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Arsenic Transformation Predisposes Human Skin Keratinocytes To UV-induced DNA Damage Yet Enhances Their Survival Apparently by Diminishing Oxidant Response

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Abstract

Inorganic arsenic and UV, both human skin carcinogens, may act together as skin co-carcinogens. We find human skin keratinocytes (HaCaT cells) are malignantly transformed by low-level arsenite (100 nM, 30 weeks; termed As-TM cells) and with transformation concurrently undergo full adaptation to arsenic toxicity involving reduced apoptosis and oxidative stress response to high arsenite concentrations. Oxidative DNA damage (ODD) is a possible mechanism in arsenic carcinogenesis and a hallmark of UV-induced skin cancer. In the current work, inorganic arsenite exposure (100 nM) did not induce ODD during the 30 weeks required for malignant transformation. Although acute UV-treatment (UVA, 25 J/cm²) increased ODD in passage-matched control cells, once transformed by arsenic to As-TM cells, acute UV actually further increased ODD (> 50%). Despite enhanced ODD, As-TM cells were resistant to UV-induced apoptosis. The response of apoptotic factors and oxidative stress genes were strongly mitigated in As-TM cells after UV exposure including increased *Bcl2/Bax* ratio and reduced *Caspase-3*, *Nrf2*, and *Keap1* expression. Several *Nrf2*-related genes (*HO-1*, *GCLs*, *SOD*) showed diminished responses in As-TM cells after UV exposure consistent with reduced oxidant stress response. UV-exposed As-TM cells showed increased expression of *cyclin D1* (proliferation gene) and decreased *p16* (tumor suppressor). UV exposure enhanced the malignant phenotype of As-TM cells. Thus, the co-carcinogenicity between UV and arsenic in skin cancer might involve adaptation to chronic arsenic exposure generally mitigating the oxidative stress response, allowing apoptotic by-pass after UV and enhanced cell survival even in the face of increased UV-induced oxidative stress and increased ODD.

Keywords

ultraviolet; arsenic; skin cancer; apoptosis; oxidative DNA damage; keratinocytes

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Introduction

Solar ultraviolet (UV) irradiation is well-recognized as a skin carcinogen that has long been implicated in the formation of squamous cell carcinomas (SCCs) (De Gruijl, 1999). UVA (315–400 nm), which is not absorbed by the ozone layer, constitutes more than 95% of the solar UV radiation that reaches the Earth's surface (De Gruijl, 1999; Pourzand and Tyrrell, 1999). UV can induce mutations in the basal layer of the skin and UVA signature mutations have been detected in SCCs (Agar et al., 2004).

Inorganic arsenic is a naturally occurring metalloid and the world-wide contamination of drinking-water has been recognized as a major human health issue (IARC, 2004). Oral exposure to inorganic arsenic is strongly associated with skin cancer (IARC, 2004). In particular, chronic oral exposure to arsenic in humans results in SCCs of the skin (IARC, 2004). A case-control study in an area of southwestern Taiwan where high arsenic exposure is endemic found that arsenic-associated skin cancer was also associated with sun exposure (Chen et al., 2003). Interestingly, inorganic arsenic alone does not seem sufficient to cause skin cancer in animals. In mouse skin it appears that inorganic arsenic acts as a co-carcinogen by enhancing skin cancer induced by UV irradiation when the metalloid is simultaneously added to the drinking water (Rossman et al., 2001; Rossman et al., 2002). In a similar fashion, co-treatment of transgenic (Tg.AC) mice, which overexpress the v-Ha-ras oncogene in skin, with dermal 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and oral inorganic arsenic also greatly enhances skin tumor development (Germolec et al., 1997; Germolec et al., 1998). Additional work has shown *in utero* arsenic exposure predisposes mice to subsequent formation of chemically-induced SCCs in Tg.AC mice and that these carcinomas are more highly aggressive than usual (Waalkes et al., 2008). In none of these mouse skin models is arsenic very effective as a carcinogen when given alone (Rossman et al., 2001; Rossman et al., 2002; Germolec et al., 1997; Germolec et al., 1998; Waalkes et al., 2008).

Many studies have shown that arsenic contributes to carcinogenesis very likely through multiple potential mechanisms (Kitchin, 2001; Rossman, 2003; Schoen et al., 2004; Huan et al., 2004; Kojima et al., 2009). Among them, oxidative DNA damage (ODD) is widely recognized as a possible mechanism for arsenic carcinogenesis in some cases (Kojima et al., 2009; Hughes, 2002). Indeed, evidence of ODD has been observed in the urine of arsenic-exposed humans (Yamauchi et al., 2004) and can be produced in various *in vitro* model systems (Kojima et al., 2009; Pi et al., 2005). *In vitro*, we find ODD occurs only in model target cells systems of arsenic carcinogenesis that biomethylate the metalloid, although both arsenic biomethylation competent and deficient cells can undergo malignant transformation (Kojima et al., 2009). Production of reactive oxygen species (ROS) during biomethylation may be responsible for ODD in cells exposed to not only inorganic arsenic, but also UV irradiation (Hei et al., 1998; Shi et al., 2004). Increased ROS combined with a defective or suppressed antioxidative defense system could certainly leave cells more vulnerable to ODD (Karbownik et al., 2001; Ray et al., 2002). NF-E2-related factor 2 (*Nrf2*) is a transcription factor shown to be an essential component of the antioxidant response element (ARE)-binding transcriptional machinery (Kobayashi and Yamamoto, 2006). Induction of ARE-mediated genes, such as heme oxygenase 1 (*HO-1*), glutamate-cysteine ligase catalytic subunit (*GCLC*) and regulatory subunit (*GCLM*) play a critical role in the cellular defense against damage caused by ROS and are similarly activated by arsenic exposure (Pi et al., 2003; Liu et al., 2001; Prestera et al., 1993).

In a previous study from our group, we developed an *in vitro* model of arsenic skin carcinogenesis using a normally non-tumorigenic human skin keratinocyte (HaCaT) cell line which has been malignantly transformed by chronic, low-level (100 nM) of sodium arsenite

for up to 30 weeks (Pi et al., 2008). These cells (termed arsenic-transformed malignant; As-TM) produce aggressive SCC in mouse xenograft study, and become adapted to arsenic, in the respect that they become resistant to both apoptosis and oxidative stress response, but are not resistant to ODD, induced by high concentrations ($> 20 \mu\text{M}$ arsenite) of inorganic arsenic (Pi et al., 2005; Pi et al., 2008). The measurement technique we used for ODD in this prior work (8-oxo-dG levels by HPLC and electrochemical detector) (Pi et al., 2008) was prone to high baseline levels that can make oxidant-induced levels of ODD very small relative to control base-line, as we indeed observed with arsenic in these cells (Pi et al., 2008). None-the-less, it appeared that As-TM cells adapted to arsenic such that high concentration arsenic-induced apoptosis was reduced and *Nrf2* related oxidant response was greatly diminished even in the face of increased ODD (Pi et al., 2005; Pi et al., 2008). This might allow cells with DNA damage to undergo apoptotic by-pass even with compromised DNA. The question becomes if this arsenic adaption would be a general response to carcinogenic oxidants, like UV.

Thus, in this study HaCaT cells were first assessed for ODD during sodium arsenite (100 nM) transformation to form As-TM cells using the immuno-spin trapping (IST) method (Ramirez et al., 2006; Ramirez et al., 2007). Since skin cells do not methylate inorganic arsenic we predicted they would not show significant ODD during transformation. Additionally, once converted to malignant As-TM cells we tested the hypothesis that arsenic adaptation, which reduces oxidative stress response and apoptosis, but not ODD, from high, acutely toxic levels of inorganic arsenic (Pi et al., 2005), might cause cross-adaptation to UV irradiation, a physical agent thought to act as a co-carcinogen with inorganic arsenic in the skin (Chen et al., 2003; Rossman et al., 2001; Rossman et al., 2002; Kessel et al., 2002). We also wanted to see if arsenic adaptation impacted UV-induced ODD by mitigating oxidant response. We found inorganic arsenic did not induce ODD during malignant transformation of these non-methylating cells, and that arsenic-induced transformation blocked UV-induced oxidant stress response and apoptosis while increasing UV-induced ODD. The latter could add significantly to a co-carcinogenic effect between inorganic arsenic and UV irradiation in the skin by allowing apoptotic by-pass of cells with significant DNA damage.

Materials and Methods

Chemicals and antibodies

Sodium arsenite (NaAsO_2) was obtained from Sigma Chemical Co. (St. Louis, MO). The primers for real-time RT-PCR analysis were synthesized by Sigma-Genosys (The Woodlands, TX). They include: *Caspase-3*, *Bcl2*, *Bax*, *Cyclin D1*, *p16*, *Nrf2*, *Keap1*, *HO-1*, *GCLC*, *GCLM*, *SOD1* and *SOD2*. The antibodies against cyclin D1 was from Abcam Inc. (Cambridge, MA), and antibodies against *Nrf2*, *HO-1*, *NQO1* and *p16* were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture and treatments

The HaCaT cell line was originally derived from normal human adult skin, and is nontumorigenic (Boukamp et al., 1988). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), antibiotics (100 U/ml penicillin and 100 g/ml streptomycin). Cultures were maintained at 37°C and in a humidified 5% CO_2 atmosphere and passaged once a week subconfluent. For chronic arsenic exposure, cells were maintained continuously in a medium containing 100 nM of arsenic for 30 weeks. The media was refreshed with passage. Passage-matched control cells were used in all assessments.

For UV treatment, the medium was removed and cells were washed twice with PBS. After the addition of PBS, the cells were irradiated with fluorescent lamps (Houvalite F20T12BL-HO PUVA, National Biological Corp., Twinsburg, OH) with the dish lid on. The UV dose (UVA; 25 J/cm²) was monitored with a Goldilux UV meter equipped with a UVA detector (Oriel Instruments, Stratford, CT). Passage-matched control cells were kept in the dark under the same conditions. After UV treatment, fresh medium containing 1% serum was added and the cells were kept at 37°C and in a humidified 5% CO₂ atmosphere for 18 hours. Hydrogen peroxide (H₂O₂, 5 mM) was added directly into the dishes with fresh serum-free medium for 24 hours. The medium was then removed and various aspects of exposure were assessed.

ODD Measurement by the Immuno Spin-Trapping (IST) Method

The IST method directly measures DNA radicals by conversion *in situ* into stable nitrones after combination with the spin trap agent DMPO prior to DNA isolation (Ramirez et al., 2006; Ramirez et al., 2007), thus avoiding adventitious artifactual DNA oxidation during isolation and reducing base-line levels. For perspective, the IST method is approximately 50-fold more sensitive than ELISA methods for 8-oxo-dG (Ramirez et al., 2006). Cells were incubated with 20 mM DMPO for 30 min at 37°C, and then harvested by trypsinization and washed three times with PBS. The DMPO step effectively changes short-lived oxidative DNA radicals into stable DNA nitrones (Ramirez et al., 2006; Ramirez et al., 2007). Cells were incubated in 0.5 ml digestion buffer (1% sodium dodecyl sulfate, 100 mM NaCl, 25 mM diethylenetriaminepentaacetic acid, and 10 mM Tris-HCl; pH 8.0) containing 25 µl proteinase K (20 mg/ml proteinase K in 50 mM Tris-HCl containing 1 mM CaCl₂) for 1 hour at 52°C. After the addition of 10 µl RNase A (20 mg/ml), the incubation continued for 1 hour at 37°C. High purity DNA resulted from a three-step purification procedure using ultrapure buffer-saturated phenol (Invitrogen, Carlsbad, CA) containing 1 mM diethylenetriaminepentaacetic acid, phenol:chloroform:isoamyl alcohol (25:24:1), and chloroform:isoamyl alcohol (24:1). DNA was resuspended in 100 µl TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA], and purity and concentration determined by absorbance at 260 and 280 nm. This method produced DNA preparations with an Abs_{260/280} ratio between 1.8–2.0. The purified DNA containing the nitron adducts was diluted to 5 µg/ml in PBS. 25 µl DNA solution were mixed with 25 µl React-bind DNA coating solution (Pierce Chemicals, Rockford, IL) in each well of flat-bottom 96-well microtiter plates (PGC Scientifics, San Diego, CA), and incubated for 4 hours at 37°C. After DNA binding, wells were washed once with 300 µl washing buffer (PBS containing 0.05% non-fat dry milk and 0.1% Tween-20). For blocking nonspecific binding sites, 120 µl blocking solution (PBS containing 3% non-fat dry milk) was added and incubated for 1.5 hours at 37°C. Each well was then washed once with 300 µl washing buffer for 5 min on an orbital shaker at room temperature. Then 100 µl of rabbit anti-DMPO polyclonal serum (1:10,000; Cayman Chemical, Ann Arbor, MI) in washing buffer was added and incubated for 1 hour at 37°C. Plates were washed three times with 300 µl washing buffer, and 100 µl of goat anti-rabbit IgG conjugated to horse-radish peroxidase (1:10,000; Pierce) was added and incubated for 1 hour at 37°C. After washing the plates three times with 300 µl washing buffer, 50 µl LumiGLO chemiluminescent substrate (Upstate, Temecula, CA) was added to each well, incubated for 30 sec, and luminescence was read using Xfluor4 Software (Tecan, Männedorf, Switzerland).

Determination of apoptosis by flow cytometry

Cells were seeded in 25 cm² flasks and grown to ~80% confluence. After the cells were exposed to hydrogen peroxide for 24 hours or 18 hours post-UVA exposure, floating and attached cells were harvested for apoptosis analysis. Detection of phosphatidylserine on the outer leaflet of apoptotic cells was carried out using Annexin V and propidium iodide according to the TREVIGEN[®] manufacturer's recommendations. For each sample, 10,000

cells were examined by flow cytometry using a Becton Dickinson FACSsort (Becton Dickinson, San Jose, CA). The percentage of apoptotic cells was determined by statistical analysis of the various dot plots using CellQuest software (Vermes et al., 1995).

Real time RT-PCR analysis

Total RNA was isolated from cells with TRIzol® (Invitrogen, Carlsbad, CA) and purified with RNeasy Mini kit (Qiagen, Valencia, CA). The quality of RNA was determined by the 260/280 ratios (1.7–1.8). RNA was reverse transcribed with MuLV reverse transcriptase and oligo-d(T) primers. The primers for selected genes were designed using Primer Express Software (Applied Biosystems, Foster City, CA). The SYBR Green Master Mix (Applied Biosystems, Foster City, CA) was used for real time PCR analysis. Relative differences in gene expression between groups were expressed using cycle time (Ct) values. These Ct values were first normalized with that of β -actin in the same sample and then expressed as fold with control set to 1.0. Real time fluorescence detection was carried out using a MyiQ™ SingleColor Real-Time PCR Detection System (Bio-Rad, Hercules, CA).

Immunocytochemistry

As described previously (Sun *et al.*, 2009), cells were plated on Lab-Tek chambered cover glass chamber slides (Nunc, Rochester, NY). Cells were washed twice with PBS and fixed for 2 minutes in acetone:methanol (1:1). The cells were incubated for 1 hour with 3% bovine serum albumin (BSA) in PBS to block nonspecific antibody binding followed by incubation for 1 hour with primary antibodies against cyclin D1 (ABcam, Cambridge, MA), Nrf2, HO-1, NQO1 and p16 (Santa Cruz, CA), each diluted 1:100 in 3% BSA in PBS. The cells were washed three times in PBS and then incubated in dark for 1 hour at 37°C with Alexa Fluor 488 goat anti-rabbit IgG (H+L) and Alexa Fluor 568 goat anti-mouse IgG (H+L) secondary antibodies (Invitrogen, Carlsbad, CA) diluted 1:200 in PBS buffer containing 3% BSA. The cells were washed three times with PBS and incubated in DAPI (4',6-diamidino-2-phenylindole) solution (1:1000 in PBS, Invitrogen, Carlsbad, CA) for 5 minutes in the dark. After washing with PBS twice, cells were imaged by fluorescent microscopy.

Zymographic analysis of metalloproteinase-9 (MMP-9) activity

Cells at 70–80% confluence were washed three times with PBS, and the medium was changed to serum-free DMEM. After 48 hrs, the conditioned medium was collected on ice for zymographic analysis of metalloproteinase-9 (MMP-9). MMP-9, a member of matrix-degrading enzyme family, plays a crucial role in tumor invasion (Bernhard *et al.*, 1994). Elevated expression levels of MMP-9 is strongly correlated with malignant phenotype in SCC (Bernhard *et al.*, 1994) and are characteristic of malignant transformation of arsenic-induced cells (Pi *et al.*, 2008). MMP-9 activity was detected as described previously (Sun *et al.*, 2009). After staining with SimplyBlue Safestain (Invitrogen, Carlsbad, CA), the bands were quantified with Image J software.

In vitro invasion assay

The in vitro invasive ability of As-TM and UV-treated As-TM cells was examined as a malignant phenotype using a modified Boyden chamber assay (Bello et al., 1997). Briefly, 2×10^5 cells per chamber were seeded in top chamber on the filter coated with Matrigel (BD Biosciences, Bedford, MA) as the matrix barrier. 10% FBS medium was loaded in bottom chamber beneath the filter as the chemoattractant. Cells were incubated at 37 °C in 5% CO₂. After 48 hours cells on the upper side of the filter were removed mechanically, and those that migrated onto the lower side of the filter were fixed and stained with HEMA 3 stain set (Fisher Scientific Company, Kalamazoo, MI), and then dissolved in 0.1 N HCl. The absorbance of this solution was read at 595 nm for quantification.

Statistical analysis

Data are expressed as Mean $\pm$ SEM of 3–6 determinations. An ANOVA followed by Duncan's multiple comparison tests was performed for comparisons between treatment groups and passage-matched control. The level of significance was set at $P < 0.05$ in all cases.

Results

ODD during arsenic transformation

To examine oxidative DNA damage (ODD) during arsenic-induced malignant transformation, HaCaT cells were continuously exposed to a low-level (100 nM) of sodium arsenite for up to 30 weeks. This is a level and time point is known to cause malignant transformation as confirmed by production of SCCs upon inoculation of cells into nude mice (Pi et al., 2008). At periodic points in this process, ODD levels were measured. Elevated ODD was not observed in the cells at any point during chronic arsenic exposure, even when cells achieved malignant qualities and became as termed 'arsenic-transformed malignant' (As-TM) cells (Fig. 1A). This is likely because the HaCaT cells poorly methylate arsenic (Trouba et al., 2002), which appears required for inorganic arsenic to induce ODD (Kojima et al., 2009).

ODD and apoptosis induced by UV or H₂O₂ exposure

However, ODD is a hallmark of UV carcinogenesis. The As-TM cells were further exposed to acute high level UV irradiation. Although this acute UV irradiation (25 J/cm²) increased ODD in passage-matched control cells, in As-TM cells UV-induced ODD was actually markedly increased over UV-treated passage-matched control cells (Fig. 1B). Thus, arsenic transformation predisposed cells to UV-induced ODD. Similarly increased ODD was observed in As-TM cells when exposed to the direct oxidant, H₂O₂, while passage-matched control cells showed no increases in ODD (Fig. 1B).

UV can effectively induce apoptosis in many kinds of cells. As-TM cells are tolerant to apoptosis induced by various stimuli including high concentrations of inorganic arsenic (Pi et al., 2005). In the present study, passage-matched control and As-TM cells were exposed to UV and apoptosis was determined. Even with the additional ODD burden (see Fig. 1B), the As-TM cells showed diminished UV-induced apoptosis compared to passage-matched control (Fig. 1C). Thus, despite the increased ODD after UV, As-TM cells were still more resistant to apoptosis. Similar results with apoptotic resistance were seen after exposure to H₂O₂. As-TM cells were resistant to H₂O₂-induced apoptosis (Fig. 1C) despite increased ODD production after H₂O₂ exposure compared to passage-matched control (see Fig. 1B).

Resistance to UV-induced apoptosis in As-TM cells was consistent with observed *Caspase-3* expression and *Bcl2/Bax* ratio data (Fig. 2A&B). *Caspase-3*, a gene expressing a key enzyme in cellular dedication to apoptotic cell death, was reduced by 40% in As-TM cells in response to UV irradiation (Fig. 2A). The ratio of the anti-apoptotic gene *Bcl2* and pro-apoptotic gene *Bax* also supported a similar apoptotic resistance to UV-induced apoptosis in As-TM cells compared to passage-matched control (Fig. 2B).

Expression of Nrf2 and its related genes

Nuclear factor E2-related factor 2 (*Nrf2*) is the primary transcription factor that controls antioxidant response through transcriptional up-regulation of an array of target genes, such as glutamate cysteine ligases (*GCLs*) and heme oxygenase 1 (*HO-1*) (Lau et al., 2008). Kelch-like ECH associating protein 1 (*Keap1*) is a key regulator of *Nrf2*. An increase in expression of these two genes was found in passage-matched control cells after acute UV

exposure (Fig. 3A&B). However, *Nrf2* and *Keap1* showed significantly diminished responses in expression in UV-treated As-TM cells compared to UV-treated passage-matched control cells. Arsenic induces *HO-1* expression in many cell types and has been suggested as a biomarker of arsenic exposure (Rossman, 2003). The expression of *HO-1* was increased about 5-fold in UV-treated passage-matched control cells compared with untreated passage-matched control cells (Fig. 3C). As-TM cells acutely treated with UV actually showed a marked decrease in expression in *HO-1* compared to UV-treated passage-matched control. A diminished expression of *GCLC* and *GCLM* was found in As-TM cells compared with passage-matched control cells after acute UV treatment (Fig. 3D&E). Similarly, expression of superoxide dismutase 1 and 2 (*SOD1* and *SOD2*), which are ROS scavengers, also showed less increase in As-TM cells after exposure to UV compared to UV-treated passage-matched control cells (Fig. 4A&B). Similar changes in *Nrf2*, *HO-1* and *NQO1* were seen at the protein level by fluorescent immunostaining micrographic analysis (Fig. 5). Thus, it appears the generalized response to oxidant stress is tamped down in As-TM cells, whether it comes indirectly from UV or inorganic arsenic or directly from H_2O_2 . This does not obviate the production of ODD but seems to block apoptosis secondarily to this damage.

Enhanced malignant phenotype in UV-treated As-TM cells

Increased secreted MMP-9 activity is an excellent marker for malignant phenotype (Pi *et al.*, 2008). As-TM cells showed elevated secreted MMP-9 activity compared to passage-matched control cells (Fig. 6A). Furthermore, As-TM cells after UV exposure showed even greater elevated MMP-9 activity than As-TM cells. Invasion ability is another marker for malignant phenotype. Again, As-TM cells showed a significant increase compared to passage-matched control cells (Fig. 6B). Moreover, UV-treated As-TM cells showed significantly higher invasiveness than As-TM cells alone (Fig. 6B). Increased *Cyclin D1* and decreased *p16* have been associated with mouse skin carcinogenesis and, importantly, *cyclin D1* appears to be up-regulated in mouse skin by inorganic arsenic in co-carcinogenicity or co-treatment experiments (Rossman *et al.*, 2001; Waalkes *et al.*, 2008). The expression of *cyclin D1* in As-TM cells was 2.3-fold of passage-matched control cells and was further increased in As-TM cells after UV exposure (Fig. 7A). The expression of the tumor suppressor gene, *p16* in As-TM cells was 57% of passage-matched control cells and was only 30% of passage-matched control cells in As-TM cells after UV treatment (Fig. 7B). Similar changes in *cyclin D1* and *p16* were seen at the protein level by fluorescent immunostaining micrographic analysis (Fig. 7C). This further supports an enhanced malignant phenotype when inorganic arsenic exposure is subsequently combined with UV exposure.

Discussion

There is accumulating evidence that arsenic may act as a skin co-carcinogen that requires another agent for full activity *in vivo* (Rossman *et al.*, 2001; Rossman *et al.*, 2002; Germolec *et al.*, 1997; Germolec *et al.*, 1998; Waalkes *et al.*, 2008) although it appears able to act alone in cell model systems (Pi *et al.*, 2005). For instance, oral inorganic arsenic together with UV irradiation induces advanced SCCs in mice, while arsenic alone has minimal effects (Rossman *et al.*, 2001; Rossman *et al.*, 2002). SCC is a common tumor type in humans associated with arsenic exposure (IARC, 2004). In fact, when inorganic arsenic is given orally to mice with UV irradiation, the resulting skin tumors are much larger and more invasive compared to UV alone (Rossman *et al.*, 2001; Rossman *et al.*, 2002) and this enhanced progression is dose-related (Burns *et al.*, 2004). Inorganic arsenic exposure can also occur *in utero* and then be followed by dermal TPA in adulthood, when all arsenic from intentional exposure would be gone, and still increase multiplicity and progression of resulting SCCs in mice (Waalkes *et al.*, 2008). The molecular mechanisms involved in these

processes are not known, but ODD likely plays a significant role in UV-induced skin carcinogenesis (Agar et al., 2004; Hughes, 2002; Kessel et al., 2002) and sometimes in arsenic-induced acquired malignant phenotype (Kojima et al., 2009). However, in this present study, we found no increase in ODD induced by inorganic arsenite alone during the basic process of malignant transformation by the metalloid (100 nM, up to 30 weeks), which allows As-TM cells to produce highly aggressive SCCs after inoculation into nude mice (Pi et al., 2005). Our work in non-skin target cells of arsenic carcinogenesis shows low-level, long-term arsenite exposure can cause increased ODD but only in arsenic biomethylation-competent cells (Kojima et al., 2009). Other studies provide evidence against a direct genotoxic mode of action for arsenic-induced skin cancer (Klein et al., 2007). In the present work ODD was not observed in HaCaT cells during malignant transformation to As-TM cells, likely because HaCaT cells are very poor methylators of arsenic (Trouba et al., 2002).

UV irradiation has been shown to produce ROS and DNA damage, especially in skin cells (Pi et al., 2005; Ichhashi et al., 2003). Enhanced ODD could be a plausible mechanism of arsenic and UV cocarcinogenesis, although arsenic alone did not cause ODD during the malignant transformation process. In prior, more limited work we found UV increased ODD in passage-matched control cells and As-TM cells, but no apparent difference occurred between these two groups (Pi et al., 2005). However, in the present work, which is more detailed and uses what is probably a more sensitive method (Ramirez et al., 2006; Ramirez et al., 2007), production of ODD was markedly increased by acute UV exposure in As-TM cells compared to passage-matched control cells, indicating arsenic transformation predisposed these cells to UV-induced DNA damage, and perhaps more importantly, by mitigating subsequent responses allows them to survive with the damage. In this regard, As-TM cells are also fully adapted to arsenic intoxication, including programmed cell death (Pi et al., 2005; this study). This appears to be due to a generalized reduction in the response to oxidant stress, and, although ODD still occurs, it does not lead to apoptotic cell death. Hence, As-TM cells may survive UV-induced DNA damage better than passage-matched control cells because they “tolerate” it better. This provides a plausible co-carcinogenic mechanism for skin with inorganic arsenic and UV co-exposure. Whether or not the ODD carries over a significant level of mutational burden will require further study, but the combination of arsenic and UV exposures clearly did enhance malignant phenotype based on increased MMP-9 secretion, increased invasiveness, increased *cyclin D1* expression and loss of *p16* expression.

Apoptosis normally functions to control the integrity of cell populations by eliminating aberrant clones, whereas failure of apoptosis is a hallmark of skin cancer (Hanahan and Weinberg, 2000). ROS and oxidative stress have been clearly linked to the apoptotic processes (He et al., 2003; Afaq et al., 2007). As-TM cells are resistant to acute, high concentration of inorganic arsenite (> 20 μ M) induced apoptosis, but not resultant the ODD (Pi et al., 2005). In this present study, even with this additional oxidative DNA damage, As-TM cells were still dramatically resistant to UV-induced apoptosis when compared to passage-matched control cells, indicating a cross-adaptation between high concentration inorganic arsenic and UV irradiation in As-TM cells. Similar acquired apoptotic resistance and increased ODD production were seen after direct oxidant H₂O₂ treatment in As-TM, indicating specific factors directed at UV irradiation were not responsible. The fact that a reduction in UV-induced apoptosis occurred in the face of an increase in UV-induced ODD likely indicates an apoptotic by-pass allowed cells to survive with damaged genetic material that would normally be eliminated from the population. This is a major step in explaining the co-carcinogenic effects of arsenic and UV irradiation in mouse skin model systems (Rossman et al., 2006; Rossman et al., 2007) and would potentially be a very important aspect of the role of arsenic in dermal cancer. It appears that arsenic can do this for carcinogens/tumor promoter other than UV, like TPA (Germolec et al., 1997; Germolec et

al., 1998; Waalkes et al., 2008), and that the arsenic insult does not have to be contemporaneous with the other agent but rather can come before (Waalkes et al., 2008). Indeed, an ingrained oxidant adaptation during inorganic arsenic exposure in fetal life would help to explain the temporal difference in the study where fetal arsenic predisposes mice to adulthood TPA-induced SCC (Waalkes et al., 2008). Again, TPA is known to act in the skin through production of oxidative stress (Kausar et al., 2003).

Accumulated evidence suggests that oxidative stress, resulting from an imbalance between cellular ROS production and antioxidative defense systems, can occur in response to arsenic exposure (Pi et al., 2003) and may be one factor in dermal arsenic carcinogenesis. Keratinocytes contain high levels of antioxidants involved in antioxidant defense, including superoxide dismutase and catalase (Liuchev and Fridovich, 1994). *Nrf2*, which is negatively regulated by *Keap1*, is critical in the regulation of antioxidant genes in response to the oxidative stress (Ishii et al., 2000). Nevertheless, generation of high levels of ROS by UV irradiation can overwhelm normal defenses against oxidative damage, leading eventually to cell death either by apoptosis or necrosis (Pourzand and Tyrrell, 1999; Morita and Krutmann, 2000). *Nrf2/Keap1* is highly expressed with acute exposure to UV irradiation in keratinocytes (Pi et al., 2003). However, A diminished *Nrf2* and *Nrf2*-mediated antioxidant (*HO-1*, *NQO1*, *GCLC*) response was observed in As-TM cells when exposed to high level arsenic in contrast to increased expression in control HaCaT cells (Pi et al., 2008). Furthermore, the expression of *Nrf2* and *Keap1* showed little or no increases after UV in As-TM cells compared to marked increases in passage-matched control cells, and *Nrf2*-regulated genes such as *HO-1* and *GCLs* were at control levels indicating adaptation to arsenic modified response of *Nrf2/Keap1* antioxidant system in these As-TM cells and this shows cross adaption to UV. The *SODs* are decreased in human skin cancers (Kobayashi et al., 1991), and expression of *SOD1* and 2 in the present study were minimally increased in As-TM cells after UV, suggesting that requirements for activation of ROS-scavenging enzymes are increased after chronic arsenic exposure, and again this shows a cross-adaption to UV exposure. In contrast to markedly increased expression of *Nrf2* and its target genes in passage-matched control cells with UV treatment, the diminished activation of *Nrf2* and its related genes in response to UV exposure observed in As-TM cells, coupled with their acquired apoptotic resistance to UV irradiation, point towards enhanced survival of cells with more ODD via apoptotic by-pass. This may help explain the remarkable co-carcinogenic effects of arsenic plus oxidant carcinogen observed in mouse models of skin carcinogenesis but the absence of activity in the same models for arsenic alone (Rossman et al., 2001; Germolec et al., 1997; Germolec et al., 1998).

Elevated expression of MMP-9 strongly correlates with malignant phenotype in SCC (Bernhard et al., 1994) and more specifically, hypersecretion of MMP-9 is often seen in cells malignantly transformed with arsenic (Pi et al., 2008). The abilities of migration and invasion are also important characteristics in cancer cells. In the present study, UV-treated As-TM cells showed a marked increase in secreted MMP-9 activity and invasion ability compared with As-TM or passage-matched control cells, fortifying oncogenic potential of co-exposure of UV and inorganic arsenic. Cell proliferation as a consequence of cell cycle progression is a critical process leading to expansion of initiated cells. In this regard, overexpression of *cyclin D1* is frequently observed in arsenic-transformed cells and arsenic-induced cancers (Liu et al., 2006; Waalkes et al., 2004). This includes skin cancer induced by inorganic arsenic and UV or TPA co-exposure (Rossman et al., 2001; Waalkes et al., 2008). *Cyclin D1* also plays an important role in G₁-S transitions induced by UV exposure (Trouba et al., 2002). Expression of *p16*, a tumor suppressor gene, is suppressed by arsenic and TPA co-exposure in a mouse skin cancer model (Waalkes et al., 2008). The further increase of *cyclin D1* expression and decrease of *p16* expression seen with UV-exposure in As-TM cells supports an enhanced cancer phenotype induced by the combined treatment

which is fortified by the aggressiveness of the skin SCC seen after *in vivo* combined treatment with arsenic and UV or other oxidants (Rossman et al., 2001; Waalkes et al., 2008).

In summary, the present study shows As-TM cells acquire resistance to UV-induced apoptosis despite increased ODD induced by UV irradiation. Thus, the potential co-carcinogenicity between UV irradiation and arsenic for skin could be due to an adaptive phenomenon to chronic arsenic exposure, which, for survival, generally mitigates the oxidative stress response, but then allows apoptotic by-pass even in the face of enhanced UV-induced oxidative stress and increased ODD.

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Abbreviations

UV	ultraviolet
H ₂ O ₂	hydrogen peroxide
TPA	12- <i>O</i> -tetradecanoylphorbol-13-acetate
ROS	reactive oxygen species
ODD	oxidative DNA damage

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Highlights

Arsenic transformation adapted to UV-induced apoptosis;
Arsenic transformation diminished oxidant response;
Arsenic transformation enhanced UV-induced DNA damage

Figure 1.

A) ODD during malignant transformation with arsenic in HaCaT cells. Cells were exposed to 100 nM sodium arsenite for 30 weeks and ODD was measured by IST (See Method). **B) UV or H₂O₂ exposure and ODD.** ODD 18 hours after UV (25 J/cm²) or 24 hours after hydrogen peroxide (H₂O₂, 5 mM) exposure, as assessed by the IST method in passage-matched control or As-TM cells. **C) UV or H₂O₂-induced apoptosis.** Apoptosis 18 hours after UV (25 J/cm²) or 24 hours after hydrogen peroxide (H₂O₂, 5 mM) exposure, as accessed by flow cytometry after annexin-V and propidium iodide staining. An asterisk (*) indicates a significant difference ($P < 0.05$) from passage-matched control. A dagger (†) indicates a significant difference versus UV-treated passage-matched control. A delta (Δ) indicates a significant difference versus H₂O₂-treated passage-matched control.

Figure 2. Apoptosis related gene expression after UV exposure

RNA was collected 18 hours after UV (25 J/cm²) exposure and transcript expression was examined by real time RT-PCR. **A)** Transcriptional expression of *Caspase-3*. **B)** Ratio of *Bcl* to *Bax*. An asterisk (*) indicates a significant difference ($P < 0.05$) from passage-matched control. A dagger (†) indicates a significant difference versus UV-treated passage-matched control.

Figure 3. Expression of *Nrf2/Keap1* and *Nrf2*-related genes after UV exposure

RNA was collected 18 hours after UV (25 J/cm²) exposure and transcript expression was examined by real time RT-PCR. **A)** Transcriptional expression of *Nrf2*. **B)** Transcriptional expression of *Keap1*. **C)** Transcriptional expression of *HO-1*. **D)** Transcriptional expression of *GCLC*. **E)** Transcriptional expression of *GCLM*. An asterisk (*) indicates a significant difference (P < 0.05) from passage-matched control. A dagger (†) indicates a significant difference versus UV-treated passage-matched control.

Figure 4. Expression of genes downstream of *Nrf2* after UV exposure

RNA was collected 18 hours after UV (25 J/cm²) exposure and transcript expression was examined by real time RT-PCR. **A)** Transcriptional expression of *SOD1*. **B)** Transcriptional expression of *SOD2*. An asterisk (*) indicates a significant difference ($P < 0.05$) from passage-matched control. A dagger (†) indicates a significant difference versus UV-treated passage-matched control.

Figure 5. Immuno-fluorescence images of Nrf2, HO-1, and NQO1 expression

Expression of Nrf2 and its related genes at the protein level by fluorescence microscopic imaging (Bar = 20 microns) with or without UV exposure in As-TM cells or passage-matched control.

Figure 6. Assessment of Malignant phenotype in UV-treated As-TM cells

Secreted activity of MMP-9 and invasion assay as indicators of cancer phenotype. **A)** Secreted MMP-9 activity. **B)** Invasion assay. An asterisk (*) indicates a significant difference ($P < 0.05$) from passage-matched control. A dagger (†) indicates a significant difference versus UV-treated passage-matched control. A dollar sign (\$) indicates a significant difference versus As-TM cells.

Figure 7. Expression of *Cyclin D1* and *p16*

RNA was collected 18 hours after UV (25 J/cm²) exposure and transcript expression was examined by real time RT-PCR. **A)** Transcriptional expression of *Cyclin D1*. **B)** Transcriptional expression of *p16*. **C)** Immuno-fluorescence images of cyclin D1 and p16 expression. Expression of the markers at the protein level by fluorescence microscopic imaging (Bar = 20 microns). An asterisk (*) indicates a significant difference ($P < 0.05$) from passage-matched control. A dagger (†) indicates a significant difference versus UV-treated passage-matched control. A dollar sign (\$) indicates a significant difference versus As-TM cells.