR-90 cells, sc-28353 (Santa Cruz Biotechnology), and for XPA in mouse keratinocytes cells, ab65963 (Abcam). Both cell types were treated with a range of arsenite concentrations or mock treated as described. After the treatment period, cells were washed with PBS and trypsinized. Approximately five million cells were collected by centrifugation for each treatment, washed with PBS and stored at -20° or -80° . Each sample of frozen cells was thawed on ice, resuspended in 200 μ l RIPA buffer (50 mM Tris (7.4), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.25% Na-Deoxycholate) containing 0.6 mM PMSF, 1% Protease Cocktail Inhibitor (Sigma) and 2 units of DNase 1 (New England BioLabs), lysed by sonication, centrifuged at 13,500 G to remove debris, and the amount of protein in each of the supernatants was quantified using the Bradford method. Samples containing 100 μ g of protein were mixed with loading dye, boiled at 100°C for 5 min, resolved in 8% (XPC/ β -actin) or 12% (XPA/ β -actin) SDS-PAGE gels and resolved proteins were transferred onto a PVDF membrane (Bio-Rad). The membranes were incubated for one hour with 5% nonfat dry milk in TBST for blocking. For XPC/ β -actin, the upper half of the membrane was probed with a 1:1000 dilution of XPC antibody and the lower half with was probed with a 1:100,000 dilution of β -actin antibody overnight at 4°C . The membrane was then washed extensively with TBST, and incubated for two hours with 1:5,000 HRP-conjugated goat anti-mouse antibodies (GE Healthcare Bio-Sciences Corp) at room temperature. After extensive washing with TBST, the binding of primary antibody was detected using enhanced chemiluminescence (ECL – Plus, GE Healthcare Bio-Sciences Corp) and phosphorimaging. For XPA/ β -actin, the full membrane was probed with 1:1,000 dilution of XPA antibody overnight at 4°C . Washing and development for XPA signal were performed in the same manner as was performed for XPC. The membrane was then stripped using the stripping buffer (Pierce) at 37°C for 15 min, washed with TBST, blocked and probed with β -actin antibody as described for XPC/ β -actin. The amount of XPC or XPA in each sample was normalized to β -actin in the same lane.

2.7 Real-time PCR

The effect of arsenite on the expression of *XPC*, *XPA*, *DDB2* and *GAPDH* RNA in IMR-90 cells was measured using Real-Time PCR. Confluent cells were incubated with arsenite or mock treated as described, washed with PBS, trypsinized, centrifuged, washed with PBS and pelleted. Total RNA was isolated from one million cells for each treatment using a GenElute mammalian total RNA Miniprep kit (Sigma). The RNA 6000 Nano Chip kit from Agilent was used to determine the quality and concentration of RNA isolated from the cells. For each treatment, 1.5 μ g of RNA was reverse transcribed to cDNA using random primers and a

high capacity cDNA RT kit (Applied Biosystems). Primer sets specific to each gene were identified using Roche Universal Probe Library (<http://www.universalprobelibrary.com>) and synthesized by Integrated DNA Technologies. Sequences of the primers are given in Table S1. Reactions were carried out on a Roche LightCycler 480 system using a 96-well plate format. Each reaction (20 μ l) contained 1 $\times$ Master Mix, 100 nM fluorescent reporter probe, 200 nM of each forward and reverse primer and 5 μ l of cDNA (diluted 1:10). Samples were first incubated for 10 min at 95°C followed by 40 cycles of amplification (95° C for 15 s denaturation and 60° C for 1 min annealing). An equal volume of 1:10 diluted cDNA from each treatment was mixed together to prepare the standard mix. A standard curve was created using serial dilutions (1:1, 1:10, 1:100, 1:1000, 1:10,000) of mixed cDNA. The expression level of RNA in the treated cells were determined by comparing their CP values with the CP values of the same RNA in the standard curve. CP is the crossing point, the amplification cycle at which the fluorescence of a sample rises above the background fluorescence. The amounts of *XPC*, *XPA*, and *DDB2* RNA were normalized to the amount of *GAPDH* for each treatment.

2.8 Statistical Considerations

All statistical evaluations were done using Graph Pad Prism 6. For the experiments comparing multiple treatments to a control, a 1-way ANOVA with a Holm-Sidak test for multiple-comparison was employed. For repair assays comparing one treatment to a control, a Student's T-test was employed. For all analyses, $p < 0.05$ was used as the threshold for significance. Statistical significance is described in the results section and figure legends and presented on the images.

3. Results

3.1 Arsenite inhibits NER in primary mouse keratinocyte cells

We investigated the impact of treatment with arsenite on NER in primary mouse keratinocytes. Arsenic can be a co-carcinogen in skin cancer development and squamous cell and basal cell carcinoma originate from keratinocytes located in the skin. First, the effect of arsenite on mouse keratinocyte cell viability was evaluated across a range of arsenite exposures (Fig. 1A). Toxicity was moderate across the treatment range, with the highest exposure producing ~50% toxicity. After examining the effects of arsenite on cell viability, the effect of arsenite on NER was studied using an immuno-slot blot assay that uses lesion-specific antibodies to measure the removal of 6-4 PPs or CPDs introduced by UV irradiation. These lesions are removed exclusively by NER in rodent and human cells, although CPDs are a poorer substrate for NER and require a longer period of time to be removed from DNA [66, 67]. In cells exposed to arsenic, they were pretreated with arsenic for 24 h, irradiated with UV and arsenic was added to the medium for the duration of the repair interval. In keratinocytes not treated with arsenite, 6-4 PPs were rapidly removed from DNA, with the majority of lesions removed within 4 h after UV irradiation. In contrast, treatment with arsenite resulted in a concentration-dependent inhibition of the removal of 6-4 PPs compared to untreated cells (Fig. 1B). 20 μ M arsenite significantly inhibited 6-4 PP removal at the 8 and 24 h timepoints, while 40 μ M arsenite significantly inhibited 6-4 PP removal at the 4, 8, and 24 h timepoints. Arsenite treatment also inhibited the removal of

CPDs in mouse keratinocytes (Fig. 1C). The UV-C dose used to measure the removal of CPDs was considerably lower than that used to measure removal of 6-4 PPs (2 J/m^2 compared to 20 J/m^2) since CPDs are introduced at a much higher frequency than 6-4 PPs. $20 \text{ }\mu\text{M}$ arsenite significantly inhibited the removal of CPDs at the 48 h timepoint, and the $40 \text{ }\mu\text{M}$ treatment significantly inhibited the removal of CPDs across all of the timepoints measured.

3.2 Arsenite reduces the abundance of XPC protein, but not XPA, in primary mouse keratinocyte cells

The effect of arsenite on the abundance of XPC and XPA was determined by western blotting using whole cell lysates isolated from mouse keratinocytes treated for 24 h with the indicated concentrations of arsenite (Fig 2A). The amounts of XPC and XPA at each concentration are presented in reference to their amounts in untreated cells, with normalization for the amount of β -actin present in the same lane (Fig. 2B–C). The abundance of XPC was reduced in cells treated with increasing concentration of arsenite (Fig. 2B); XPC levels were reduced by 40% in cells treated with $10 \text{ }\mu\text{M}$ arsenite, the lowest concentration tested. The reduction increased to 59% and 81% for the 20 and $40 \text{ }\mu\text{M}$ arsenite treatments. The amount of reduction compared to untreated cells was statistically significant for each concentration of arsenite administered. In contrast, the abundance of XPA was not significantly affected by arsenite treatment, except at the highest exposure (Fig. 2C).

3.3 Arsenite inhibits NER in IMR-90 cells

We also investigated the effects of arsenite on the NER pathway by studying the human lung fibroblast cell line, IMR-90. First, the effect of arsenite on cell viability was evaluated by treating cells with the indicated concentrations for 24 h (Fig. 3A). Treatment with arsenite produced some moderate toxicity at exposures $15 \text{ }\mu\text{M}$, with $40 \text{ }\mu\text{M}$ producing an approximate 50% reduction in viability. NER was studied as described. Treatment with $20 \text{ }\mu\text{M}$ arsenite resulted in a concentration-dependent inhibition of the removal of 6-4 PPs compared to untreated cells. The removal of 6-4 PP lesions was significantly slowed in all the arsenite treatments, across all timepoints measured (except As-20 at 2, 4 and 6 h) (Fig. 3B). No significant effect on the removal of 6-4 PPs was observed when cells were treated with $10 \text{ }\mu\text{M}$ arsenite for 24 h (data not shown). Second, IMR-90 cells were treated for a longer period of time, 48 h, with a reduced range of arsenic concentrations (0 – $15 \text{ }\mu\text{M}$ arsenite) and cell viability and the removal of 6-4 PPs was measured (Supplemental Fig. 1). When cells were treated with arsenic for 48h, the medium was removed after 24 h of treatment and replaced with fresh medium containing the same concentration of arsenic. Treatment of cells with 10 or $15 \text{ }\mu\text{M}$ arsenite for 48 h produced little toxicity (Supplemental Fig. 1A) but resulted in a significant inhibition of the removal of 6-4 PPs compared to untreated cells at all timepoints except the 3 h timepoint of the $10 \text{ }\mu\text{M}$ arsenite treatment (Supplemental Fig 1B). At these lower doses, the amount of inhibition observed was similar, likely due to technical limitations of the assay to differentiate small differences in NER efficiency. Thus, we observe that arsenic inhibits the removal of 6-4 PPs after treatment with higher concentrations for 24 h and after treatment with lower concentrations for 48 h. The impact of arsenite on the removal of CPDs was also investigated. When cells were treated with 20 or $40 \text{ }\mu\text{M}$ arsenite for 24 h and irradiated with 2 J/m^2 UV light, the removal of CPDs

was inhibited in a statistically significant fashion at multiple timepoints (12 and 48 h timepoints for 20 μ M arsenite, all timepoints for 40 μ M arsenite) (Fig. 3C).

3.4 Arsenite reduces the abundance of XPC, but not XPA, protein in IMR-90 cells

The effect of arsenite on the abundance of XPC and XPA was determined by western blotting using whole cell lysates isolated from IMR-90 cells that were treated for 24 h with different concentrations of arsenite. Whole cell lysates isolated from untreated cells were used as controls. The amounts of XPC and XPA were normalized to the amount of β -actin in the same lane. The abundance of XPC was reduced in IMR-90 cells treated with increasing concentrations of arsenite (Fig. 4A); XPC levels were reduced by 40% in cells treated with 10 μ M arsenite, the lowest exposure tested. Inhibition increased to 70 and 85% for the 20 and 40 μ M arsenite treatments. The reduction in the abundance of XPC in all three treatments compared to untreated cells was statistically significant (Fig 4B). XPA levels were also examined after arsenite treatment (Fig 4C). In contrast to XPC, only the highest concentration of arsenite produced a modest but significant 25% reduction in the abundance of XPA (Fig 4D).

3.5 Arsenite reduces XPC, XPA, and DDB2 RNA levels in IMR-90 cells

The effect of arsenite treatment on the abundance of *XPC*, *XPA*, and *DDB2* RNA in IMR-90 cells was measured by real-time PCR, using the same treatment conditions used to evaluate its effect on protein levels. Arsenite significantly reduced RNA expression of all three genes in a concentration-dependent fashion (Fig. 5). Changes in the abundance of *XPC* and *XPA* RNA were observed at 20 and 40 μ M arsenite, whereas *DDB2* expression was severely reduced even at the lowest exposure, 10 μ M arsenite.

3.6 The reduction of XPC protein in IMR-90 cells by arsenite treatment is partially mediated through the proteasome

After observing that treatment of IMR-90 cells with arsenite results in a significant reduction in the abundance of XPC protein, we investigated the potential influence of arsenite treatment on ubiquitin-mediated degradation of XPC. As XPC is modified by ubiquitin [68–70], we hypothesized that arsenite may inhibit XPC abundance in a manner that is regulated by the proteasome. To test this hypothesis, we treated IMR-90 cells with arsenite in the presence or absence of the proteasomal inhibitor MG-132 for 16 h and measured XPC by Western blotting (Fig. 6A). In cells treated with arsenic alone, we observed a reduction in XPC expression similar to the levels seen in Fig. 4A. When cells were co-exposed to arsenic and MG-132, the reduction in XPC expression was eliminated, and expression levels were maintained at a level similar to untreated cells (Fig. 6B). These findings suggest that proteasomal degradation of XPC is at least partially responsible for the observed reduction in XPC protein levels upon treatment with arsenite.

4. Discussion

In this study, we use an immuno slot-blot method to directly measure the removal of UV photoproducts and we find that arsenic, in the trivalent form of sodium arsenite, inhibits the efficiency of NER. Treatment with arsenite significantly inhibits the removal of 6-4 PPs and

CPDs in two different cell types; human IMR-90 lung fibroblasts and primary mouse keratinocytes. In addition, we find that treatment with arsenite reduces the abundance of XPC protein and *XPC*, *XPA* and *DDB2* RNA. Others have investigated the effects of arsenic on the efficiency of NER using a variety of different assays. Arsenite has been found to interfere with the production of repair-related DNA strand breaks in general and alters DNA incision and ligation frequencies in human fibroblasts exposed to UV light, more indirect measurements of NER [59–62]. An early observation of an inhibitory effect of arsenite on the efficiency of NER was made studying the removal of thymine dimers using chromatography in a Chinese hamster ovary (CHO) cell line measured 24 h after UV irradiation [26]. More recently, arsenite treatment of human lymphoblastoid TK6 cells was found to slow the removal of UV-induced DNA damage as measured by the Comet assay 2 h after UV irradiation [27] and arsenite treatment of a mouse keratinocyte cell line was found to delay the removal of 6-4 PPs introduced by solar simulated UV radiation [25]. Therefore, our findings extend and add to the understanding of how arsenic alters NER and possibly contributes to arsenic-mediated carcinogenesis.

We observed some increase in cellular toxicity when cells were treated with different concentrations of arsenic. While the inhibition of NER can sensitize cells to cell death and apoptosis [71–73], it is unclear how apoptosis or cell death may impact the efficiency of NER. There was a 10 fold difference in the doses of UV light used in our experiments to measure the removal of 6-4 PP and CPD lesions (20 J/m² for 6-4 PPs and 2 J/m² for CPDs). However, we found that the inhibition of NER found after treatment with arsenic was significant regardless of whether the cells were treated with a lower or higher dose of UV light; this suggests that the inhibition of NER in response to treatment with arsenic was not solely brought about changes in toxicity. In addition, when we treated cells with lower concentrations of arsenic for a longer period of time, 48 h instead of 24 h, toxicity was markedly reduced, but we still observed a significant inhibition in removal of 6-4 PPs.

Our observations of the impact of arsenic on cellular expression of NER proteins and RNA levels also extend previous studies. Treatment of human skin fibroblasts with arsenic reduced the expression of XPC and DDB2 at both the protein and RNA levels, but the efficiency of NER was not measured [58]. We observe that treatment with arsenic significantly reduces XPC protein in human IMR-90 lung fibroblasts and primary mouse keratinocytes but we were unable to measure DDB2 protein since we were unsuccessful in identifying an antibody that yielded results specific to the protein. We also observe a reduction in *XPC*, *XPA*, and *DDB2* RNA levels in IMR-90 cells treated with arsenic. Our observation of a reduction in *XPA* RNA but not XPA protein after treatment with some concentrations of arsenic might be due to a relatively high abundance of XPA protein or low turnover of the protein. Interestingly, arsenic exposure has also been linked to a reduction in RNA levels of several NER genes in a human population, including *ERCC1*, *XPF*, and *XPB*, but not *XPA* (*XPC* and *DDB2* RNA levels were not measured) [74].

Several studies have proposed mechanisms by which arsenic can inhibit NER function. One study observed that arsenic reduces the expression of XPC (and DDB2) protein and RNA in human skin fibroblasts, and these reductions also correlated with a reduction in the recruitment of XPC to sites of DNA damage [58]. The mechanism by which arsenic reduced

XPC expression or whether treatment with arsenic impacted the efficiency of the removal of DNA damage was not explored, in contrast to our study. Additionally, arsenic inhibits the removal of BPDE adducts in A549 human lung cells and it can displace zinc from a peptide representing the zinc finger domain of XPA at high exposures (as judged by 19% zinc release when the peptide was treated with 100 μ M arsenite) [33]. Since the zinc finger domain has been shown to be important for the function of XPA in NER [75], arsenic may disrupt NER by altering the structure of XPA, but additional studies are needed to explore these findings. In addition, methylated metabolites of arsenite were found to be considerably more efficient than the parental arsenical in displacing zinc from XPA, and inhibited the removal of BPDE lesions at lower concentrations [33].

We observe that treatment with arsenic inhibits NER function and reduces XPC expression. XPC is ubiquitinated in response to UV-induced DNA damage [68–70]. There are multiple ubiquitin modifications to XPC following the introduction of UV-induced DNA damage. These include a lysine-48-linked polyubiquitin linkage that appears to promote degradation or recycling of XPC [76] and a lysine-63-linked ubiquitination of XPC that can be critical for the removal of XPC from the damaged site to allow downstream NER factors access to the DNA damage [77]. The deubiquitinating enzyme USP-7 is responsible for removing a UV-dependent ubiquitin linkage to XPC, and this promotes XPC stability [78]. We tested whether the reduction of XPC protein expression that we observe after treatment of IMR-90 cells with arsenic is related to proteasomal turnover of XPC. We observe that the proteasomal inhibitor MG-132 abrogated the effects of arsenite in reducing XPC protein levels. This indicates that the reduction in the abundance of XPC produced by exposure to arsenic is, at least in part, a consequence of enhanced proteasome-dependent turnover of the protein that is mediated by ubiquitination. Additional studies are needed to determine if arsenic-mediated turnover of XPC functions by promoting ubiquitination or inhibiting deubiquitination and to characterize the sites of ubiquitination. Furthermore, arsenic's effects on the proteasome may not be limited to altering the expression of XPC and may have an influence on the stability of other proteins and pathways.

Given our observation that XPC RNA is also reduced by arsenite treatment, arsenic-mediated reduction in XPC expression may not be solely a result of increased proteasomal turnover of XPC. However, inhibiting proteasomal activity with MG-132 fully restored XPC expression in the presence of arsenic, indicating that proteasomal turnover of XPC is responsible for the majority of the observed reduction of XPC protein abundance. Had the reduction in XPC levels by arsenic treatment been primarily a consequence of reduced transcript levels, MG-132 would not have been able to restore XPC expression to levels seen in untreated cells. Additionally, treatment with MG-132 alone did not result in an increase in XPC abundance. This suggests that the steady state turnover of XPC is fairly low in undamaged cells, and this observation is consistent with results of a previous study where XPC levels were unchanged for up to nine hours when XPC turnover was inhibited [79]. Consequently, the increase in XPC protein in cells treated with MG-132 and arsenic relative to cells treated with arsenic alone is not a result of MG-132 increasing XPC above basal levels, but rather a function of inhibiting the turnover of XPC mediated by arsenic. Nevertheless, the relative contributions of the reduction in XPC RNA and the increase in XPC turnover to the overall arsenite-dependent reduction in XPC needs further study.

We studied the effects of arsenic on the NER pathway in light of previous associations made between exposure to arsenic and elevated frequencies of lung and skin cancer in humans [8, 11] and elevated frequencies of skin cancer in UV-exposed mice [41, 42]. The primary risk factor for lung cancer is exposure to tobacco smoke and the primary risk factor for skin cancer is exposure to solar UV irradiation. Tobacco smoke introduces many types of DNA damage, including the highly studied PAHs such as B[a]P. BPDE, the ultimate metabolite of B[a]P, produces large bulky adducts that are recognized and removed by the NER pathway [44, 80, 81]. BPDE adducts produce a characteristic mutational signature, and this signature is found more frequently in lung cancers of smokers than non-smokers [82–84]. Sun exposure introduces DNA lesions which are also recognized by NER [43, 49]. In non-melanoma skin cancer, mutational signatures characteristic of exposure to UV light are observed in the p53 gene and are present at mutational hotspots [85], indicating that these NER substrates can be causative in the production of non-melanoma skin cancer. Melanocytes are more resistant to UV induced cytotoxicity than are keratinocytes, the epidermal skin cells responsible for most non-melanoma skin cancers. This difference may explain the observation that arsenic increases the cancer incidence of non-melanoma skin cancers [86] while the relationship between arsenic and melanoma development is less clearly established [87, 88]. Since deficiencies in the removal of BPDE adducts or UV-induced photolesions by NER result in increased mutation levels, the inhibition of NER by arsenite could explain how arsenic can act as a co-carcinogen in smoking-related lung and UV-related skin cancers.

In general, humans are exposed to arsenic for a substantial period of time, such as when they are exposed to contaminated drinking water (which can occur for years), or when they are treated with arsenic-based chemotherapies (which can occur for several weeks [21]). In contrast, treatment of cells in culture usually involves a single, much shorter exposure to arsenic which often necessitates the use of higher than physiological concentrations to observe measurable effects. Chronic treatment of cells with low concentrations of arsenite can induce cellular transformation [89–92], global DNA hypomethylation [90–93], local DNA hypermethylation [93, 94], alterations in chromatin structure and splicing patterns [89], and increases in oncogenic gene expression [91, 95]. Recent research has indicated that chronic arsenic exposure alters methylation patterns of DNA repair factors involved in NER, leading to reduced RNA expression of ERCC1 and ERCC2 [96]. These epigenetic changes likely contribute to arsenic-mediated carcinogenesis, but chronic treatment of cells in culture with lower, physiological concentrations would likely complicate investigations and interpretations of direct effects on DNA repair. Additional studies in human populations are important to determine if chronic exposure to lower concentrations of arsenic through contaminated drinking water or arsenic-based chemotherapy inhibits the efficiency of NER in a human populations.

Several previous studies, some described above, and our study have investigated the effects of arsenic on NER using single treatments of at least 10 μ M arsenic [26, 33, 34, 58]. These concentrations are higher than what humans would usually encounter during long term chronic exposure to contaminated drinking water. However, they are within the range of concentrations used to sensitize chemo-resistant cells to killing by chemotherapeutic agents such as doxorubicin [97], paclitaxel [98], erlotinib [99], and cisplatin [100]. Of particular

interest is the finding that arsenic treatment sensitizes chemo-resistant cancer cells to killing by cisplatin. Cisplatin generates DNA adducts that are substrates for NER. Hence, co-treatment with arsenic may sensitize cells to cisplatin by inhibiting the NER pathway [100]. The same study also found that treatment with arsenic reduced expression of XPC, and that disruption of the *XPC* gene in the absence of arsenic treatment sensitized the chemo-resistant cancer cells to killing by cisplatin. These studies and our study suggest that disruption of the NER pathway by treatment with arsenic should be explored to enhance the efficacy of chemotherapeutic agents used to treat cancer.

While several studies have indicated that arsenic increases the mutation frequency of other carcinogens such as UV-light [27–30] and components of tobacco smoke [35, 37], relatively few studies have investigated the impact arsenic plays on the types of mutations found in co-exposed systems. While not a measurement of mutagenesis, co-exposure of arsenic and benzo[a]pyrene increased the frequency of adducts produced by benzo[a]pyrene in mice [38]. In cultured CHO cells, co-treatment with arsenic and UV-light increases mutation frequency by a factor of two relative to cells exposed to UV-light alone, and the mutational spectrum in the co-treated cells retained a UV-signature [101]. Very few studies have examined the spectra of mutations produced in human skin cancers related to human exposure to arsenic. In one single study of an arsenic-exposed population of skin cancer patients, the mutations found in p53 were at different codons than the normal hotspots seen in the p53 gene of UV-induced skin cancers [102]. However, all the tumor samples in this single study were obtained from areas of the body not generally exposed to sunlight, and hence it is unlikely that UV light played any major role in the etiology of these tumors. Consequently, it is clear that additional studies are important to investigate the mutational spectra found in skin tumors derived from sun exposed areas of the body in individuals exposed to arsenic. These studies should aid in understanding whether the alterations to the efficiency of NER that we have observed in our study also occur in humans exposed to arsenic, and if so, whether they play a role in increasing mutational burden and the risk of developing cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

6-4 PP	6-4 Photoproduct
B[a]P	Benzo(a)pyrene

BPDE	Benzo(a)pyrene diol epoxide
CPD	Cyclobutane pyrimidine dimer
DDB2	Damaged DNA Binding Protein
NER	Nucleotide Excision Repair
PAH	polycyclic aromatic hydrocarbon
XP	Xeroderma Pigmentosum

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Figure 1.

Figure 2.

A

B

C

Figure 3.

Figure 4.

Figure 5.

Figure 6.