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Aberrant Cytokeratin Expression During Arsenic-induced Acquired Malignant Phenotype in Human HaCaT Keratinocytes Consistent with Epidermal Carcinogenesis

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Abstract

Inorganic arsenic is a known human skin carcinogen. Chronic arsenic exposure results in various human skin lesions, including hyperkeratosis and squamous cell carcinoma (SCC), both characterized by distorted cytokeratin (CK) production. Prior work shows the human skin keratinocyte HaCaT cell line, when exposed chronically for >25 weeks to a low level of inorganic arsenite (100 nM) results in cells able to produce aggressive SCC upon inoculation into nude mice. In the present study, CK expression analysis was performed in arsenic-exposed HaCaT cells during the progressive acquisition of this malignant phenotype (0 to 20 weeks) to further validate this model as relevant to epidermal carcinogenesis induced by arsenic in humans. Indeed, we observed clear evidence of acquired cancer phenotype by 20 weeks of arsenite exposure including the formation of giant cells, a >4-fold increase in colony formation in soft agar and a ~2.5-fold increase in matrix metalloproteinase-9 secretion, an enzyme often secreted by cancer cells to help invade through the local extra-cellular matrix. During this acquired malignant phenotype, various CK genes showed markedly altered expression at the transcript and protein levels in a time-dependent manner. For example, CK1, a marker of hyperkeratosis, increased up to 34-fold during arsenic-induced transformation, while CK13, a marker for dermal cancer progression, increased up to 45-fold. The stem cell marker, CK15, increased up to 7-fold, particularly during the later stages of arsenic exposure, indicating a potential emergence of cancer stem-like cells with arsenic-induced acquired malignant phenotype. The expression of involucrin and loricrin, markers for keratinocyte differentiation, increased up to 9-fold. Thus, during arsenic-induced acquired cancer phenotype in human keratinocytes, dramatic and dynamic alterations in CK expression occur which are consistent with the process of epidermal carcinogenesis helping validate this as an appropriate model for the study of arsenic-induced skin cancer.

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Keywords

Cytokeratins; arsenic; skin cancer; HaCaT cells

1. Introduction

Inorganic arsenic is a human carcinogen and a common drinking water contaminant potentially affecting millions of people worldwide (IARC 2004; NRC 1999; NRC 2001). Chronic health effects of arsenic include skin and internal cancers, peripheral vascular disease, ischemic heart disease, diabetes mellitus and hypertension (Bates et al., 1992; Tseng et al., 1968; Chen, 1990; Engel et al., 1994; Rahman et al., 1999a; Rahman et al., 1999b; Yu et al., 2002). The skin is thought to be one of the most sensitive tissues to chronic arsenic exposure. In humans, chronic exposure to arsenic results in various epidermal lesions, including hyperpigmentation, hyperkeratosis, squamous cell carcinomas (SCC) and basal cell carcinomas (IARC 2004; NRC 1999; NRC 2001). Indeed, SCC is one of the more common cancers seen in chronic arsenic exposed humans (IARC 2004; NRC 1999; NRC 2001). Arsenic-induced human skin cancers typically arise in areas of arsenic-induced hyperkeratosis and often occur as multiple lesions (Tseng et al., 1968; Yu et al., 2001). In a previous study from our group, continuous exposure of HaCaT cells, a nontumorigenic human skin keratinocyte cell line, to a low level (100 nM) of inorganic arsenite for 28 weeks induced malignant transformation, as clearly established by production of aggressive SCC after inoculation of these transformed cells into mice (Pi et al., 2008). This cell line (Pi et al., 2008) could prove useful for in depth study of arsenic-induced human skin cancer, although additional evidence, such as defining molecular events in acquired arsenic-induced malignant phenotype as similar to epidermal carcinogenesis, would be invaluable to further validate this model.

Keratins are the major structural proteins of the epidermis and represent some of the most abundant proteins in epithelial cells (Coulombe and Omary, 2002). The expression of keratin intermediate filaments appears tightly linked with tissue-specific and cell-specific differentiation of epithelial cells (Osborn and Weber, 1982). Keratins make up the two largest subgroups of intermediate filament proteins, which are the epithelial (cytokeratin [CK], type I) and trichocyte (hair/hard, type II) keratins (Rugg and Leigh, 2004). The intermediate filament types are largely conserved during malignant conversion and thus their expression in a tumor can be useful in identifying its origin (Osborn and Weber, 1982). Progressive alterations in keratin expression are clearly involved in the development of skin pathology, such as hyperkeratosis, as well as skin cancers (Tseng et al., 1968). Skin cancers frequently exhibit alterations in keratin expression which are often related to tumor grade or progression (Leigh et al., 1993; Markey et al., 1991).

Thus, SCC is common in arsenicosis patients (IARC 2004; NRC 1999; NRC 2001), the *in vitro* arsenic-transformed human keratinocyte HaCaT cell line can duplicate this tumor type in xenograft studies (Pi et al., 2008), and keratin expression is frequently altered in skin cancer relative to tumor stage (Leigh et al., 1993; Markey et al., 1991). Therefore, we explored the temporal keratin alterations in our *in vitro* model of malignant transformation of human skin keratinocytes induced by chronic low-level exposure to inorganic arsenite (Pi et al., 2008) to help further validate this as a model of arsenic-induced epidermal carcinogenesis. Various time points during transformation were tested and 25 CKs were examined at both the transcript and protein level. Our results show that during the acquisition of malignant phenotype, marked and dynamic alterations in CKs occur with arsenic exposure that are consistent with the process of epidermal carcinogenesis, making this an excellent and realistic model for further in depth study of the molecular processes of arsenic-induced human skin cancer *in vitro*.

2. Materials and Methods

2.1. Chemicals and antibodies

Sodium arsenite (NaAsO_2 ; 97% pure) 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). Antibodies against CK1, CK5, CK6, CK13, CK14, CK15, CK7/17, loricrin, filaggrin (Abcam Inc., Cambridge, MA), CK8 (Sigma Chemical Co., St. Louis, MO), CK18 and involucrin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used.

2.2. Cell culture

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), and antibiotics (100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin). Cultures were maintained at 37 °C and in a humidified 5% CO_2 atmosphere. For chronic exposure, cells were maintained continuously in medium containing 100 nM of arsenic for 20 weeks.

2.3. Soft agar colony assay

Cells were subcultured and passed through a 40 μm cell strainer (BD Biosciences) to get a single cell suspension. Plates were prepared by adding 2 ml of agar medium (0.8 ml of 1.25% agar and 1.2 ml of DMEM with 10% FBS and 10% DPBS) to each plate. HaCaT cells (12,500 cells/35 mm plate) were suspended in 1 ml DMEM with 10% FBS and 0.33% agar and layered on top of the hardened agar medium. Plates were maintained at 37 °C for 21 days. On the final day of the assay, 1 ml of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT, Sigma) was added to each plate and incubated at 37 °C for 24 hrs. Colonies were then counted using an automated colony counter. Only colonies ≥ 0.5 mm in diameter were counted as positive.

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

Cells at 70-80% confluence were washed three times with phosphate-buffered saline (PBS), and the medium was changed to serum-free DMEM. After 48 hrs, the conditioned medium was collected and kept on ice until zymographic analysis of MMP-9. MMP-9 activity was detected as described previously (Pi et al., 2008). After staining with GelCode Blue (Pierce Corp., Rockford, IL), the bands were quantified using Bio-Rad Gel Doc 2000™ Systems with Bio-Rad TDS Quantity One software.

2.5. Real time RT-PCR analysis

Total RNA was isolated from cells with TRIzol (Invitrogen, Carlsbad, CA) and purified with the 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 (C_t) values. These C_t values were first normalized with that of β -actin in the same sample and then expressed as fold change compared to control (100%). Real time fluorescence detection was carried out using a MyiQ™ SingleColor Real-Time PCR Detection System (Bio-Rad, Hercules, CA).

2.6. Western Blot analysis

After washing three times with ice-cold PBS, whole cell extracts were obtained using Cell Lysis Buffer (Cell Signaling, Technology, Inc. Beverly, MA) with 0.5% Protease Inhibitor

Cocktail (Sigma, St. Louis, MO) and 1% PMDF. Protein fractions were stored at -70°C until use. Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA) with BSA as a standard. Proteins were separated by Novex 4-12% Nupage Gel (Invitrogen, Carlsbad, CA) and transferred onto nitrocellulose membranes. The blots were probed with the primary antibodies (1:1000), followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Antibody incubations were carried out in BlockerTM BLOTTO in TBS (Pierce, Rockford, IL). Immunoreactive proteins were detected by chemiluminescence using ECL reagent (Amersham Pharmacia, Piscataway, NJ) and subsequent autoradiography. Quantitation of the results was carried out by Bio-Rad Gel Doc 2000 Systems with Bio-Rad TDS Quantity One software. After the blots were stripped using Restore Western Blot Stripping Buffer (Pierce, Rockford, IL), they were probed for β -actin (Cell Signaling Technology, Inc. Beverly, MA), which was used as the loading control.

2.7. Confocal imaging of fluorescence measurement of CKs

Cells were washed three times with PBS and fixed with 3% paraformaldehyde/0.25 % glutaraldehyde in PBS for 10 min, followed by permeabilization with 0.5% Triton X-100 for 30 min at room temperature. After washing with PBS, cells were blocked for 1 hr with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Cells in the coverglass chambers were incubated overnight at 4°C with each of the primary antibodies (CK10, CK13, involucrin and CK8/18) diluted with 1% BSA in PBS (1:100). After rinsing three times in PBS, secondary antibodies [Alexa Fluor 488 conjugated goat anti-mouse IgG (H+L) (Invitrogen) for CK10 and CK8/18, Alexa Fluor 488 conjugated rabbit anti-goat IgG (Invitrogen) for CK13 and Alexa Fluor 543 conjugated goat anti-rabbit IgG (H+L) (Invitrogen) for involucrin (1:500) diluted in PBS containing 1% BSA] were added to the cells and incubated at 37°C for 1 hr. Propidium iodide ($1\mu\text{g}/\text{ml}$) in 1% BSA was used to stain cell nuclei (1:1000) at room temperature for 10 min. After further washing with PBS, cells were transferred to Teflon microscope imaging chamber for confocal microscopy. Negative controls were treated similarly except they were not exposed to primary antibody. Negative control experiments showed no signal at the settings used to image specific fluorescence.

To acquire images, the chamber containing the specimen was mounted on the stage of a Zeiss Model 510 inverted confocal laser-scanning microscope (Carl Zeiss Inc., Thornwood, NY) and viewed through a $40\times$ water-immersion objective (numeric aperture = 1.2), with a 488-nm laser line for excitation (Ar-ion laser), a 510 nm dichroic filter and a 500-550 nm band-pass emission filter. A 543-nm laser line for excitation was also set up for viewing nuclear staining. Low laser intensity was used to avoid photobleaching. Confocal images ($512\times 512\times 8$ bits, 4 frames averaged) were acquired and saved to disk. For each tissue sample, 5-10 areas of cells were selected for image collection.

2.8. Statistical analysis

Data are expressed as mean $\pm$ SEM of 3-6 determinations. Dunnett's multiple comparison tests after ANOVA were performed for comparisons between treatment groups and control. The level of significance was set at $P \leq 0.05$ in all cases.

3. Results

3.1. Low level, chronic arsenic exposure of HaCaT cells induces oncogenic phenotype

To drive cells towards oncogenic phenotype, normal HaCaT cells were continuously exposed to a low level (100 nM) of sodium arsenite for 20 weeks, a level known to produce malignant transformation in HaCaT cells by 28 weeks or earlier based on SCC production after inoculation of cells into nude mice (Pi et al., 2008). The arsenic concentration used is comparable to the blood levels from a population which suffered chronic arsenic intoxication

in China (Pi et al., 2000) and for which arsenic-induced skin lesions and epidermal cancers were common. After 20 weeks of arsenic exposure, morphological differences were observed between the arsenic-treated cells and the passage-matched control cells. Control cells maintained an epithelial-like morphology, while arsenic-treated cells exhibited morphological alterations with the frequent occurrence of giant multinuclear cells (Fig. 1A). The soft agar colony assay is an important method to identify malignantly transformed cells *in vitro*. Colony formation was 4.4-fold higher in the arsenic-treated cells than in passage-matched control cells (Fig. 1B). In addition, a marked increase in the secretion of active MMP-9 from arsenic-treated cells occurred (Fig. 1C). Extracellular MMP-9 activity was 2.4-fold higher in the arsenic-treated cells compared to the passage-matched untreated control cells. These are common characteristics in cells with an acquired cancer phenotype. Thus, the cells tested in the present work after 20 weeks of arsenic exposure likely represent a malignant transformant.

3.2. Alterations of CK genes with arsenic treatment

CKs are major structural proteins in epithelial cells, and progressive alterations in CK expression are closely associated with the development of a variety of skin cancers, including SCC (Leigh et al., 1993; Markey et al., 1991). Thus, we looked in greater detail at CK expression at various times during arsenic-induced transformation. In this regard, HaCaT cells were exposed to inorganic arsenic for up to 20 weeks and compared to passage-matched, untreated cells as controls. At various time points, a battery of 25 CK genes was assessed for expression. Approximately half of the CK genes showed significantly altered transcript levels in a time-dependent manner. These alterations in CK gene expressions generally occurred as early as 4 weeks of arsenic exposure, progressively increased with time, and often peaked at 20 weeks of exposure.

For instance, the transcript level for CK1, a CK often associated with hyperkeratosis, progressively increased up to a maximum of 34-fold during 20 weeks of arsenic exposure (Fig. 2A). CK 10, which is typically co-expressed with CK1, was also markedly increased at the transcript level up to 67-fold by arsenic exposure (Fig. 2B). Arsenic also increased the transcript level of CK13, a biomarker for dermal cancer progression, up to 45-fold (Fig. 2C). These increases in CK1, 10 and 13 with arsenic were confirmed at the protein level by Western blot analysis (Fig. 2D) and confocal laser scanning micrographic analysis (Fig. 2E) at 20 weeks.

The transcript levels of CK5 and CK14, which are expressed in the basal layer of skin, increased up to 6-fold and 4-fold, respectively, during arsenic-induced transformation (Fig. 3A and 3B). The transcript level of the stem cell marker, CK15, increased up to 7-fold, particularly during the later stages of exposure indicating the potential emergence of a stem cell-like population with chronic arsenic treatment (Fig. 3C). The increases in these genes were also confirmed at the protein level by Western blot (Fig. 3D) at 20 weeks of arsenic exposure.

CK8 and CK18 are expressed together, are often the earliest keratin genes expressed during embryogenesis, and are differentiation marker genes in simple epithelial tissues. CK8 and CK18 increased at the transcript and protein levels after 20 weeks of arsenic exposure (Fig. 4A-C). Confocal laser scanning micrographic analysis also showed that the protein levels of CK8 and CK18 were also much higher after arsenic exposure (Fig. 4D).

Loricrin and filaggrin are used in the assembly of the corneocyte membrane in the granular layer. Involucrin is expressed in the spinous layer as an early differentiation marker, while loricrin is thought of as a late differentiation gene and filaggrin appears to be linked to proliferation. Loricrin, filaggrin and involucrin increased up to 3-fold, 9-fold and 7-fold, respectively (Fig. 5A-C). The increases in these 3 genes were also confirmed by Western blot analysis (Fig. 5D). Confocal scanning analysis showed that involucrin expression in cells was much higher after arsenic exposure (Fig. 5E).

CK6 is normally induced in response to stressful stimuli, such as wounding. Stress response CKs 6/16 and 7/17 are rapidly induced upon injury or inflammation. In HaCaT cells chronically exposed to low-level arsenic, the expressions of CKs 6/16 and 7/17 were increased at both the transcript and protein levels (Fig. 6), suggesting CKs 6/16 and 7/17 are expressed as part of an adaptive response to arsenic.

CKs 3, 4, 9, 12 and 20 did not significantly change at any time in arsenic-treated HaCaT cells (not shown).

4. Discussion

Our prior work shows malignant transformation occurs with continuous arsenic exposure of HaCaT cells to a low, environmentally relevant level of arsenic for ~28 weeks, as indicated by production of SCC in xenograft study, and MMP-9 hyper-secretion and multinuclear giant cell formation *in vitro* (Pi et al., 2008). MMPs degrade the extracellular matrix, aid in invasion and are often hyper-secreted by aggressive tumors (Liotta et al., 1980). MMP-9 hyper-secretion is commonly observed after malignant transformation with arsenic (Pi et al., 2008; Benbrahim-Tallaa et al., 2005; Achanzar et al., 2002). In the present study, HaCaT cells exposed to the same level of arsenic (Pi et al., 2008) showed marked increases in colony formation, MMP-9 secretion and multinuclear giant cells at 20 weeks, indicating they had likely already acquired a malignant phenotype. In fact, secreted MMP-9 activity at 20 weeks was essentially the same as when these cells produce SCC upon inoculation (Pi et al., 2008). Thus, it appears that the HaCaT cells exposed to arsenic in the present work have already acquired a malignant phenotype by 20 weeks of arsenic exposure. With this acquisition of malignant phenotype there was a dramatic and dynamic series of changes in CK expression in this study that were consistent with the process of epidermal carcinogenesis (Tseng et al., 1968; Leigh et al., 1993). This fortifies the use of these transformed cells as a valid *in vitro* model of human skin cancer induced by arsenic.

Progressive alterations in keratin expressions are closely associated with the development of a variety of tumors including skin malignancies (Chu and Weiss, 2002; Casanova et al., 2004). Study of chronic arsenic poisoning in Taiwan indicates progressive alterations in CK expression occurs in various skin lesions, like hyperkeratosis and SCC (Yu et al., 1993). However, an integrated investigation of CKs during chronic arsenic-induced acquired malignant phenotype in a specific target cell of concern is not available. The present study was performed to systematically assess the expression of CKs during arsenic-induced malignant transformation in a human skin keratinocyte line, and to help define the expression pattern of various CKs in arsenic-induced skin cancer in a relevant human model. Dramatic and dynamic alterations in CK expressions occurred with arsenic that are largely consistent with the process of epidermal carcinogenesis, and support and expand on the limited available human data with arsenic (Yu et al., 1993).

Hyperkeratosis is one of the most common skin lesions with chronic arsenic poisoning (Pi et al., 2000), and considered a sign of aberrant cell proliferation, and likely a precursor skin lesion of SCC (Alain et al., 1993). The over-expression of CK1 and CK10 signal over-production of keratinocytes. Pathologically, keratinizing SCCs are consistently positive for CK1 and CK10, while nonkeratinizing SCCs are negative (Remotti et al., 2001). In the present study, the time-dependent increases of CK1 and CK10 with arsenic exposure clearly indicate these two CKs are linked to arsenic-induced malignant transformation. This would be consistent with hyperkeratotic lesions and keratin positive SCC in arsenic-exposed patients, and the fact that arsenic-associated SCC typically arise in areas of arsenic-induced hyperkeratosis (Tseng et al., 1968; Yu et al., 2001). Furthermore, the SCC formed after inoculation of these arsenic-exposed cells are similarly keratin positive (Pi et al., 2008).

Over-expression of CK13 appears to be a marker for skin tumor progression (Warren et al., 1993; Slaga et al., 1995), and occurs in HaCaT cells malignantly transformed with UV irradiation (He et al., 2006). Although not normally expressed in the epidermis, CK13 is expressed in epidermal tumors (Caulin et al., 1993). Again these findings suggest chronic arsenic exposure has initiated changes in CK expression in potential target cells consistent with similar CK expression seen in epidermal carcinogenesis.

Evolving theory in carcinogenesis proposes tumors originate from pluripotent stem cells with self-renewal capacity and conditional immortality, characteristics required for accumulating the genetic alterations needed for acquisition of cancer phenotype (Perez-Losada and Balmain, 2003). CK5, 14 and 15 are all considered markers for epidermal stem cells (Gerdes and Yuspa, 2005; Lyle et al., 1998; Liu et al., 2003). Recent evidence indicates that arsenic exposure in the fetal mouse facilitates cancer response in adulthood by distorting skin tumor stem cell signaling and population dynamics, creating an over-abundance of cancer stem cells in resulting SCC (Waalkes et al., 2008). This implicates stem cells as a primary target of arsenic in the fetal basis of skin cancer in adulthood (Waalkes et al., 2008). Other work (Patterson and Rice, 2007) shows arsenic *in vitro* delays exit of human epidermal stem cell into differentiation pathways, thus increasing stem cells and potentially increasing target cell number for carcinogenic insult. CK15 is expressed in various skin tumors (Misago and Narisawa, 2006; Kanitakis et al., 1999), while UV irradiation increases CK5 and CK14 expression of human keratinocytes (Kinouchi et al., 2002). In the present study, arsenic increased CK5, CK14 and CK15. Together with the increased expression of additional stem cell markers in arsenic-treated HaCaT cells, such as p63 (~2-fold at 20 weeks; not shown), this indicates arsenic transformation might be involved at the level of stem cells. Additional study on chronic arsenic exposure and stem cells dynamics is required to elucidate this relationship, however.

CK8 and CK18 are often over-expressed in SCC, particularly when poorly differentiated (Markey et al., 1991) or invasive (Schaafsma et al., 1993). Again, the over-expression of CK8 and CK18 in arsenic-treated HaCaT cells in the present work is consistent with a molecular pathology of SCC.

Loricrin transcription can be up-regulated when keratinocytes differentiation is stimulated (Hohl et al., 1991). Filaggrin is a filament-associated protein which binds to keratin fibers in epidermal cells. Involucrin expression is increased in cultured keratinocytes following treatment with TPA, a tumor promoter (Efimova et al., 1998). The increased expressions of loricrin, filaggrin and involucrin in arsenic-treated HaCaT cells indicate that chronic arsenic exposure may also be involved in aberrant terminal differentiation of the epidermis.

Keratinocyte activation after wounding involves cellular changes at the wounds edge, which are accompanied by induction of stress response CKs 6, 16 and 17 (Mansbridge and Knapp, 1987). Increases in CKs 6, 16, and 17 are considered a hallmark of keratinocyte activation and are seen in hyperproliferative skin disorders (Weiss et al., 1984). CK6 and CK16 are found in SCC while CK17 is widely expressed in invasive SCC (Leigh et al., 1993). CKs 6, 16 and 17 are also found in arsenic-induced SCC in human populations (Yu et al., 1993). The expression of CK6/16 and 7/17 were increased by arsenic transformation in the present work, suggesting they are part of an adaptive response to chronic injury or stress. Again our *in vitro* system appears to duplicate the responses seen *in vivo* during epidermal oncogenesis with many of the specific changes seen with arsenic.

In conclusion, chronic arsenic exposure of human keratinocytes at environmentally relevant levels drives them towards malignant transformation. Multiple molecular events are likely associated with this arsenic-induced transformation. Dynamic and dramatic alterations in CK expression occur that frequently duplicate epidermal carcinogenesis in general and arsenic skin

cancer in particular. This further establishes these transformed cells as an important model for in-depth molecular analysis of the events associated with arsenic-induced skin cancer.

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References

- Achanzar WE, Brambila EM, Diwan BA, Webber MM, Waalkes MP. Inorganic arsenite-induced malignant transformation of human prostate epithelial cells. *J. Natl. Cancer Inst* 2002;94:1888–1891. [PubMed: 12488483]
- Alain G, Tousignant J, Rozenfarb E. Chronic arsenic toxicity. *Int. J. Dermatol* 1993;32:899–901. [PubMed: 8125698]
- Bates MN, Smith AH, Hopenhayn-Rich C. Arsenic ingestion and internal cancers: a review. *Am. J. Epidemiol* 1992;135:462–476. [PubMed: 1570813]
- Benbrahim-Tallaa L, Waterland RA, Styblo M, Achanzar WE, Webber MM, Waalkes MP. Molecular events associated with arsenic-induced malignant transformation of human prostatic epithelial cells: aberrant genomic DNA methylation and K-ras oncogene activation. *Toxicol. Appl. Pharmacol* 2005;206:288–298. [PubMed: 16039940]
- Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J. Cell. Biol* 1988;106:761–771. [PubMed: 2450098]
- Casanova ML, Bravo A, Martínez-Palacio J, Fernández-Aceñero MJ, Villanueva C, Laucher F, Conti CJ, Jorcano JL. Epidermal abnormalities and increased malignancy of skin tumors in human epidermal keratin 8-expressing transgenic mice. *FASEB J* 2004;18:1556–1558. [PubMed: 15319370]
- Caulin C, Bauluz C, Gandarillas A, Cana A, Quintanilla M. Changes in keratin expression during malignant progression of transformed mouse epidermal keratinocytes. *Exp. Cell Res* 1993;204:11–21. [PubMed: 7677983]
- Chen CJ. Blackfoot disease [Letter]. *Lancet* 1990;336:442. [PubMed: 1974970]
- Chu PG, Weiss LM. Keratin expression in human tissues and neoplasms. *Histopathol* 2002;40:403–439.
- Coulombe PA, Omary MB. ‘Hard’ and ‘soft’ principles defining the structure, function and regulation of keratin intermediate filaments. *Curr. Opin. Cell Biol* 2002;14:110–122. [PubMed: 11792552]
- Efimova T, LaCelle P, Welter JF, Ecker RL. Regulation of human involucrin promoter activity by a protein kinase C, Ras, MEKK1, MEK3, p38/RK, AP1 signal transduction pathway. *J. Biol. Chem* 1998;273:24387–24395. [PubMed: 9733728]
- Engel RR, Hopenhayn-Rich C, Receveur O, Smith AH. Vascular effects of chronic arsenic exposure: a review. *Epidemiol. Rev* 1994;16:184–209. [PubMed: 7713176]
- Fuchs E. Keratins and skin. *Annu. Rev. Cell Dev. Biol* 1995;11:123–153. [PubMed: 8689554]
- Gerdes MJ, Yuspa SH. The contribution of epidermal stem cells to skin cancer. *Stem Cell Rev* 2005;1:225–232. [PubMed: 17142859]
- Gonsebatt ME, Del-Razo LM, Cerbon MA, Zúñiga O, Sanchez-Peña LC, Ramírez P. Arsenite induced oxidative damage in mouse liver is associated with increased cytokeratin 18 expression. *Arch. Toxicol* 2007;81:619–626. [PubMed: 17340120]
- He YY, Pi J, Huang JL, Diwan BA, Waalkes MP, Chignell CF. Chronic UVA irradiation of human HaCaT keratinocytes induces malignant transformation associated with acquired apoptotic resistance. *Oncogene* 2006;25:3680–3688. [PubMed: 16682958]
- Hohl D, Lichti U, Bretkreutz D, Steinert PM, Roop DR. Transcription of the human loricrin gene in vitro is induced by calcium and cell density and suppressed by retinoic acid. *J. Invest. Dermatol* 1991;96:414–418. [PubMed: 2007780]

- IARC. International Agency for Research on Cancer Monographs on Evaluation of Carcinogenic Risk to Humans. Some Drinking Water Disinfectants and Contaminants, including Arsenic. Lyon, France, 2004;84:269–477.
- Kanidakis J, Bouchany D, Faure M, Claudy A. Expression of the hair stem cell-specific keratin 15 in pilar tumors of the skin. *Europ. J. Dermatol* 1999;9:363–365.
- Kinouchi M, Takahashi H, Itoh Y, Ishida-Yamamoto A, Iizuda H. Ultraviolet B irradiation increases keratin 5 and keratin 14 expression through epidermal growth factor receptor of SV40-transformed human keratinocytes. *Arch. Dermatol. Res* 2002;293:634–641. [PubMed: 11875647]
- Leigh IM, Purkis PE, Markey A, Collins P, Neill S, Proby C, Glover M, Lane EB. Keratinocyte alterations in skin tumor development. *Recent Results Cancer Res* 1993;128:179–191. [PubMed: 7689238]
- Liotta LA, Tryggvason K, Garbisa S, Hart I, Foltz CM, Shafie S. Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature* 1980;284:67–68. [PubMed: 6243750]
- Liu Y, Lyle S, Yang Z, Cotsarelis G. Keratin 15 promoter targets putative epithelial stem cells in the hair follicle bulge. *J. Invest. Dermatol* 2003;121:963–968. [PubMed: 14708593]
- Lyle S, Christofidou-Solomidou M, Liu Y, Elder DE, Albelda S, Cotsarelis G. The C8/144B monoclonal antibody recognizes cytokeratin 15 and defines the location of human hair follicle stem cells. *J. Cell Sci* 1998;111:3179–3188. [PubMed: 9763512]
- Mansbridge J, Knapp A. Changes in keratinocyte maturation during wound healing. *J. Invest. Dermatol* 1987;89:253–263. [PubMed: 2442269]
- Markey AC, Lane EB, Churchill LJ, McDonald DM, Leigh IM. Expression of simple epithelial keratins 8 and 18 in epidermal neoplasia. *J. Invest. Dermatol* 1991;97:763–770. [PubMed: 1717607]
- Misago N, Narisawa Y. Cytokeratin 15 expression in apocrine mixed tumors of the skin and other benign neoplasms with apocrine differentiation. *J. Dermatol* 2006;1:2–9. [PubMed: 16469077]
- NRC. Arsenic in the drinking water. National Research Council, National Academy; Washington, DC: 1999. p. 1-320.
- NRC. Arsenic in the drinking water. National Research Council, National Academy; Washington, DC: 2001. p. 1-244.
- Osborn M, Weber K. Intermediate filaments: cell-type specific markers in differentiation and pathology. *Cell* 1982;31:303–306. [PubMed: 6891619]
- Patterson TJ, Rice RH. Arsenite and insulin exhibit opposing effects on epidermal growth factor receptor and keratinocyte proliferative potential. *Toxicol. Appl. Pharmacol* 2007;221:119–128. [PubMed: 17400267]
- Perez-Losada J, Balmain A. Stem-cell hierarchy in skin cancer. *Nat. Rev. Cancer* 2003;3:434–443. [PubMed: 12778133]
- Pi J, Diwan BA, Sun Y, Liu J, Qu W, He YY, Styblo M, Waalkes MP. Role of protein kinase CK2 in perturbed Nrf2-mediated oxidative stress response in human keratinocytes during arsenic-induced malignant transformation. *Free Radic. Bio. Med* 2008;45:651–658. [PubMed: 18572023]
- Pi J, Kumagai Y, Sun G, Yamauchi H, Yoshida T, Iso H, Endo A, Yu L, Yuki K, Miyauchi T, Shimojo N. Decreased serum concentrations of nitric oxide metabolites among Chinese in an endemic area of chronic arsenic poisoning in Inner Mongolia. *Free Radic. Biol. Med* 2000;28:1137–1142. [PubMed: 10832076]
- Rahman M, Tondel M, Ahmad SA, Chowdhury IA, Faruquee MH, Axelson O. Hypertension and arsenic exposure in Bangladesh. *Hypertension* 1999a;33:74–78. [PubMed: 9931084]
- Rahman M, Tondel M, Chowdhury IA, Axelson O. Relations between exposure to arsenic, skin lesions, and glucosuria. *Occup. Environ. Med* 1999b;56:277–81. [PubMed: 10450246]
- Remotti F, Fetsch JF, Miettinen M. Keratin 1 expression in endothelia and mesenchymal tumors: an immunohistochemical analysis of normal and neoplastic tissues. *Hum. Pathol* 2001;32:873–879. [PubMed: 11521233]
- Rugg EL, Leigh IM. The keratins and their disorders. *Am. J. Med. Genet. Part C (Semin. Med. Genet)* 2004;131C:4–11. [PubMed: 15452838]
- Schaafsma HE, Van Der Velden LA, Manni JJ, Peters H, Link M, Rutter DJ, Ramaekers FC. Increased expression of cytokeratins 8, 18 and vimentin in the invasion front of mucosal squamous cell carcinoma. *J. Pathol* 1993;170:77–86. [PubMed: 7686975]

- Shannon RL, Strayer DS. Arsenic-induced skin toxicity. *Hum. Toxicol* 1989;8:99–104. [PubMed: 2663702]
- Slaga TJ, DiGiovanni J, Winberg LD, Budunova IV. Skin carcinogenesis: characteristics, mechanisms, and prevention. *Prog. Clin. Biol. Res* 1995;391:1–20. [PubMed: 8532708]
- 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]
- Yu HS, Chiou KS, Chen GS, Yang RC, Chang SF. Progressive alterations of cytokeratin expressions in the process of chronic arsenism. *J. Dermatol* 1993;20:741–745. [PubMed: 7509818]
- Yu HS, Lee CH, Chen GS. Peripheral vascular diseases resulting from chronic arsenical poisoning. *J. Dermatol* 2002;29:123–130. [PubMed: 11990246]
- Yu HS, Lee CH, Jee SH, Ho CK, Guo YL. Environmental and occupational skin diseases in Taiwan. *J. Dermatol* 2001;28:628–631. [PubMed: 11770720]
- Waalkes MP, Liu J, Germolec DR, Trempus CS, Cannon RE, Tokar EJ, Tennant RW, Ward JM, Diwan BA. Arsenic exposure in utero exacerbates skin cancer response in adulthood with contemporaneous distortion of tumor stem cell dynamics. *Cancer Res* 2008;68:8278–8285.
- Warren BS, Naylor MF, Winberg LD. Induction and inhibition of tumor progression. *Proc. Soc. Exp. Biol. Med* 1993;202:9–15. [PubMed: 8093814]
- Weiss RA, Eichner R, Sun TT. Monoclonal antibody analysis of keratin expression in epidermal diseases: a 48- and 56-kdalton keratin as molecular markers for hyperproliferative keratinocytes. *J. Cell Biol* 1984;98:1397–1406. [PubMed: 6201492]

Fig. 1. Chronic low-level arsenic exposure induced malignant transformation in HaCaT cells

Cells were exposed to 100 nM arsenic for 20 weeks and compared to untreated control cells. A) Morphological alterations in arsenic-exposed HaCaT cells (Bottom) compared to control cells (top; 200 x). Arrow shows multinuclear in the giant arsenic-transformed HaCaT cell. B) Colony formation was measured by soft agar colony assay in arsenic-treated cells and passage-matched control cells. C) Analysis MMP-9 activity from arsenic-treated cells and passage-matched control cells in conditioned medium by zymogram gel. Representative zymogram (top); Bar graph of data after quantification by densitometric analysis of the bands. In all cases an asterisk (*) indicates a significant difference ($P < 0.05$) from control.

Fig. 2. Increased expressions of CK1, 10 and 13 in arsenic-treated HaCaT cells

Cells were exposed to 100 nM sodium arsenite for 4, 10 and 20 weeks and compared to untreated control cells. Transcriptional expression was examined by real time RT-PCR, and protein expression was assessed by either Western blot analysis or immunofluorescence. A) Transcriptional expression of CK1. B) Transcriptional expression of CK10. C) Transcriptional expression of CK13. D) Protein expression of CK1 and 13 by Western blot analysis. E) Protein expression of CK10 and 13 as detected by immunofluorescence. In all cases an asterisk (*) indicates a significant difference ($P < 0.05$) from passage matched control.

Fig. 3. Increased expressions of CK5, 14 and 15 in arsenic-treated HaCaT cells

Cells were exposed to 100 nM sodium arsenite for 4, 10 and 20 weeks and compared to untreated control cells. Transcriptional expression was examined by real time RT-PCR, and protein expression was assessed by either Western blot analysis or immunofluorescence. A) Transcriptional expression of CK5. B) Transcriptional expression of CK14. C) Transcriptional expression of CK15. D) Protein expression of CK5, 14 and 15 on protein level by Western blot analysis. In all cases an asterisk (*) indicates a significant difference ($P < 0.05$) from passage matched control.

Fig. 4. Increased expressions of CK8 and 18 in arsenic-treated HaCaT cells

Cells were exposed to 100 nM sodium arsenite for 4, 10 and 20 weeks and compared to untreated control cells. Transcriptional expression was examined by real time RT-PCR, and protein expression was assessed by either Western blot analysis or immunofluorescence. A) Transcriptional expression of CK8. B) Transcriptional expression of CK18. C) Protein expression of CK8 and 18 by Western blot analysis. D) Protein expression of CK8/18 as detected by immunofluorescence. In all cases an asterisk (*) indicates a significant difference ($P < 0.05$) from passage matched control.

Fig. 5. Increased expressions of loricrin, filaggrin and involucrin in arsenic-treated HaCaT cells Cells were exposed to 100 nM sodium arsenite for 4, 10 and 20 weeks and compared to untreated control cells. Transcriptional expression was examined by real time RT-PCR, and protein expression was assessed by either Western blot analysis or immunofluorescence. A) Transcriptional expression of loricrin. B) Transcriptional expression of filaggrin. C) Transcriptional expression of involucrin. D) Protein expression of loricrin, filaggrin and involucrin by Western blot analysis. E) Protein expression of involucrin as detected by immunofluorescence. In all cases an asterisk (*) indicates a significant difference ($P < 0.05$) from passage matched control.

Fig. 6. Increased expressions of CK6/16, and 7/17 in arsenic-treated HaCaT cells

Cells were exposed to 100 nM sodium arsenite for 4, 10 and 20 weeks and compared to untreated control cells. Transcriptional expression was examined by real time RT-PCR, and protein expression was assessed by Western blot analysis. A) Transcriptional expression of CK6. B) Protein expression of CK6 and 7/17 by Western blot analysis. C) Transcriptional expression of CK7 and 17. D) Transcriptional expression of CK16. In all cases an asterisk (*) indicates a significant difference ($P < 0.05$) from passage matched control.