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Induction of Cytoplasmic Accumulation of p53: A Mechanism for Low Levels of Arsenic Exposure to Predispose Cells for Malignant Transformation

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Abstract

Although epidemiologic studies have linked arsenic exposure to the development of human cancer, the mechanisms underlying the tumorigenic role of arsenic remain largely undefined. We report here that treatment of cells with sodium arsenite at the concentrations close to environmental exposure is associated with the up-regulation of Hdm2 and the accumulation of p53 in the cytoplasm. Through the mitogen-activated protein kinase pathway, arsenite stimulates the P2 promoter-mediated expression of Hdm2, which then promotes p53 nuclear export. As a consequence, the p53 response to genotoxic stress is compromised, as evidenced by the impaired p53 activation and apoptosis in response to UV irradiation or 5FU treatment. The ability of arsenite to impede p53 activation is further demonstrated by a significantly blunted p53-dependent tissue response to 5FU treatment when mice were fed with arsenite-containing water. Together, our data suggests that arsenic compounds predispose cells to malignant transformation by up-regulation of Hdm2 and subsequent p53 inactivation.

Introduction

Exposure to arsenic is a major public health concern throughout the world. Whether the source is naturally occurring deposits or industrial and agricultural runoff, the primary route of human exposure is contaminated ground water. Long-term arsenic exposure is associated with increased risks of numerous human cancers (1,2), and so, arsenic has been classified as a human carcinogen by both the U.S. Environmental Protection Agency⁴ and the IARC.⁵ Although this classification is based on the results of multiple epidemiologic studies, there is still debate as to whether arsenic is a carcinogen by itself, or instead, only acts synergistically to predispose cells to transformation by complete carcinogens. This controversy is due in part to a lack of

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

⁴<http://www.epa.gov/>

⁵<http://www.iarc.fr/>

consistent success in inducing cancer in animal models through arsenic exposure (3). Some researchers have been able to show arsenic-mediated transformation in certain cells and assays (4–6), but again, the data are not completely consistent and other results merely show hyperproliferation (7) or survival (8,9). Arsenic is at least well accepted as a cocarcinogen (10).

Available data showing the effects of arsenic on p53 are often conflicting. Although high concentrations of arsenic compounds almost always induce an increase in p53 protein levels as well as activity, lower concentrations yield various results dependent on concentration, compound, duration of treatment, and cell type (10,12). Because p53 almost likely is functionally inactivated before a cell can undergo transformation, the present study aimed to determine how the low level of arsenic affects the p53 response in nontransformed human cells and in mice.

Materials and Methods

Cell culture and transfection

MCF-10A (American Type Culture Collection), keratinocytes and p53^{-/-} mouse embryo fibroblasts (MEFs) were cultured as described (13). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer.

Preparation of whole-cell extracts and Western blotting

Preparation of whole-cell lysates and Western blot analysis have been previously described (13). Samples were boiled in the loading dye and resolved by SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and probed with the indicated antibodies: anti-p53 (Ab-6; Calbiochem), anti-Flag (M5; Sigma), anti- β -actin (AC-15; Sigma), anti-MDM2 (Ab-1, Ab-3 Oncogene), and anti-pS15p53 (Cell Signaling). Proteins were visualized with an enhanced chemiluminescence detection system (NEN).

Subcellular distribution assay

Cells were grown on coverslips, treated, and followed by 12 h of incubation with sodium arsenite or mock treatment, fixed with 4% paraformaldehyde, and stained as described (13). Slides were mounted with Fluoromount-G (Southern Biotechnology Associates). Specimens were examined under a fluorescent microscope (Zeiss).

Mice studies

C57BL/6 mice at age of 12 weeks were fed with water with or without sodium arsenite (1.0 mg/L) for 3 days. 5-Fluorouracil (5FU; 30 mg/kg) was then administered i.v. Animals were sacrificed 12 h later and whole-body perfusion with 4% paraformaldehyde was performed. Cryosections (10 μ m) were prepared on harvested organs and stained with standard procedures.

Results

In the United States, the current maximum contaminant level for arsenic in drinking water is 10 μ g/L, which is equal to ~0.5 μ mol/L of arsenic. A 0.5 or 1.0 μ mol/L concentration of arsenic was used for long-term treatment of cells. In light of the fact that concentrations of arsenic in certain areas may be much higher, and in addition, arsenic compounds accumulate within the body upon repeated exposure (14). A higher concentration of arsenic (10 μ mol/L) was selected for a short exposure (12 hours or less), which is comparable to a dose achievable through prolonged exposure to arsenic in contaminated drinking water. We chose untransformed human mammary epithelial cell line MCF-10A cells and TERT-immortalized human keratinocytes in this study for assessing the p53 response. MCF-10A cells were treated for 12 hours with 10

μmol/L of sodium arsenite. 5FU, a DNA-damaging agent able to activate p53, was included as a control. As expected, MCF-10A cells responded to 5FU by marked elevation of p53 abundance and induction of Hdm2 (Fig. 1A, left, lane 3). Interestingly, whereas arsenic treatment was associated with an increase in Hdm2, there was no noticeable change in the p53 protein level (Fig. 1A, left, lane 2). Considering the possibility that the p53 protein can, under certain circumstances, be activated by phosphorylation without a significant increase in protein abundance, we examined serine 15 phosphorylation, a well-documented biochemical marker for stress-induced p53 activation (15). Probing the membrane with an anti-pS15p53 antibody revealed that this residue was heavily phosphorylated in 5FU-treated cells, whereas no such phosphorylation was detectable in arsenic-treated cells (Fig. 1A, left, lane 2). A similar result was also seen in keratinocytes (Fig. 1A, right). Together, it seems that contrary to 5FU, arsenic treatment resulted in the up-regulation of Hdm2 without apparent p53 activation.

Although *Hdm2* is a well-documented gene controlled by p53, its expression can also be regulated by mechanisms independent of p53. The apparent discrepancy of Hdm2 up-regulation without accompanying p53 activation in arsenic-treated cells prompted us to test a p53-independent mode of regulation. We assessed the effect of arsenic in p53-null MEFs. Indeed, the results confirmed a p53-independent mechanism, as evidenced by a marked up-regulation of Hdm2 levels in arsenic-treated p53-null cells (Fig. 1B, left). To substantiate these results, we determined the effects of arsenic on the Hdm2 mRNA level. There are two well-defined promoters, P1 and P2, that control the expression of Hdm2 (Fig. 1C), of which P1 is independent and P2 can be either dependent or independent of p53 (15). Primers corresponding to the sequences of each promoter were generated for probing Hdm2 mRNAs. Arsenic treatment induced the mRNA levels driven by the P2 promoter, whereas no detectable effect on P1 promoter was observed (Fig. 1B, right). This result is further confirmed by a luciferase-based assay (Fig. 1D). Together, the results indicate that arsenic induces the expression of Hdm2 via the P2 promoter in a p53-independent fashion.

Hdm2 is an ubiquitin E3 ligase targeting p53 for ubiquitination, which can result in either proteasome-mediated degradation or nuclear export (15). The p53 abundance did not significantly change in arsenic-treated cells (Fig. 1); we thus analyzed p53 distribution in cells. Immunostaining with an anti-p53 antibody indicated that untreated MCF-10A cells exhibited relatively low levels of nuclear p53 (Fig. 2A). As expected, 5FU treatment resulted in an increase in p53 staining that is exclusively nuclear localized (Fig. 2A). Interestingly, in arsenic-treated cells, this nuclear localization was markedly reduced with an accompanying increase of cytoplasmic p53 staining (Fig. 2A). An almost identical observation was also evident in keratinocytes (Fig. 2B). Given the fact that the increased cytoplasmic p53 localization could be attributed to either increased nuclear export or reduced nuclear import, we treated cells with leptomycin B (LMB), which inhibits the activity of the exportin protein Crm1 (16), to differentiate these two possibilities. Examination of p53 distribution revealed that this nuclear export inhibitor completely abrogated the cytoplasmic p53 accumulation in arsenic-treated cells (Fig. 2C). Together, our data show that arsenic exposure is associated with increased p53 nuclear export, leading to its accumulation in the cytoplasm.

It is well documented that Hdm2 promotes p53 ubiquitination and subsequent nuclear export. However, the phosphorylation of S15 can also regulate p53 subcellular localization (17). The serine 15 residue locates within the second nuclear export signal (NES), the phosphorylation of which could result in the inactivation of this NES and thereby p53 nuclear accumulation. Because the S15 of p53 was not detectably phosphorylated in arsenic-treated cells (Fig. 1A), the increased p53 nuclear export could be due to the active NH₂-terminal NES. To test this, we examined p53 phosphorylation when arsenic-induced p53 nuclear export was blocked by LMB. Interestingly, in the presence of LMB, arsenic-treated cells displayed strong S15 phosphorylation, whereas LMB alone induced little p53 phosphorylation (Fig. 2D, left). These

results suggest that p53 nuclear export was a consequence of arsenic-induced Hdm2 expression and as a consequence, the p53 protein became inaccessible to nuclear localized protein kinases. In agreement with this notion, treatment of cells with wortmannin, an inhibitor of ATM, but not the phosphoinositide-3-kinase (PI3K) inhibitor, suppressed LMB-induced p53 phosphorylation in arsenic-treated cells (Fig. 2D, *right*).

It has been previously reported that arsenic treatment can cause changes in gene expression via activating various cellular signaling pathways (2). To elucidate the cellular pathway that mediates arsenic-induced Hdm2 expression, we selectively inhibited mitogen-activated protein kinase (MAPK), p38, nuclear factor κ B, or PI3K pathways by using specific inhibitors. Whereas the inhibitors specific to the p38, nuclear factor κ B, and PI3K pathways did not have any detectable effect, the MAPK inhibitor almost completely abrogated arsenic-induced stimulation of the Hdm2 P2 promoter (Fig. 3A), suggesting a role for the MAPK pathway in mediating the effects of arsenic. Indeed, arsenic treatment resulted in marked activation of the MAPK pathway, as evidenced by ERK phosphorylation (Fig. 3B). Our result is consistent with published data showing activation of ERK by arsenic exposure (18). To substantiate the result derived from using pharmacologic inhibitors, we used a dominant-negative mutant of MEKK, an upstream kinase of ERK, as a complement approach to block the activity of this pathway. Consistent with the results obtained from the use of chemical inhibitors, expression of dominant-negative MEKK, but not dominant-negative AKT, diminished the effect of arsenic on Hdm2 levels (Fig. 3C). Our data are in good agreement with the induction of the P2 promoter of MDM2 by the Raf/MEK/MAPK pathway (19). Together, our results indicate that the MAPK pathway mediates arsenic-induced up-regulation of Hdm2, which then promotes p53 nuclear export.

We next investigated the effect of arsenic on p53 for its biological significance. The ability of arsenic to prevent p53 from nuclear distribution would likely interfere with its function. To test this, we examined the effect of arsenic on the p53 response to UV and 5FU treatment. Significantly, both UV- and 5FU-induced p53 activation were severely compromised in the presence of arsenic, as evidenced by the p53 protein levels and S15 phosphorylation (Fig. 4A). To examine the cellular effects of arsenic-mediated interference of stress-induced p53 activation, we determined UV- or 5FU-induced cell killing in the presence or absence of arsenic. As expected, exposure of keratinocytes to 5FU or UV irradiation was associated with the induction of apoptosis. Although sodium arsenite at a concentration of 1 μ mol/L did not have a detectable effect on cell viability, pretreatment of cells with arsenic for 24 h significantly suppressed both 5FU- and UV-induced apoptosis (Fig. 4B). To further substantiate this observation, we examined the effect of arsenic on p53 response in mice. C57BL/6 mice at an age of 12 weeks were fed with water with or without sodium arsenite (1.0 mg/L) for 5 days. 5FU (30 mg/kg) was then administered i.v. Animals were sacrificed 12 hours later and tissues were harvested. Given the fact that the small intestine is one of the most sensitive tissues in response to DNA damage by undergoing p53-dependent apoptosis (20), we focused on this tissue to assess the effect of arsenic. To facilitate the visualization of cellular morphology, we stained the tissue with phalloidin (actin) and 4',6-diamidino-2-phenylindole (nucleus). As shown in Fig. 4C, the small intestines are highly organized lumen structures comprised of two single layers of epithelial cells (*a* and *e*). Mice fed with sodium arsenite-containing water did not show any signs of damage in the small intestine (*b* and *f*). In contrast, 5FU treatment resulted in marked damage in the small intestine, as evidenced by the drastic disruption of the lumen structures and loss of integrity of the outer epithelium layer (*c* and *g*). Significantly, the effect of 5FU was almost completely mitigated in mice that had been fed with arsenic-containing water. To determine whether the 5FU-induced damage was caused by apoptosis, we performed the terminal nucleotidyl transferase-mediated nick end labeling assay. The results indicate massive apoptotic cell death in the small intestines isolated from 5FU-treated mice, whereas such 5FU-induced cell death was significantly reduced in arsenic-pretreated animals (Fig. 4D).

Discussion

Epidemiologic studies have long supported a link between human exposure to inorganic arsenic and an increased incidence of cancers, which have led to intensive investigations of the underlying mechanism. Studies with various *in vitro* and *in vivo* models have revealed many insights implicating a number of mechanisms such as induction of oxidative stress and DNA damage, stimulation of cell proliferation, inhibition of DNA repair, and deregulation of DNA methylation in arsenic-induced carcinogenesis (1,2). These mechanisms are not mutually exclusive and may participate to different extents in arsenic-induced carcinogenesis. Of note is that these events can all activate p53, a tumor suppressor that functions as a transcription factor. p53 is readily activated in response to various forms of stress and increased cell proliferation, leading to the induction of its target genes the products of which mediate cell cycle arrest, DNA repair, or apoptosis. Regardless what modes of action are taken by arsenic compounds, the tumor suppressor function of p53 almost has to be compromised before a cell can undergo transformation. We now show that arsenic up-regulates the expression of Hdm2, resulting in increased p53 nuclear export, and thereby, its functional inactivation. Our study thus provides an important mechanism by which arsenic compounds predispose cells to malignant transformation.

Available evidence indicates that arsenic induces pleiotropic cellular effects. Dependent on the dosages, arsenic can be either pro-growth or pro-death; a low level of arsenic usually leads to the stimulation of cell proliferation, whereas high doses are often cytotoxic. In keeping with the biphasic dose-response of arsenic (11,12), the activity of p53 would be activated by high doses and suppressed by low levels of arsenic. Indeed, high concentrations of arsenic compounds (>20 $\mu\text{mol/L}$) almost always activate the p53 response via induction of DNA damage (18). Low levels of arsenic stimulate cell proliferation, which is incompatible with the growth-inhibitory activity of p53. Thus, the function of p53 almost has to be inactivated before arsenic can stimulate cell proliferation. Data from the present study shows that a low level of arsenic does so via ERK-dependent up-regulation of MDM2 expression. Although MAPK may regulate the level of MDM2 by multiple mechanisms, our data seems to be consistent with a mode of transcription regulation. In agreement with our finding, it has been shown that the MEK-ERK pathway stimulates the P2 promoter of MDM2 by binding to the Ets/AP1 motif (19). As one of the major p53 E3 ubiquitin ligases, MDM2 targets p53 for ubiquitination leading to either nuclear export or proteasome-mediated degradation. Although no significant reduction of steady state p53 levels was seen in arsenic-treated cells, the p53 protein was found mainly in the cytoplasm, indicating increased p53 nuclear export as the dominant consequence to arsenic-induced up-regulation of MDM2. Reactive oxygen species, the major mediator of low levels of arsenic-induced cellular effects (1,2,12), can activate p53, which, however, is not evident until the nuclear export is abrogated. The data implicates that arsenic-induced nuclear export of p53 is an early event, which prevents p53 from nuclear localized protein kinases-mediated phosphorylation/activation. In addition, the cytoplasm distribution prevents p53 from acting as a transcription factor, functionally disables this tumor suppressor, which not only makes arsenic-induced cell proliferation possible but also impedes p53 activation in response to additional stress. The functional significance of such an effect with arsenic is highlighted by the compromised apoptotic response to DNA damage agents both *in vitro* and *in vivo*. Failure to eliminate damaged cells would likely lead to the accumulation of mutations and genomic instability. Our data supports a model in which arsenic functionally disables tumor suppressor p53, which is in concert with other proposed mechanisms promoting malignant transformation.

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Figure 1.

Arsenic induces Hdm2 expression via stimulating the P2 promoter in a p53-independent manner. *A*, MCF-10A cells (*left*) or keratinocytes (*right*) were treated with sodium arsenite (10 $\mu\text{mol/L}$) or 5FU (375 $\mu\text{g/mL}$) for 12 h. Cells were lysed in radioimmunoprecipitation assay buffer, analyzed by SDS-PAGE, and Western blot with the indicated antibodies. *B*, p53-null MEFs were treated as in (*A*) and divided into two aliquots; one subjected to Western analysis (*left*) and the other reverse transcription-PCR using primers specific for P1 or P2 promoter of *hdm2* (*right*). *C*, schematic representation of the *hdm2* gene and its promoters. *D*, plasmid-encoding luciferase driven by the P1 or P2 *hdm2* promoter along with *Renilla* (pRL-TK) as a transfection efficiency control. Sodium arsenite or 5FU was added 24 h after transfection and luciferase expression was measured 36 h posttransfection. *Columns*, mean luciferase activities from experiments done in triplicate; *bars*, SD.

Figure 2.

Arsenic induces p53 accumulation in the cytoplasm. **A**, MCF-10A cells were treated as in Fig. 1 and were stained with an anti-p53 antibody as described in Materials and Methods. Cells were visualized by using a fluorescence microscope, and representative fields were photographed. 4',6-Diamidino-2-phenylindole (*DAPI*) staining is used to locate the nucleus. **B**, keratinocytes were analyzed as in **A**. **C**, MCF-10A cells were treated with 10 μ mol/L of sodium arsenite for 12 h. LMB (50 nmol/L) was added to cultures 6 h before fixing. Cells were analyzed as in **A**. **D**, MCF-10A cells were treated with as in **C** and subjected to Western analysis. 5FU-treated cells were included as a control.

Figure 3.

Arsenic induces Hdm2 expression by the ERK pathway. *A*, p53-null MEFs were transfected with the *hdm2* P2 promoter-driven luciferase vector. Cells were treated with 10 μ mol/L of sodium arsenite 24 h after transfection, and the indicated inhibitors (Calbiochem) were added 6 h later. The cells were incubated for another 6 h and then harvested for luciferase assay. *Columns*, mean luciferase activities from experiments done in triplicate; *bars*, SE. *B*, MCF-10A cells were serum starved overnight and then treated as indicated for 3 h. Cells were analyzed by Western blotting using the indicated antibodies. *C*, MCF-10A cells were treated with 10 μ mol/L of sodium arsenite for 6 h and the indicated inhibitors were added to cultures for another 6 h. Cells were analyzed by Western blotting using the indicated antibodies. *D*, MCF-10A cells were transfected with plasmids encoding dominant-negative mutant of MEKK (*MEKKDN*) or AKT (*AKTDN*). Cells were treated with arsenic for 24 h, after transfection for 12 h, and then analyzed by Western blot.

Figure 4.

Low levels of arsenic exposure impedes DNA damage-induced p53 response. *A*, keratinocytes were pretreated with or without 1.0 $\mu\text{mol/L}$ of sodium arsenite for 24 h and then treated as indicated for 12 h. Cells were analyzed by Western blotting using the indicated antibodies. *B*, keratinocytes were treated as in *A* and harvested 24 h later for FACS analysis of sub- G_1 population. *Columns*, mean percentages of sub- G_1 cells from experiments done in triplicate; *bars*, SD. *C*, C57BL/6 mice at 10 wk of age were randomly divided into four groups (three mice/group) and fed with water with or without sodium arsenite (1.0 mg/L) for 3 d. 5FU (30 mg/kg) was then administered by tail vein injection. Animals were sacrificed 12 h later and tissues were harvested. Images are representative staining of small intestine. *D*, terminal nucleotidyl transferase-mediated nick end labeling assay was performed according to the instructions of the manufacturer (Invitrogen). Apoptotic cells are shown in green.