Published in final edited form as:

Toxicol Lett. 2014 January 30; 224(3): 407–415. doi:10.1016/j.toxlet.2013.11.010.

Melanocytes and keratinocytes have distinct and shared responses to ultraviolet radiation and arsenic

KL Cooper^a, JW Yager^b, and LG Hudson^{a,c}

^aDept. of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico, Albuquerque, NM

^bDivision of Epidemiology, Biostatistics and Preventive Medicine, University of New Mexico, Albuquerque, NM

Abstract

The rise of melanoma incidence in the United States is a growing public health concern. A limited number of epidemiology studies suggest an association between arsenic levels and melanoma risk. Arsenic acts as a co-carcinogen with ultraviolet radiation (UVR) for the development of squamous cell carcinoma and proposed mechanisms include generation of oxidative stress by arsenic and UVR and inhibition of UVR-induced DNA repair by arsenic. In this study, we investigate similarities and differences in response to arsenic and UVR in keratinocytes and melanocytes. Normal melanocytes are markedly more resistant to UVR-induced cytotoxicity than normal keratinocytes, but both cell types are equally sensitive to arsenite. Melanocytes were more resistant to arsenite and UVR stimulation of superoxide production than keratinocytes, but the concentration of arsenite necessary to inhibit the activity of the DNA repair protein poly(ADP-ribose)polymerase and enhance retention of UVR-induced DNA damage was essentially equivalent in both cell types. These findings suggest that although melanocytes are less sensitive than keratinocytes to initial UVR-mediated DNA damage, both of these important target cells in the skin share a mechanism related to arsenic inhibition of DNA repair. These findings suggest that concurrent chronic arsenic exposure could promote retention of unrepaired DNA damage in melanocytes and act as a co-carcinogen in melanoma.

Keywords

melanocytes; keratinocytes; arsenic; UVR; ROS; DNA damage; co-carcinogenesis

1. Introduction

The skin is a major target organ for arsenic toxicity and chronic exposure has been linked to increased incidence of keratinocytic tumors, basal cell carcinoma (BCC¹) and squamous cell carcinoma (SCC) (Chervona et al., 2012; Morton and Dunnette, 1994; Platanias, 2009;

© 2013 Elsevier Ireland Ltd. All rights reserved.

^cCorresponding author: MSC09 5360, 1 University of New Mexico, Albuquerque, NM 87131; 1-505-272-2482; lhudson@salud.unm.edu.

Financial statement: Authors have no conflicts of interest to declare.

Conflict of Interest Statement: The authors declare that there are no conflicts of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Shannon and Strayer, 1989; Tokar et al., 2010). There is less evidence for potential contributions of arsenic exposure to the development of melanoma. However, many populations associated with areas of high endemic arsenic, such as Taiwan and Bangladesh, are highly resistant to ultraviolet radiation (UVR)-induced melanoma. Thus, the many epidemiological studies focused on these global sites would be unlikely to detect increases in the rate of malignant melanoma, leaving the question of arsenic involvement in melanoma risk unresolved.

There is emerging evidence that environmental or occupational exposures to arsenic may contribute to malignant melanoma risk. A small increase in melanoma risk was detected in socioeconomically disadvantaged areas of Australia and associated with soil arsenic exposure (Pearce et al., 2012). A suggested, but not statistically significant, association between melanoma and exposure to arsenic-containing pesticides in the U.S. was also noted (Dennis et al., 2010). These studies corroborate a previous report of a significant positive association between toenail arsenic levels and melanoma risk in a predominantly Caucasian Iowa population (Beane Freeman et al., 2004).

Exposure to UVR is well recognized as the major etiologic factor for development of nonmelanoma skin cancer and melanoma (IARC, 1992; Pfeifer and Besaratinia, 2012). Epidemiologic data indicate an association between sun exposure and nonmelanoma skin cancer in arsenic exposed populations (Chen et al., 2006; Chen et al., 2003; Melkonian et al., 2011; Watanabe et al., 2001) and there is strong *in vivo* evidence that arsenic acts as a co-carcinogen with UVR for development of SCC in mice (Rossman and Klein, 2011; Rossman et al., 2004). Nude mice chronically exposed to arsenite in drinking water develop significantly more skin tumors following UVR exposure than mice exposed to arsenite or UVR alone (Rossman et al., 2004). Potential mechanisms to account for these observations include generation of oxidative stress by arsenic and UVR (Cooper et al., 2009; Rossman and Klein, 2011; Wiencke et al., 1997; Yager and Wiencke, 1997) and inhibition of UVR-induced DNA repair (Beyersmann and Hartwig, 2008; Cooper et al., 2013; Ding et al., 2008; Ebert et al., 2011; Piatek et al., 2008; Zhou et al., 2011). Poly(ADP-ribose)polymerase (PARP)-1 is a recognized direct molecular target for arsenic and a key protein in the base excision repair arm of DNA repair which is responsible for resolution of oxidative lesions and strand breaks (Beyersmann and Hartwig, 2008; Cooper et al., 2013; Zhou et al., 2011). Oxidative DNA damage was greatly increased in the skin and tumors of the mice exposed to both arsenite and UVR, suggesting that both proposed mechanisms may be involved in the enhanced carcinogenesis (Rossman and Klein, 2011).

Melanin is an important regulator of the balance of reactive oxygen species in melanocytes that could alter response of melanocytes to arsenic and UVR when compared to keratinocytes (Cunha et al., 2012; Jenkins and Grossman, 2013; Suzukawa et al., 2012). In this study, we investigate similarities and differences between purported mechanisms underlying arsenic and UVR-induced DNA damage in these two important target cells within the skin. We find that normal melanocytes are markedly more resistant to UVR-induced cytotoxicity than normal keratinocytes, whereas cell viability following arsenite exposure is similar in the two cell types. Melanocytes are also more resistant to arsenite and UVR stimulation of superoxide with greater exposure levels required to generate responses comparable to keratinocytes. In contrast, the arsenite concentration dependence for zinc loss from PARP-1 and inhibition of PARP-1 enzyme activity was essentially equivalent in both cell types. These findings suggest that although melanocytes are less sensitive to initial UVR-mediated genotoxic insult, if UVR exposure is sufficient to generate DNA damage, melanocytes and keratinocytes are equally sensitive to arsenite inhibition of DNA repair mediated by PARP-1. The interaction between UVR-induced DNA damage and inhibition

of DNA repair by arsenic could account, in part, for the epidemiologic findings suggesting increased risk of melanoma upon exposure to arsenic in non-Hispanic whites.

2. Materials and methods

2.1. Cell culture and treatment

Normal human neonatal epidermal keratinocytes (HEKn), normal human neonatal epidermal melanocytes (HEMn) and DermaLife culture medium with supplements were purchased from Lifeline Cell Technologies (Oceanside, CA). This medium contains no serum or phenol red indicator and is clear with little UV absorptive properties. Cells were cultured at 37°C in 95% air/5% CO₂-humidified incubators. All experiments were performed on cells at passage 9 or less. 10 mM stock solution of sodium arsenite (99%; Fluka Chemie, Buchs, Germany) was prepared in milliQ water and sterilized using a 0.22-μm syringe filter. Working solutions were prepared by diluting the stock with complete cell growth medium. Cells were rinsed and placed in complete medium containing arsenite, then exposed to solar simulated (ss)UVR at doses and times indicated in the figure legends. Cell viability for both cell lines and all treatment conditions and exposure times was performed using the CellTiter 96 Non-radioactive cell proliferation assay kit following the manufacturer's instructions (Promega, Madison, WI).

2.2. UV source

UVR exposures were performed using an Oriel 1000 W Watt Solar Ultraviolet Simulator (Oriel Corp., Stratford, CT). This solar simulator produces a high intensity UVR beam in both the UVA (320-400 nm) and UVB (280-320 nm) spectrum with an emission ratio of 14:1 (UVA: UVB). The proportion and intensity of UVA/UVB was measured using a radiospectrometer (Optronics laboratories, Inc.; Orlando, FL) and exposure times were calculated to give the desired doses. Measurements were made with Erythema UV and UVA intensity meter (Solar Light Co., Inc., Philadelphia, PA) in order to verify daily lamp output consistency and to estimate minimum erythema dose (MED). The dose of 3 kJ/m² used for the keratinocytes is approximately 1 MED and the highest dose used for melanocytes (10 kJ/m²) is about 1.5 MED. These values are supported by measurements reported by Ciren and Li showing that the average daily human exposures are 3-5 kJ/m² for 1 MED exposure at mid-latitudes (Ciren and Li, 2003).

2.3. Reactive oxygen species (ROS) detection

Cells were cultured in 96 well plates in complete medium. When cells reached approximately 40% of confluent density, cultures were placed in fresh media and treated with arsenite (1 or 5 μM), UVR (3 or 5 kJ/m²) or both for the times indicated in the figure legends. Thirty minutes prior to collection, dihydroethidium (DHE, 5 μM) was added as a fluorescent indicator of ROS generated in response to the described treatment. Cells were rinsed with PBS and relative fluorescence intensity was quantified by measuring the intensity of fluorescence emission using a Wallac Victor 2 fluorescence spectrophotometer equipped with 390 nm excitation and 410 nm emission filters. A minimum of 3 independent samples were analyzed per treatment and time point. Values were normalized to total DNA fluorescence as previously described (Rago et al., 1990). Briefly, plates previously analyzed for ROS were rinsed with Krebs Ringer buffer (20 mM HEPES, 10 mM dextrose, 127 mM NaCl, 5.5 mM KCl, 1 mM CaCl₂, 2 mM MgSO₄, pH 7.4) then frozen at -80°C overnight. Plates were thawed for at least 2 hrs at room temperature, stained with Hoechst dye (10 μg/ml bis Benzimide) overnight and fluorescence determined using a Wallac Victor 2 fluorescence spectrophotometer equipped with 350nm excitation and 460nm emission filters. This method of fluorescence quantification was validated by comparison with data

obtained using Metamorph software (version 6.3r6) as previously described (Cooper et al., 2007).

2.4. NADPH oxidase activity

Cells were cultured in 12-well plates and treated as described for ROS experiments. NADPH oxidase activity was detected using the Lucigenin Illumination method. Briefly, following treatment and incubation time cells were rinsed thoroughly with PBS, removed by scraping, resuspended in 500 μ l PBS and placed in microfuge tubes. Cells were frozen at -80°C overnight to lyse cells. Samples were aliquoted (100 μ l) in quadruplicate, placed in luminometer tubes and incubated with diethyldithiocarbamate (DTC, 1 M) for 20 min at 37°C to inhibit superoxide dismutase activity. Immediately before measuring, 1 μ l lucigenin (0.5 mM) was added to the sample followed by 1 μ l NADPH (10mM) and mixed. Luminescence was measured in a TD20/20 Luminometer (Turner Designs, Sunnyvale, CA) with ten 30 sec. counts. Sample values were integrated and average units calculated. Parallel samples were analyzed with the addition of Tiron (1 M) to scavenge superoxide and confirm measurement of NADPH oxidase activity.

2.5. Assessment of PARP-1 activity

Cells were treated with arsenite for 24 hrs, then exposed to ssUVR (3 kJ/m²) and incubated for an additional 1 hr. Whole cell extracts were assayed for PARP activity using the HT Colorimetric PARP/Apoptosis Assay kit (Trevigen, Inc., Gaithersburg, MD) according to manufacturer's instructions. Briefly, PARP standards and total protein (100 ng) were placed in duplicate in rehydrated, histone coated wells of a 96 well plate and incubated for 30 min. Samples were incubated with the substrate cocktail containing activated DNA and NAD for an additional 30 min., washed and PAR antibody added. HRP conjugated secondary antibody followed by TACS-Sapphire reagent was subsequently added and the reaction measured using a SpectraMax M2 plate reader (Molecular Devices, Sunnydale, CA) at 450 nm. Sample absorbance was compared to the standard curve generated and activity reported as mUnits per mg protein.

2.6. Isolation of zinc finger proteins and zinc content measurement

PARP-1 was isolated from treated cells by immunoprecipitation. Cells were cultured as described above and treated as described in the figure legend (Fig. 5). Cells were no more than 75 % confluent at the time of collection. Immunoprecipitation and determination of zinc content was performed as previously described (Cooper et al., 2013; Zhou et al., 2011). Briefly, total protein was collected and PARP-1 isolated via immunoprecipitation (500 μ g in 500 μ l) with 5 μ l of rabbit polyclonal antibody (PARP-1; Cell Signaling #9542). Protein samples and zinc standards (100 μ l) were transferred to 96 well plates and zinc content measured by adding 10 μ l of 1 mM 4,(2-pyridylazo)-resorcinol and determining absorbance at 493 nm was determined by a SpectraMax M2 plate reader (Molecular Devices; Sunnyvale, CA) with comparison to the standard curve. The relative zinc content was normalized to protein concentration.

2.7. Immunocytochemistry for DNA damage assessment

DNA damage was assessed by indirect immunofluorescence using anti-8-OHdG (N45.1, Abcam, Cambridge, MA), anti-phospho-H2Ax (Cell Signaling Technologies cat # 2577, Danvers, MA), and anti-cyclobutane pyrimidine dimers (CPDs, Thymine clone KTM53, Kamiya Laboratories, Seattle, WA) antibodies in combination with either anti-mouse or anti-rabbit FITC conjugated secondary antibodies (Abcam, Cambridge, MA) diluted in blocking buffer (PBS containing 5% horse serum and 0.05% triton X-100). Cells were mounted with VectaShield +DAPI (Vector Laboratories, Burlingame, CA) and visualized

with an Olympus IX70 fluorescence microscope equipped with a DP72 digital camera and imaging software. A minimum of 10 images per slide were obtained per treatment and average fluorescence intensity of nuclei from at least 3 separate experiments was quantified using Image J image analysis software (<http://rsb.info.nih.gov/ij/index.html>).

2.8. Statistical analyses

Data from a minimum of 3 separate experiments were pooled and statistical significance assessed by one-way ANOVA with Tukey's multiple comparison tests conducted using Graphpad Prism 5.03 (San Diego, CA). Results are reported as mean $\pm$ SEM of all experiments; $n = 3$.

3. Results

3.1. Cell viability

Cell types differ in sensitivity to arsenite and UVR- induced cytotoxicity. To compare responses in normal human keratinocytes and normal human melanocytes, cell viability was measured following arsenite, UVR and dual exposure. Cells were treated with increasing concentrations of arsenite (0 – 30 μ M) or ssUVR (0 – 30 kJ/m²) and incubated up to 24 hrs. At 24 hrs post-exposure, arsenite treatment produced similar levels of cytotoxicity in both cell types (Fig. 1A) with LD50 of 9.5 μ M for keratinocytes and 8 μ M for melanocytes. In contrast, melanocytes were markedly more resistant to ssUVR than keratinocytes with greater than 60% viability at exposures up to 30 kJ/m² ssUVR (Fig. 1B) with LD50's of 4.5 kJ/m² for keratinocytes and >30 kJ/m² for melanocytes. Viability was also assessed over 24 hrs for combined exposures and demonstrated no additional loss of viability in the combined exposure versus As alone (melanocytes, Fig 1C) or UVR alone (keratinocytes, Fig 1D).

3.2. ROS generation and NADPH Oxidase activity

UVR exposure causes oxidative DNA lesions due to the production of reactive oxygen species (ROS) (Ahmed et al., 1999). UVA is primarily associated with elevated levels of ROS, but both UVA and UVB contribute to ROS production in keratinocytes (Nishigori et al., 2004). Arsenite exposure also stimulates ROS production leading to increased cellular oxidative stress (Shi et al., 2004a) and oxidative DNA damage (Ding et al., 2005; Huang et al., 2004; Rossman et al., 2004). Because ROS production is a shared consequence of UVR and arsenic exposure, we investigated whether responses were similar in normal keratinocytes and melanocytes. In keratinocytes, 1 μ M arsenite induced sustained production of ROS with peak production at 15 min. and little increase in response when arsenite concentration was increased to 5 μ M (Fig. 2A). Melanocytes showed similar magnitude and kinetics of ROS production following treatment with 5 μ M arsenite, but only transient ROS production was detected at 1 μ M arsenite concentration (Fig. 2B). Cell-type differences were also noted with ssUVR exposure. In keratinocytes, ssUVR induced a transient spike of ROS production 15 min. post exposure (Fig. 2C) with no increase evident with UVR dose elevation. Exposure of melanocytes to ssUVR exposure resulted in a rapid and sustained response that was further enhanced with increased ssUVR dose (Fig. 2D). In contrast to previously published observations in keratinocytes where combined arsenite and UV exposures produced synergistic levels of ROS (Cooper et al., 2009), co-exposure in melanocytes had minimal impact on ROS production compared to ssUVR alone (Fig. 2E).

ROS induction was rapid in both cell types, but duration differed so we determined whether changes in ROS corresponded to NADPH oxidase activity. Arsenite rapidly induced NADPH oxidase activity in both keratinocytes and melanocytes. The arsenite response in keratinocytes was significantly greater than melanocytes, and NADPH oxidase activity remained elevated up to 6 hrs after exposure (not shown) whereas the response was transient

in melanocytes (Fig. 3A). ssUVR exposure transiently induced NADPH oxidase activity in keratinocytes and these findings are comparable to those reported for transformed human keratinocytes (HaCaT cells) (Cooper et al., 2009). We reported previously that while activation of NADPH oxidase was a major contributor to arsenic-induced ROS in HaCat cells, NADPH oxidase was only partially involved in ROS induction by UVR (Cooper et al., 2009). In contrast, a higher ssUVR exposure was required to stimulate NADPH oxidase activity in normal human melanocytes (Fig. 3B) and the response persisted compared to keratinocytes. The lack of synergistic increases in ROS by ssUVR and arsenite in melanocytes suggests that an alternate mechanism is likely to be required for the co-carcinogenic actions of arsenic.

3.3. Inhibition of PARP activity by arsenite

Another mechanism implicated in arsenic co-carcinogenesis is inhibition of DNA repair proteins. PARP-1 is protein involved in repair of strand breaks and oxidative DNA damage, and more recently has been shown to participate in repair of direct DNA damage such as CPDs (Ding et al., 2009; King et al., 2012; Qin et al., 2008; Wnek et al., 2011; Zhao et al., 2012; Zhou et al., 2011). Importantly, PARP-1 is a very sensitive target for arsenic with inhibition evident at submicromolar arsenic concentrations in some cell types, but there is no information on arsenite actions on PARP activity in melanocytes (Beyersmann and Hartwig, 2008; Cooper et al., 2013; Ding et al., 2005; Qin et al., 2008; Shi et al., 2004b). Melanocytes and keratinocytes were treated with increasing concentrations of arsenite for 24 hrs, DNA damage was initiated by exposure to ssUVR (10 and 3 kJ/m², respectively), and then PARP-1 activity was measured. PARP-1 activity is stimulated by DNA damage in both cell types. There was little impact of arsenite exposure on basal (no DNA damage) PARP activity levels (data not shown). However, arsenite treatment caused a dose-dependent decrease in DNA damage-induced PARP activity in both melanocytes and keratinocytes with melanocytes displaying greater magnitude of inhibition at the lowest arsenite concentrations (Fig. 4).

PARP-1 binding to damaged DNA requires two zinc finger motifs. We demonstrated previously that arsenite treatment causes loss of zinc from PARP-1 protein isolated from arsenic treated cells with consequent loss of enzyme activity (Zhou et al., 2011). Endogenous PARP-1 was immunoprecipitated from keratinocytes and melanocytes treated with varying concentrations of arsenite for 24 hrs and zinc content was measured in the isolated PARP-1 protein. As a positive control for zinc release, cells were treated with the zinc chelator, TPEN. Zinc content of PARP-1 was decreased in an arsenite dose-dependent manner from both melanocytes and keratinocytes (Fig. 5). The findings in Figs. 4 and 5 demonstrate that melanocytes and keratinocytes are similar with regard to arsenite disruption of PARP-1 zinc binding and enzyme activity.

3.4. Retention of ssUVR DNA damage

Low concentrations of arsenite inhibit DNA repair leading to retention of UVR-induced DNA damage in keratinocytes and other cell types (Beyersmann and Hartwig, 2008; Cooper et al., 2013; Ding et al., 2008; Shi et al., 2004a), but responses of melanocytes are unknown. Consistent with findings in Figs. 1 and 2, a greater dose of ssUVR was required to produce equivalent DNA damage in melanocytes (10 kJ/m²) when compared to keratinocytes (3 kJ/m²) (Fig. 6A). This was true for three types of DNA damage, 8-hydroxy-3-deoxyguanosine (8-OHdG; oxidative damage), cyclobutane pyrimidine dimers (CPDs; direct damage) and phospho-H2AX (pH2AX; strand breaks). Melanocytes treated with arsenite alone did not exhibit significant increases in any of the DNA damage markers investigated (data not shown). Modest, but not statistically significant, differences in DNA damage were observed 1 hr post ssUVR exposure in the presence of arsenite. Inhibition of DNA repair causes

retention of lesions, and cell treatment with 1 μ M arsenite significantly elevated ssUVR-induced DNA damage 6 hr post ssUVR for all three types of DNA damage (Fig. 6 B and C). Melanocytes treated with arsenite showed retention of DNA damage at 6h post ssUVR with 8-OHdG 23 fold, pH2AX 2.6 fold and CPDs 2.5 fold greater than cells exposed to ssUVR alone. The degree of DNA damage retained at 6 hr post ssUVR was comparable to that seen for oxidative lesions (8-OHdG and pH2AX, 16 and 2.6 fold respectively) and direct DNA damage (CPDs) 5.6 fold over UV alone as we reported for keratinocytes (Cooper et al., 2009). These findings indicate that under UVR exposures sufficient to generate DNA damage, arsenite is capable of increasing DNA damage retention in melanocytes as has been reported for keratinocytes.

4. Discussion

Skin tumors account for nearly 50% of cancers in the United States with approximately 2 million cases diagnosed every year. Melanoma is the least common form of skin cancer and accounts for only about 4% of all skin cancer cases (Siegel et al., 2012). There is significant experimental and epidemiological evidence implicating solar UVR as the most important etiological factor in the development of skin tumors (Pfeifer and Besaratinia, 2012). Exposure to solar UVR results in direct DNA damage (such as CPDs) and oxidative lesions (such as 8-OHdG) in both keratinocytes and melanocytes. While squamous and basal cell carcinomas typically have signature DNA mutations caused by direct DNA damage, these mutations are rare in primary melanomas. The dose-dependent increase in oxidative DNA damage due to UVR appears to have a dominant role in the development of melanoma highlighting the differences between cell types within the skin (Abdel-Malek, 2010).

Pigmentation is a major photo-protective mechanism provided by melanocytes and pigment content of the skin correlates negatively with skin cancer risk. The pigments produced by melanocytes also provide protection by directly and indirectly scavenging ROS (Abdel-Malek, 2010; Kadekaro et al., 2012; Maresca et al., 2008). This may provide some explanation for the need for higher doses of UVR to produce ROS at levels comparable to those induced in keratinocytes (Figs. 2 C and D), induction of measurable DNA damage (Fig. 6A) and cytotoxicity (Fig. 1B). Alternatively, differences in the As-induced ROS response of melanocytes compared to keratinocytes may be due to different levels of antioxidant proteins such as glutathione (GSH) (data not shown) or alternative mechanisms of response to ROS. However with the similar levels of oxidative damage seen in both cell types (Fig. 6B&C), there appears to be adequate antioxidant response to compensate for the increased oxidative stress. The differences in UVR-induced responses have been proposed to be, at least in part, due to the UV absorptive properties of melanin and the ROS generated due to the production of melanin itself (Jenkins and Grossman, 2013). The evidence that ROS was not significantly increased by the combined treatment, but both oxidative (8-OHdG and strand breaks) and direct (CPDs) DNA damage was increased by the presence of arsenite (Fig. 6) suggests that ROS is less likely the mechanism accounting for the observed increase in DNA damage. Experiments conducted in more highly pigmented melanocytes did not display significant differences in ROS or DNA damage, but displayed similar retention of DNA lesions as the lighter pigment melanocytes (data not shown) providing further evidence that ROS is unlikely to be the mechanism for increased DNA damage. The notable differences between keratinocytes and melanocytes with regard to UVR-induced responses may be an important factor when considering potential mechanisms of co-carcinogenesis.

Because the skin is exposed to a variety of environmental challenges, the interactions between UVR and environmental carcinogens in the genesis of skin tumors is receiving increasing attention. Arsenic is of particular interest due to epidemiologic evidence showing increased risk in arsenic exposed populations (Karagas et al., 2001; Leonardi et al., 2012;

Tseng et al., 1968) and compelling experimental data demonstrating the co-carcinogenic relationship between UVR and arsenic (Beyersmann and Hartwig, 2008; Germolec et al., 1989). While previous investigations reported that melanocytes derived from human melanoma are more sensitive to arsenic cytotoxicity than non-tumor derived melanocytes (Graham-Evans et al., 2004; Ivanov and Hei, 2004), we have demonstrated that normal human melanocytes display nearly identical sensitivity to arsenic-induced cytotoxicity as normal keratinocytes (Fig. 1A) and experiments conducted in melanocytes with higher levels of pigmentation show similar cytotoxicity than the lighter pigmented cells under the same conditions (data not shown). This suggests that tumor cells may be more sensitive to arsenic, or alternatively may reflect variation in tumor cell response. There is evidence that there are dramatic differences between malignant melanoma cell lines in their apoptotic response to arsenic with some displaying IC₅₀ values similar to those observed for normal melanocytes and others displaying values 10-fold higher (McNeely et al., 2008). By focusing on normal cells, we find that melanocytes and keratinocytes are similar in responses to arsenic related to inhibition of DNA repair. Both cell types displayed comparable sensitivity with regard to arsenite inhibition of PARP activity and zinc loss from this target protein (Figs. 4 and 5). Inhibition of DNA repair by arsenite enhances retention of UVR-induced DNA lesions and increased mutagenesis in keratinocytes (Cooper et al., 2013). Although melanocytes require a greater dose of UVR exposure to generate DNA damage equivalent to keratinocytes (Fig. 6A), arsenite concentrations of 1 μ M were sufficient to cause retention of UVR-induced DNA damage in keratinocytes (Cooper et al., 2013; Zhou et al., 2011) and melanocytes (Fig. 6 B and C). Collectively, the findings indicate that melanocytes and keratinocytes are equally sensitive to inhibition of DNA repair by arsenic and suggest the important distinction with regard to arsenic and UVR interactions in co-carcinogenesis may be due to differential sensitivity to UVR-induced DNA damage.

The association between cutaneous melanoma and UV sun exposure specifically in populations of European origin has been established (IARC, 1992). More recent epidemiology studies indicate that high intensity intermittent sun exposure compared with chronic exposure is associated with significantly increased risk for melanoma (Gandini et al., 2005). Sunburn history was also determined to be a risk factor for melanoma. Risk of multiple primary melanomas was shown to increase significantly for the highest estimated UV exposure metric at birth and at 10 years of age (Krickler et al., 2007). It is suggested that high intermittent UV exposure that may include sunburn episodes may lead to substantial unrepaired DNA damage in melanocytes. Thus under these conditions, our results *in vitro* suggest that concurrent chronic arsenic exposure could lead to retention of unrepaired DNA damage in melanocytes with perhaps increased probability for subsequent mutational events (Gilchrist et al., 1999). Because cell types may differ in uptake and metabolism of arsenic as well as intrinsic DNA repair capacity and UV resistance as shown here, it is important to study different target cells to gain understanding of potential site specific effects of arsenic on co-carcinogenesis.

Acknowledgments

The authors would like to acknowledge LifeLine Cell Technologies for their technical expertise in the culturing of primary melanocytes.

Funding: This work was supported by the National Institutes of Health [grant number RO1 ES015826 (Hudson, L.G.)] and National Institute of Environmental Health Sciences [grant number 1R21ES018705-01A1 (Yager, J.W.)].

References

- Abdel-Malek ZA. Development of alpha-melanocortin analogs for melanoma prevention and targeting. *Adv Exp Med Biol.* 2010; 681:126–132. [PubMed: 21222265]
- Ahmed NU, Ueda M, Nikaido O, Osawa T, Ichihashi M. High levels of 8-hydroxy-2'-deoxyguanosine appear in normal human epidermis after a single dose of ultraviolet radiation. *Br J Dermatol.* 1999; 140:226–231. [PubMed: 10733270]
- Beane Freeman LE, Dennis LK, Lynch CF, Thorne PS, Just CL. Toenail arsenic content and cutaneous melanoma in Iowa. *Am J Epidemiol.* 2004; 160:679–687. [PubMed: 15383412]
- Beyersmann D, Hartwig A. Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. *Arch Toxicol.* 2008; 82:493–512. [PubMed: 18496671]
- Chen Y, Graziano JH, Parvez F, Hussain I, Momotaj H, van Geen A, Howe GR, Ahsan H. Modification of risk of arsenic-induced skin lesions by sunlight exposure, smoking, and occupational exposures in Bangladesh. *Epidemiology.* 2006; 17:459–467. [PubMed: 16755266]
- Chen YC, Guo YL, Su HJ, Hsueh YM, Smith TJ, Ryan LM, Lee MS, Chao SC, Lee JY, Christiani DC. Arsenic methylation and skin cancer risk in southwestern Taiwan. *J Occup Environ Med.* 2003; 45:241–248. [PubMed: 12661181]
- Chervona Y, Arita A, Costa M. Carcinogenic metals and the epigenome: understanding the effect of nickel, arsenic, and chromium. *Metallomics.* 2012; 4:619–627. [PubMed: 22473328]
- Ciren PB, Li ZQ. Long-term global earth surface ultraviolet radiation exposure derived from ISCCP and TOMS satellite measurements. *Agr Forest Meteorol.* 2003; 120:51–68.
- Cooper KL, King BS, Sandoval MM, Liu KJ, Hudson LG. Reduction of arsenite-enhanced ultraviolet radiation-induced DNA damage by supplemental zinc. *Toxicol Appl Pharmacol.* 2013; 269:81–88. [PubMed: 23523584]
- Cooper KL, Liu KJ, Hudson LG. Contributions of reactive oxygen species and mitogen-activated protein kinase signaling in arsenite-stimulated hemeoxygenase-1 production. *Toxicol Appl Pharmacol.* 2007; 218:119–127. [PubMed: 17196236]
- Cooper KL, Liu KJ, Hudson LG. Enhanced ROS production and redox signaling with combined arsenite and UVA exposure: contribution of NADPH oxidase. *Free Radic Biol Med.* 2009; 47:381–388. [PubMed: 19414066]
- Cunha ES, Kawahara R, Kadowaki MK, Amstalden HG, Noleto GR, Cadena SM, Winnischhofer SM, Martinez GR. Melanogenesis stimulation in B16-F10 melanoma cells induces cell cycle alterations, increased ROS levels and a differential expression of proteins as revealed by proteomic analysis. *Exp Cell Res.* 2012; 318:1913–1925. [PubMed: 22668500]
- Dennis LK, Lynch CF, Sandler DP, Alavanja MC. Pesticide use and cutaneous melanoma in pesticide applicators in the agricultural health study. *Environ Health Perspect.* 2010; 118:812–817. [PubMed: 20164001]
- Ding W, Hudson LG, Liu KJ. Inorganic arsenic compounds cause oxidative damage to DNA and protein by inducing ROS and RNS generation in human keratinocytes. *Mol Cell Biochem.* 2005; 279:105–112. [PubMed: 16283519]
- Ding W, Hudson LG, Sun X, Feng C, Liu KJ. As(III) inhibits ultraviolet radiation-induced cyclobutane pyrimidine dimer repair via generation of nitric oxide in human keratinocytes. *Free Radic Biol Med.* 2008; 45:1065–1072. [PubMed: 18621123]
- Ding W, Liu W, Cooper KL, Qin XJ, de Souza Bergo PL, Hudson LG, Liu KJ. Inhibition of poly(ADP-ribose) polymerase-1 by arsenite interferes with repair of oxidative DNA damage. *J Biol Chem.* 2009; 284:6809–6817. [PubMed: 19056730]
- Ebert F, Weiss A, Bultemeyer M, Hamann I, Hartwig A, Schwerdtle T. Arsenicals affect base excision repair by several mechanisms. *Mutat Res.* 2011; 715:32–41. [PubMed: 21782832]
- Gandini S, Sera F, Cattaruzza MS, Pasquini P, Picconi O, Boyle P, Melchi CF. Meta-analysis of risk factors for cutaneous melanoma: II. Sun exposure. *Eur J Cancer.* 2005; 41:45–60. [PubMed: 15617990]
- Germolec DR, Yang RS, Ackermann MF, Rosenthal GJ, Boorman GA, Blair P, Luster MI. Toxicology studies of a chemical mixture of 25 groundwater contaminants. II. Immunosuppression in B6C3F1 mice. *Fundam Appl Toxicol.* 1989; 13:377–387. [PubMed: 2515087]

- Gilchrist D, Schwarze U, Shields K, MacLaren L, Bridge PJ, Byers PH. Large kindred with Ehlers-Danlos syndrome type IV due to a point mutation (G571S) in the COL3A1 gene of type III procollagen: low risk of pregnancy complications and unexpected longevity in some affected relatives. *Am J Med Genet.* 1999; 82:305–311. [PubMed: 10051163]
- Graham-Evans B, Cohly HH, Yu H, Tchounwou PB. Arsenic-induced genotoxic and cytotoxic effects in human keratinocytes, melanocytes and dendritic cells. *Int J Environ Res Public Health.* 2004; 1:83–89. [PubMed: 16696182]
- Huang C, Ke Q, Costa M, Shi X. Molecular mechanisms of arsenic carcinogenesis. *Mol Cell Biochem.* 2004; 255:57–66. [PubMed: 14971646]
- IARC. Solar and ultraviolet radiation. International Agency for Research on Cancer; Lyon, France: 1992.
- Ivanov VN, Hei TK. Arsenite sensitizes human melanomas to apoptosis via tumor necrosis factor alpha-mediated pathway. *J Biol Chem.* 2004; 279:22747–22758. [PubMed: 15028728]
- Jenkins NC, Grossman D. Role of melanin in melanocyte dysregulation of reactive oxygen species. *Biomed Res Int.* 2013; 2013:908797. [PubMed: 23555101]
- Kadekaro AL, Chen J, Yang J, Chen S, Jameson J, Swope VB, Cheng T, Kadakia M, Abdel-Malek Z. Alpha-melanocyte-stimulating hormone suppresses oxidative stress through a p53-mediated signaling pathway in human melanocytes. *Mol Cancer Res.* 2012; 10:778–786. [PubMed: 22622028]
- Karagas MR, Stukel TA, Morris JS, Tosteson TD, Weiss JE, Spencer SK, Greenberg ER. Skin cancer risk in relation to toenail arsenic concentrations in a US population-based case-control study. *Am J Epidemiol.* 2001; 153:559–565. [PubMed: 11257063]
- King BS, Cooper KL, Liu KJ, Hudson LG. Poly(ADP-ribose) contributes to an association between poly(ADP-ribose) polymerase-1 and xeroderma pigmentosum complementation group A in nucleotide excision repair. *J Biol Chem.* 2012; 287:39824–39833. [PubMed: 23038248]
- Kricker A, Armstrong BK, Goumas C, Litchfield M, Begg CB, Hummer AJ, Marrett LD, Theis B, Millikan RC, Thomas N, Culver HA, Gallagher RP, Dwyer T, Rebbeck TR, Kanetsky PA, Busam K, From L, Mujumdar U, Zanetti R, Berwick M. Ambient UV, personal sun exposure and risk of multiple primary melanomas. *Cancer Causes Control.* 2007; 18:295–304. [PubMed: 17206532]
- Leonardi G, Vahter M, Clemens F, Goessler W, Gurzau E, Hemminki K, Hough R, Koppova K, Kumar R, Rudnai P, Surdu S, Fletcher T. Inorganic arsenic and basal cell carcinoma in areas of Hungary, Romania, and Slovakia: a case-control study. *Environ Health Perspect.* 2012; 120:721–726. [PubMed: 22436128]
- Maresca V, Flori E, Briganti S, Mastrofrancesco A, Fabbri C, Mileo AM, Paggi MG, Picardo M. Correlation between melanogenic and catalase activity in in vitro human melanocytes: a synergic strategy against oxidative stress. *Pigment Cell Melanoma Res.* 2008; 21:200–205. [PubMed: 18426413]
- McNeely SC, Belshoff AC, Taylor BF, Fan TW, McCabe MJ Jr, Pinhas AR, States JC. Sensitivity to sodium arsenite in human melanoma cells depends upon susceptibility to arsenite-induced mitotic arrest. *Toxicol Appl Pharmacol.* 2008; 229:252–261. [PubMed: 18328521]
- Melkonian S, Argos M, Pierce BL, Chen Y, Islam T, Ahmed A, Syed EH, Parvez F, Graziano J, Rathouz PJ, Ahsan H. A prospective study of the synergistic effects of arsenic exposure and smoking, sun exposure, fertilizer use, and pesticide use on risk of premalignant skin lesions in Bangladeshi men. *Am J Epidemiol.* 2011; 173:183–191. [PubMed: 21098630]
- Morton, W.; Dunnette, D. Health effects of environmental arsenic. In: Nriagy, JO., editor. *Arsenic in the Environment, Part II: Human Health and Ecosystem Effects.* John Wiley and Sons, Inc.; 1994.
- Nishigori C, Hattori Y, Toyokuni S. Role of reactive oxygen species in skin carcinogenesis. *Antioxid Redox Signal.* 2004; 6:561–570. [PubMed: 15130282]
- Pearce DC, Dowling K, Sim MR. Cancer incidence and soil arsenic exposure in a historical gold mining area in Victoria, Australia: a geospatial analysis. *J Expo Sci Environ Epidemiol.* 2012; 22:248–257. [PubMed: 22434113]
- Pfeifer GP, Besaratinia A. UV wavelength-dependent DNA damage and human nonmelanoma and melanoma skin cancer. *Photochem Photobiol Sci.* 2012; 11:90–97. [PubMed: 21804977]

- Piatek K, Schwerdtle T, Hartwig A, Bal W. Monomethylarsonous acid destroys a tetrathiolate zinc finger much more efficiently than inorganic arsenite: mechanistic considerations and consequences for DNA repair inhibition. *Chem Res Toxicol*. 2008; 21:600–606. [PubMed: 18220366]
- Platanias LC. Biological responses to arsenic compounds. *J Biol Chem*. 2009; 284:18583–18587. [PubMed: 19363033]
- Qin XJ, Hudson LG, Liu W, Timmins GS, Liu KJ. Low concentration of arsenite exacerbates UVR-induced DNA strand breaks by inhibiting PARP-1 activity. *Toxicol Appl Pharmacol*. 2008; 232:41–50. [PubMed: 18619636]
- Rago R, Mitchen J, Wilding G. DNA fluorometric assay in 96-well tissue culture plates using Hoechst 33258 after cell lysis by freezing in distilled water. *Anal Biochem*. 1990; 191:31–34. [PubMed: 1706565]
- Rossman TG, Klein CB. Genetic and epigenetic effects of environmental arsenicals. *Metallomics*. 2011; 3:1135–1141. [PubMed: 21976018]
- Rossman TG, Uddin AN, Burns FJ. Evidence that arsenite acts as a cocarcinogen in skin cancer. *Toxicol Appl Pharmacol*. 2004; 198:394–404. [PubMed: 15276419]
- Shannon RL, Strayer DS. Arsenic-induced skin toxicity. *Hum Toxicol*. 1989; 8:99–104. [PubMed: 2663702]
- Shi H, Hudson LG, Ding W, Wang S, Cooper KL, Liu S, Chen Y, Shi X, Liu KJ. Arsenite causes DNA damage in keratinocytes via generation of hydroxyl radicals. *Chem Res Toxicol*. 2004a; 17:871–878. [PubMed: 15257611]
- Shi H, Shi X, Liu KJ. Oxidative mechanism of arsenic toxicity and carcinogenesis. *Mol Cell Biochem*. 2004b; 255:67–78. [PubMed: 14971647]
- Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin*. 2012; 62:10–29. [PubMed: 22237781]
- Suzukawa AA, Vieira A, Winnischofer SM, Scalfo AC, Di Mascio P, Ferreira AM, Ravanat JL, Martins Dde L, Rocha ME, Martinez GR. Novel properties of melanins include promotion of DNA strand breaks, impairment of repair, and reduced ability to damage DNA after quenching of singlet oxygen. *Free Radic Biol Med*. 2012; 52:1945–1953. [PubMed: 22401857]
- Tokar EJ, Benbrahim-Tallaa L, Ward JM, Lunn R, Sams RL 2nd, Waalkes MP. Cancer in experimental animals exposed to arsenic and arsenic compounds. *Crit Rev Toxicol*. 2010; 40:912–927. [PubMed: 20812815]
- Tseng WP, Chu HM, How SW, Fong JM, Lin CS, Yeh S. Prevalence of skin cancer in an endemic area of chronic arsenicism in Taiwan. *J Natl Cancer Inst*. 1968; 40:453–463. [PubMed: 5644201]
- Watanabe C, Inaoka T, Kadono T, Nagano M, Nakamura S, Ushijima K, Murayama N, Miyazaki K, Ohtsuka R. Males in rural Bangladeshi communities are more susceptible to chronic arsenic poisoning than females: analyses based on urinary arsenic. *Environ Health Perspect*. 2001; 109:1265–1270. [PubMed: 11748034]
- Wiencke JK, Yager JW, Varkonyi A, Hultner M, Lutze LH. Study of arsenic mutagenesis using the plasmid shuttle vector pZ189 propagated in DNA repair proficient human cells. *Mutat Res*. 1997; 386:335–344. [PubMed: 9219570]
- Wnek SM, Kuhlman CL, Camarillo JM, Medeiros MK, Liu KJ, Lau SS, Gandolfi AJ. Interdependent genotoxic mechanisms of monomethylarsonous acid: role of ROS-induced DNA damage and poly(ADP-ribose) polymerase-1 inhibition in the malignant transformation of urothelial cells. *Toxicol Appl Pharmacol*. 2011; 257:1–13. [PubMed: 21925530]
- Yager JW, Wiencke JK. Inhibition of poly(ADP-ribose) polymerase by arsenite. *Mutat Res*. 1997; 386:345–351. [PubMed: 9219571]
- Zhao L, Chen S, Jia L, Shu S, Zhu P, Liu Y. Selectivity of arsenite interaction with zinc finger proteins. *Metallomics*. 2012; 4:988–994. [PubMed: 22847370]
- Zhou X, Sun X, Cooper KL, Wang F, Liu KJ, Hudson LG. Arsenite interacts selectively with zinc finger proteins containing C3H1 or C4 motifs. *J Biol Chem*. 2011; 286:22855–22863. [PubMed: 21550982]

Highlights

- Melanocytes are more resistant to UV toxicity than keratinocytes.
- Greater arsenic and UV are required for comparable ROS induction in melanocytes.
- Both cell types exhibit similar mechanism of PARP1 inhibition with As exposure.
- Melanocytes and keratinocytes retain DNA lesions after co-treatment with As and UV.

Figure 1. Cellular viability following arsenite or UVR exposure

A&B) Keratinocytes (HEK, black circles) or melanocytes (HEM, gray squares) were treated with either (A) arsenite or (B) UVR as indicated and cellular viability determined at 24 hrs post treatment via the CellTiter 96 Non-Radioactive Cell Proliferation Assay kit. C&D) Time course assessment of melanocyte (C) and keratinocyte (D) viability. Cells were treated with the indicated concentrations of arsenite (black circles), UVR (gray circles) or both (open triangles, dashed line) and viability determined as in A and B at the indicated time points. Data shown is mean $\pm$ SEM, $n=3$. * = significantly different from untreated control; $p<0.01$.

Figure 2. Arsenite and UVR induced superoxide varies by cell type and exposure level
 Keratinocytes (HEK, A&C) or melanocytes (HEM, B,D&E) were exposed to A&B) arsenite (As; 1 μ M gray circles, 5 μ M black squares) or C&D) UVR (3 kJ/m² gray circles, 5 kJ/m² black squares) for the indicated times and 5 μ M dihydroethidium (DHE) was added 30 min prior to collection as a cell permeable, fluorescent superoxide indicator. Resultant fluorescence was quantified as described in section 2.3 and normalized to total DNA content. E) Melanocytes were treated with 5 μ M arsenite (As, solid gray squares; solid line), 5 kJ/m² UVR (UVR, open gray squares; dashed line) or both (As + UVR, black diamond) for the indicated times, then DHE added as above and ROS fluorescence quantified. Cellular

viability was at least 80% for both cell types at the longest time point indicated. Data shown is mean $\pm$ SEM, n=3. * = significantly different from exposure matched sample; $p < 0.01$.

Figure 3. Activation of NADPH oxidase by arsenite and UVR correlates with observed increases in superoxide

Melanocytes (HEM, gray squares) and keratinocytes (HEK, black circles) were treated with A) arsenite (As, 5 μ M or 1 μ M) or B) UVR (5 or 3 kJ/m²) for the indicated times then NADPH oxidase (NOX) activity measured via the Lucigenin Illumination method as described in section 2.4. Cellular viability was at least 70% for both cell types at the longest time point indicated. Data shown is mean $\pm$ SEM, n=3. * = significantly different from exposure matched sample; p<0.01.

Figure 4. Arsenite inhibits PARP activity in normal melanocytes and keratinocytes

Keratinocytes (HEK, black bars) or melanocytes (HEM, gray bars) were exposed to the indicated concentrations of arsenite (As) for 24h. PARP-1 was activated by a single exposure to 3 kJ/m² ssUVR (HEK) or 10 kJ/m² ssUVR (HEM). Total protein was collected 1h post UVR treatment and PARP activity determined via the HT Colorimetric PARP/ Apoptosis Assay kit according to the manufacturer's instructions. The keratinocyte data shown here is presented for comparison and to provide intra-experiment controls. This data was generated independently and confirms previously published data (Cooper et al., 2013). Results presented are mean $\pm$ SEM of 3 separate experiments * = significantly different from untreated, activated control; $p < 0.01$. δ = significantly different from concentration matched sample; $p < 0.01$, $n = 3$.

Figure 5. Mechanism of arsenite-dependent PARP inhibition

Keratinocytes (black bars) and melanocytes (gray bars) were treated with the indicated concentrations of arsenite (As, 24h), TPEN (a zinc chelator, 4h) or left untreated. PARP protein was immunoprecipitated and the zinc release assay performed as described in section 2.6 and normalized to the untreated controls. Data shown is mean $\pm$ SEM, $n=3$. * = significantly different from untreated control; $p<0.01$.

Figure 6. UV-induced DNA damage is retained following arsenite exposure

A) Cells were cultured on 4 well slides and melanocytes (HEM) exposed to 3 or 10 kJ/m² UVR to compare to keratinocytes (HEK) exposed to only the lower dose of UVR. Cells were fixed and stained for the indicated DNA damage markers at 1h post exposure. B&C) Cells were treated with arsenite (As, 1 μ M; gray bars) or without (black bars), exposed UVR (10 kJ/m²), then fixed at 1 and 6 h post exposure and stained for the indicated DNA damage markers. B) Representative immunofluorescence images and C) quantification of average fluorescence intensity from at least 10 images per treatment per experiment (for a total of at least 100 cells per treatment) and normalized to the UVR only treatment group with Image J showing the DNA damage markers 8-OHdG, CPD and pH2Ax following treatment with UVR alone (black bars) or As+UVR (gray bars) at the indicated times post UVR. Data

shown is mean $\pm$ SEM, n=3 * = significantly different from time matched sample; $p < 0.01$,
n = 3.