

APOPTOTIC AND SIGNAL TRANSDUCTION CHANGES IN HUMAN KERATINOCYTES EXPOSED TO As(III) and As(V)

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ABSTRACT

Epidemiological studies suggest a link between the levels of arsenic (As) found in the drinking water and internal cancers of the bladder, liver, lung and kidney. Arsenic-related stress in mammalian cells includes metabolic abnormalities accompanied by growth inhibition and eventually apoptosis. Dermatological effects of chronic exposure to arsenic include hyperpigmentation and hyperkeratosis as well as cancers of skin. Laboratory studies of arsenic effects on skin are very limited. The objective of this study was to evaluate the effect of arsenic on apoptosis and signal transduction events in artificial human skin equivalents grown at the air-liquid interface developed in this laboratory.

Our previous studies demonstrated that As (V) and As (III) exposure at higher concentrations of arsenic on human keratinocytes (HK) grown in lifted cultures reproduce microscopically observed alterations found in human skin exposed to high arsenic levels *in vivo* in endemic areas. Furthermore, it destroys integrity of the cell membrane, and dramatically affects the rate of DNA, RNA and protein syntheses. In this study, we established that the effect of As(III) is significantly greater than that of As(V) on early apoptotic events in HK as evidenced by EM, the TUNEL[®] and DNA strand breaks gel assays. Consistent with the effects As resembling heat shock stress we were able to show dose-dependent changes in the pattern of expression of heat shock proteins of the HSP70 family by western blot.

INTRODUCTION

Arsenic produces a variety of stress responses in mammalian cells including metabolic abnormalities accompanied by growth inhibition and eventually apoptosis. Morphological alterations in cells exposed to arsenic often suggest underlying disruption of cytoskeletal structural elements responsible for cellular integrity, shape and locomotion. However, specifics of the ultrastructural changes produced by arsenic remain poorly understood. Various tissues and organs differ in their sensitivity to arsenic with the liver and skin being the most studied. Characteristic skin pathology related to arsenic exposure ranges from hyperkeratotic lesions to squamous cell carcinomas (Jaafar et al., 1993; Ohnishi et al., 1997). However, molecular events in the arsenic-exposed skin still remain to be elucidated.

Although mutagenicity of arsenic has not been unequivocally established, recent evidence supports the view that oncogenic mutations do occur, and that only selected enzymes related to DNA replication and repair are affected by arsenic. Sensitivity of the mitotic spindle to arsenic, particularly its organic compounds, underlies the well-documented chromosomal aberrations in arsenic-exposed populations (for a review see Bernstam and Nriagu, 2000).

Arsenite-induced stress at the molecular level shares many features with the heat shock response. This includes the differential sensitivity of the stress signal pathway elements to the magnitude of the stress, stressor-specific activation of the response elements and the protective role of the heat shock response. Oxidative stress, the central component of heat shock response, is typical of arsenic-related effects that are, in fact, regarded as the chemical paradigm of heat stress.

Similar to heat stress, arsenite induces heat shock proteins (HSPs) of various sizes. The signal cascade triggered by arsenite - like heat stress induces the activity of the mitogen-activated protein (MAP) kinases, extracellular regulated kinase (ERK), c-jun terminal kinase (JNK) and p38. Through the JNK and p38 pathways, arsenite activates the immediate early genes c-fos, c-jun and egr-1, usually activated by various growth factors, cytokines, differentiation signals and DNA damaging agents. Like other oxygen radical-producing stressors, arsenic induces nitric oxide production at the level of transcriptional activation along with induction of poly (ADP)-ribosylation, NAD depletion, DNA strand breaks and formation of micronuclei (Bernstam and Nriagu 2000).

So far these effects have been observed in tissues other than skin. Since skin is one of the major targets

BACKGROUND

Our previous findings established that lifted cultures of human epidermal keratinocytes (pseudo-epidermis) represent an excellent model for the study of arsenic effects in human cells (Bernstam and Nriagu, submitted). Exposure of such artificial skin models to arsenic in water faithfully reproduce gross morphological appearance of epidemiologically and clinically observed effects of arsenic on human skin *in vivo* in endemic areas. These gross morphological changes are reflected in the microscopically observed alterations of keratinocytes grown in lifted cultures. We observed a dramatic enhancement of mitotic activity determined in human skin fibroblasts exposed to different concentrations of arsenic as detected by conventional cytogenetic methods. Further As(III) and As(V) concentrations producing macro and microscopically observed pathology were found to interfere with the integrity of the cellular membrane, DNA, RNA and protein synthesis. Significant observations on the effects of As(V) on DNA, RNA and protein synthesis reveal the presence of both inhibitory and stimulatory consequences of exposure to different concentrations of As(V) consistent with the known effects of other stressors (eg., heat shock) (Bernstam and Nriagu, submitted).

Incorporation experiments were performed over 24-hr in cultures lifted for 13 days with samples removed to measure the level of precursor incorporation at 0.5, 1, 2, 3 and 24-hr of exposure. Changes in ³H-TdR incorporation in lifted cultures appear at 3 hr exposures to 10 to 1000µg/L. Variability in the pattern of ³H-TdR incorporation is noticeable to a greater extent than of ¹⁴C-leucine. On the other hand, protein synthesis appears to be decreased already at 1hr exposure to all concentrations of As(III) tested (10-1000µg/L). Interestingly, statistically significant enhancement (by up to 50%) of ¹⁴C-leucine incorporation is seen as early as within the first 0.5 hr of exposure to 100-1000µg/L As(III). ³H-TdR incorporation also demonstrates a noticeable stimulation (by up to 50%), but at different doses of exposure to As(III) – at 3 hr exposure to 10, 500 and 1000µg/L. ³H-TdR and ¹⁴C-leucine incorporation return to control levels by the end of 24 hr exposure to these concentrations of As(III). No changes in ³H-uridine incorporation are seen in cultures exposed to 10µg/L, while ³H-uridine incorporation stimulation (up to 50%) is observed within the first hr of exposure to 100 and 1000µg/L returning to control levels by 2 hr.

The stimulatory effect of As(V) on the synthetic activity at low doses and longer exposures suggests the involvement of adaptive mechanisms in keratinocytes exposed to As(V) (Bernstam and Nriagu, submitted).

OBJECTIVE

The main objective of this study was to establish the extent of regulation of expression of selected signal transduction components in the development of the response of HK to As(III) and their progression through apoptotic changes.

METHODOLOGY

Artificial skin model

Artificial skin models developed in our laboratory will be generated from a purified population of human germinative cutaneous keratinocytes (HK) as described by Bernstam et al. (1990).

Exposure to arsenic

Cornified, stratified cultures on nylon microporous membranes were exposed for varying lengths of time to arsenic solution by topical application of different concentrations of As(III) and As(V) in EBSS for designated periods of time and in minimal essential medium (MEM) for exposures of submerged cultures. After exposure the cultures were washed three times with balanced salt solution (PBS) to remove any residual arsenic before further “work-up”.

Evaluation of apoptotic changes

Apoptotic changes were detected using the TUNEL[®] assay (Apop-Tek, Intragen) in paraffin sections of submerged and lifted cultures exposed to 10-1000µg/L As(V) and As(III) reflecting DNA fragmentation in exposed cells *in situ*. Further analysis of effects of arsenic compounds on genomic DNA was performed using DNA fragmentation analysis. Genomic DNA was isolated from exposed cells using the Wizard kit (Promega) followed by agarose gel electrophoresis for the evaluation of the “ladder” pattern reflecting the extent of DNA fragmentation.

As(III) for 6 days revealed a dose dependent increase in HSP70 expression detected by Western Blot technique. Exposure to 10µg/L resulted in HSP70 band value of 0.80 while at 100µg/L this value was found to

of arsenic toxicity and with the emphasis placed in our research on elucidation of the effects of arsenic in water, the impact of arsenic in water on human skin is difficult to underestimate. We believe that understanding of intricate mechanisms of arsenic effects on human skin could offer practical insights into the most effective approaches to mitigating and preventing arsenic toxicity to humans.

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DNA fragmentation analysis. Genomic DNA was isolated from exposed cells using the Wizard kit (Promega) followed by agarose gel electrophoresis for the evaluation of the “ladder” pattern reflecting the extent of DNA fragmentation.

Western blot analysis of signal transduction protein expression

HK grown submerged were detached from the dish by scraping the cells into lysis buffer (RIPA buffer containing protease inhibitors aprotinin, sodium vanadate and PMSF). Thirty microgram of the isolated proteins were fractionated using PAGE, the bands were transferred to the PVDF membrane and the western blot performed. The primary HSP70 and p38 antibodies were from Santa Cruz Biotechnology. The secondary antibodies were supplied by Amersham Pharmacia Biotech. The detection of membrane-bound primary antibodies was done by the ECL Plus technique (Amersham Pharmacia Biotech) using Storm 860 Molecular Devices system.

RESULTS AND DISCUSSION

Effects of As(III) and As(V) on apoptosis related damage to DNA

HK, grown submerged for 24 hr, were exposed to As(III) and As(V) for various periods of time. No changes could be detected in cells exposed to 10-250 μ g/L As(V) and extensive differentiation and stratification were observed by 72hr in HK exposed to 500-1000 μ g/L. As(III) showed progressive deterioration of cell morphology and function as early as 36hr starting at 250 μ g/L (Fig. 1). In lifted cultures exposed to As(V) and As(III) the major findings can be summarized as follows: the progressive changes in cells exposed to increasing dosages of As(III) are manifested in the transition of apoptotic changes to necrotic alterations.

With an increase in As(III) concentration the changes observed at 6hr 10ppb exposure resemble those found in cells exposed to 100 μ g/L for 1hr (Data not shown). At exposures to higher concentrations of As(III) (500 and 1000 μ g/L) occasional apoptotic changes can be observed already within 1hr of exposure that are later replaced by necrotic destruction of cells exposed to 6hr at these concentrations. At the highest doses of exposure (500 and 1000 μ g/L for 72hr), the cultures are completely destroyed and no structural features can be detected. The dramatic difference in the effects of As(III) and As(V) is clearly seen in the comparison of the effects of these substances at 500 and 1000 μ g/L in HK exposed for 6hr as shown in Fig. 2.

In the case of As(V) exposure, no apoptotic changes were noticed at 1hr whereas significantly increased apoptotic changes could be observed at 6hr exposure to 100, 500 and 1000 μ g/L As(V) (Manuscript in preparation).

Since we established unequivocally that As(III) effects produce significantly higher toxicity in HK we further concentrated on detailed analysis of As(III). Apoptotic changes were evaluated using DNA gel electrophoresis to detect the “ladder” pattern and the Comet assay testifying to DNA fragmentation in the course of apoptosis. The earliest definitive changes in the Comet patterns of the HK and human dermal fibroblasts are observed within 2hr of exposure to 5 μ g/L of As(III) (Manuscript in preparation).

Effects of As(III) on the expression of selected stress proteins

Initially we limited the evaluation of As(III) effects on the signal transduction pathway in submerged HK cultures to estimates of HSP70 and p38 expression representative indicators. In evaluating the degree of expression of signal transduction pathway components it should be noted that exposure of HK cultures to As(III) produces a dose-dependent decrease in protein concentration with a significant decrease at 500 μ g/L (over 30% of control) and 1000 μ g/L (over 45% of control) within 48 hrs of exposure. Lower concentrations (10 and 100 μ g/L) reduced protein concentration in cells by about 10%. At longer exposures the protein loss markedly increased reaching 20% at 10 μ g/L exposure and about 45% at 100 μ g/L in cells exposed to As(III) for 3 days. Prolonging exposure of HK to As(III) up to 6 days did not further increase protein loss at these doses (10 and 100 μ g/L) in our experiments, while higher As(III) concentrations (500 and 1000 μ g/L) produced dramatic morphological changes in HK with noticeable loss of structural integrity of cells.

A very important distinction between the effects of As(III) and As(V) was observed in HK exposed to the same doses of the toxicant. Namely, while the response of HK to As(III) showed a progressive loss of protein, the cultures exposed to increasing concentrations of As(V) initially developed loss of protein (25-27% at 10 μ g/L) that was replaced by a gradual summary increase in protein concentrations at higher As(V) doses (10-15% at 100 and 500 μ g/L and 0-5% 1000 μ g/L for 2 days). This increase can be explained by the development of an adaptive response to As(V) manifested in increased differentiation and stratification of the exposed HK. Accordingly, we observed a lower protein loss in these differentiating cells. Moreover, an increasing protein accumulation could be observed in keratinocytes exposed to higher concentrations of As(V).

For instance, there was a 14% increase in protein concentration in cells exposed to 1000µg/L for 1 hr and a 53% increase at 3hr exposure to these concentrations. At the highest doses tested (1000µg/L for 48hr), the cells were losing protein (over 25%).

Analysis of the expression of heat shock proteins 70 (HSP70) in HK exposed to 10, 100 and 500µg/L As(III) for 6 days revealed a dose dependent increase in HSP70 expression detected by Western Blot technique. Exposure to 10µg/L resulted in HSP70 band value of 0.80 while at 100µg/L this value was found to be 4.06. This increase should also be considered in the context of progressive loss of protein in HK exposed to these As(III) concentrations. Taking into account the protein loss of some 45% at 100 ppb (see above), the true increase in the expression of HSP70 in these experiments is 5.89, i.e., over 7-fold.

A similar dose dependent increase in the expression of the signal transduction protein p38 was observed in HK exposed to 10µg/L of As(III) for 6 days. These effects are being further evaluated in experiments under way.

Comparison of the findings in morphological changes, permeability assays, incorporations studies and detection of apoptotic changes reveal a consistent picture of cultured human keratinocyte responses to As(III) and As(V) confirming the higher toxicity of As(III) and the ability of cultured HK to respond to this toxicity by either stimulation of some functions (cell proliferation by MTS test, LDH cytotoxicity assay, ³H-TdR and ³H-uridine incorporation) or suppression of other functions (¹⁴C-leucine incorporation) (Bernstam and Nriagu, submitted). It is significant that with longer exposures to lower doses of the toxicant, cultured HK are apparently capable of at least partially repairing the damage produced by these exposures as demonstrated by the return of incorporation of DNA, RNA and protein precursors to control levels.

The present findings related to early apoptotic changes in HK exposed to As(III) confirm our earlier observations made at the microscopy and chromosome levels about the critical distinction between responses on HK to As(III) versus As(V), i.e., progressive loss of cells exposed to As(III) contrasting with the increased differentiation and stratification of HK exposed to As(V). It is apparent that early involvement of signal transduction pathways in As-stressed HK constitutes the controlled response of HK to potentially carcinogenic effects of As exposure. Our current efforts are directed at further evaluation of regulatory events in HK exposed to very low doses of As(III) (5-100µg/L).

References

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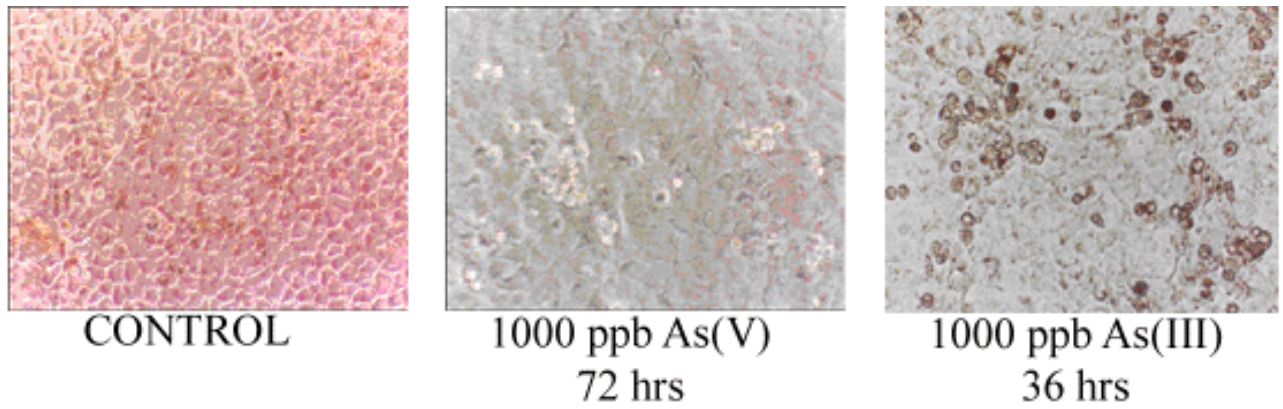


Fig.1 Phase contrast micrograph of human keratinocytes grown submerged for 24 hr cultures showing morphological changes produced by exposure to As(V) and As(III). x590

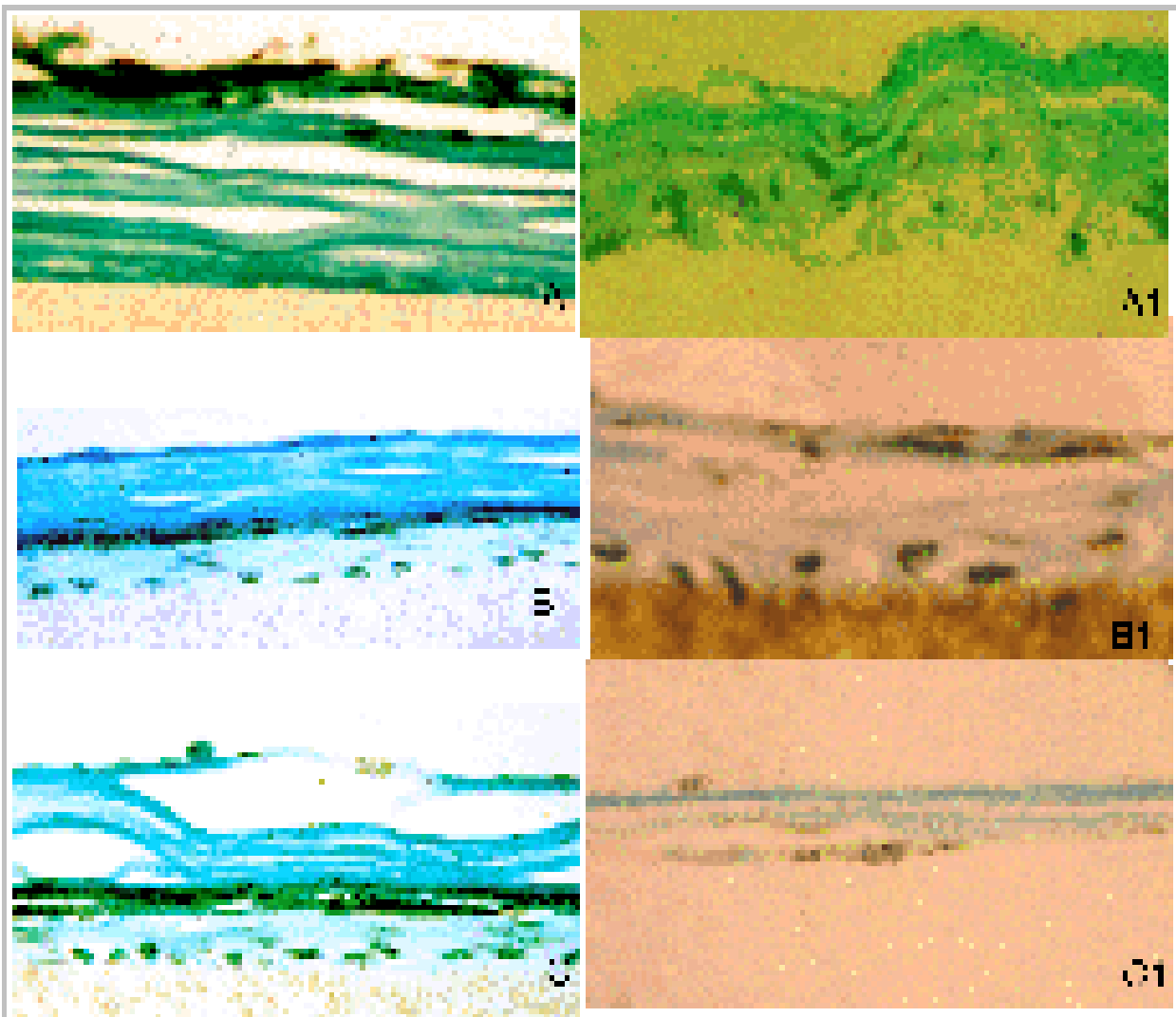


Fig.2 Photomicrographs showing dose-dependent changes in lifted cultures of human keratinocytes, typically exposed to As(V) (A,B,C) and As(III) (A1,B1,C1) for 6 hr. A,A1 -control. B,B1 - exposure to 500 µg/L. C,C1 - 1000 µg/L. Staining shows *in situ* DNA fragmentation at the single cell level.