

Genetic predisposition to the cytotoxicity of arsenic: the role of DNA damage and ATM¹

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SPECIFIC AIMS

It has been proposed that arsenic, a pervasive carcinogen in the environment, damages cells and DNA through the generation of oxidative free radicals, perhaps in a fashion similar to ionizing radiation. We sought to determine the importance of arsenic-induced DNA damage in the cytotoxicity caused by this agent, especially at environmentally relevant doses, and to reveal the genetic pathways responsible for resistance to arsenic.

PRINCIPAL FINDINGS

1. Mutation in ATM, but not other DNA repair-associated genes, renders cells hypersensitive to killing by sodium arsenite

A series of human and hamster cell lines with well-characterized deficiencies in DNA repair was subjected to the colony-forming assay for cell survival after exposure to various doses of sodium arsenite. The hamster cell line EM9 carries a mutation in the *XRCC1* gene, which plays a major role in the DNA base excision repair pathway. Mutation to *XRCC1* renders cells sensitive to agents that cause minor base damage and single-strand breaks. The clonogenic survival curves for EM9 and its nonmutated parental cell line, AA8, after 24 h exposure to 0–3000 $\mu\text{g/L}$ As(III), are very similar, as shown in Fig. 1A. [The doses of As(III) used with these cells were greater than those used with human cells because rodent cells are less sensitive to As(III) than human cells.]

The human cell lines tested included representative cell lines from the autosomal recessive disorders Xeroderma pigmentosum (XP), Fanconi anemia (FA), Bloom's syndrome (BS), and Ataxia telangiectasia (AT). These cell lines display aberrant responses to oxidative damage, in some cases to other agents such as UV (XP) and DNA cross-linking reagents (FA). The responses of the FA, BS, and XP cell lines after 24 h exposure to 0–300 $\mu\text{g/L}$ sodium arsenite are shown in Fig. 1B. Also included in this plot are survival curves for two DNA repair proficient human cell lines: lung

carcinoma cell line A549 and the normal fibroblast line, GM43. The results indicate a fairly narrow range of sensitivities, with most cells falling between 40 and 70% survival at 300 $\mu\text{g/L}$. None of the FA, BS, or XP cell lines showed any remarkable response. The most sensitive, AG06040, the BS line, had a response very similar to the repair-proficient cell line, GM43.

On the other hand, the two AT fibroblast lines, AT2BE and AT5BI, were observed to be significantly more sensitive than the other human cell lines examined (Fig. 1C). The dose of arsenic required to reduce cell survival to 50% was >2.5-fold greater for GM43 cells than either of the AT cell lines, and the surviving fraction of the AT cells at 300 $\mu\text{g/L}$ As(III) was only 0.15 compared with 0.4 for GM43. Further support for ATM involvement in the cellular response to arsenic was provided by the use of an ATM-complemented, SV40-transformed AT cell line, FT/pEBS7-YZ5, and the control AT cell line bearing the empty vector, FT/pEBS7. While FT/pEBS7 cells displayed a similar response to sodium arsenite (Fig. 1C) and radiation (Fig. 1D) as another SV40-transformed AT cell line, GM05849C, the FT/pEBS7-YZ5 cells were observed to have a normal survival response to both radiation and arsenite, which indicates that complementation with ATM restores resistance to radiation and arsenite.

Because ATM is normally associated with cellular response to DNA double-strand breaks, several additional cell lines, each carrying mutations that severely reduce repair of double-strand breaks and cause hypersensitivity to ionizing radiation, were examined. These included MO59J cells, which lack active DNA-depend-

¹ To read the full text of this article, go to <http://www.fasebj.org/cgi/doi/10.1096/fj.02-0093fje>; doi: 10.1096/fj.02-0093fje

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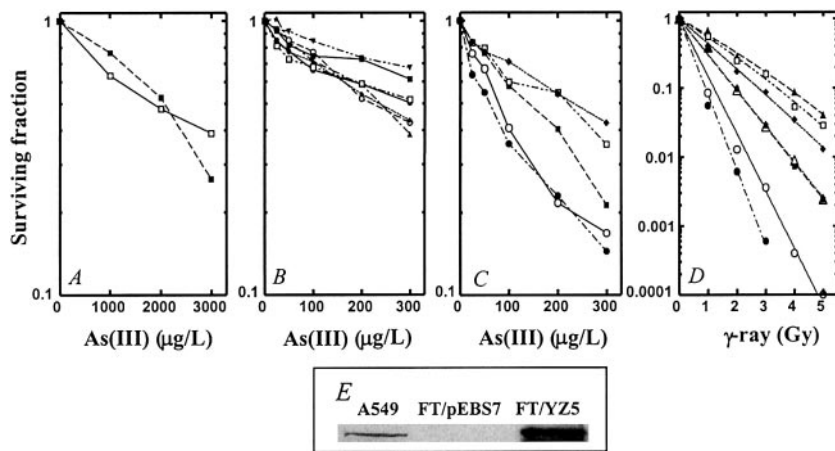


Figure 1. A) Response of Chinese hamster ovary cells to 24 h exposure to sodium arsenite. A standard colony-forming assay was used to examine the survival of (■) AA8 (wild-type); (□) EM9 (the *XRCC1* mutant). B) Clonogenic survival of human fibroblasts after 24 h exposure to increasing concentrations of As(III). The data show the results of standard colony-forming assays and are drawn from 4~8 determinations for each cell line. The following cells were examined: (■) A549 (DNA repair-proficient human lung carcinoma), (◆) GM43 (normal human fibroblasts), (○) CRL-1223 (XPA fibroblasts), (□) GM434 (XPD fibroblasts), (●) GM3021 (XPG fibroblasts), (▼) CRL-1196 (FA fibroblasts), and (▲) AG06040 (BS fibroblasts). Response of Ataxia telangiectasia

cells to 24 h exposure to sodium arsenite (C) and γ -radiation (D). Each data point represents the mean of 4–6 determinations. Cells examined include (◆) GM43 (normal human fibroblasts), (○) AT2BE (AT fibroblasts), (●) AT5BI (AT fibroblasts), (■) FT/pEBS7 (SV40-transformed AT fibroblasts transfected with the vector pEBS7), (□) FT/pEBS7-YZ5 (SV40-transformed AT fibroblasts complemented with ATM cDNA), (Δ) GM5849 (SV40-transformed AT5BI fibroblasts), and (\blacktriangle) GM0637A (SV40-transformed normal fibroblasts). E) Western blot analysis of ATM protein expression in the indicated cell lines. Used with permission from X. C. Le and M. Weinfeld (2003) *Cellular Responses to Arsenic: DNA Damage and Defense Mechanisms*, Awwa Research Foundation, Denver, CO.

dent protein kinase required for nonhomologous end-joining, and cells with mutated *NBS1*, *XRCC2*, or *XRCC3*, components of the homology-driven double-strand break repair pathway. None of these cell lines displayed a significant hypersensitive response to sodium arsenite.

2. DNA double-strand break induction by sodium arsenite

The difference in response displayed by AT cells and the double-strand break repair-deficient cell lines led us to examine the effectiveness of sodium arsenite to induce double-strand breaks. Of the various techniques currently available to assay double-strand breaks, we chose an immunochemical approach involving quantification of phosphorylation of histone H2AX because of its sensitivity. GM43 cells were either incubated with 1000 $\mu\text{g/L}$ sodium arsenite for 24 h or irradiated with 1 or 2 Gy. After cessation of treatment the cells were incubated for 30 min, then fluorescently stained for phosphorylated H2AX foci. Individual cells were chosen at random from each group of cells, and the foci detected and total fluorescence intensity per cell were quantified by confocal microscopy and specifically designed software. The results, which are presented in **Table 1**, indicate a linear dose response for irradiated cells, with 2-Gy irradiated cells displaying an almost 10-fold increase in fluorescence over untreated cells. On the other hand, no significant increase in fluorescence was observed in the As-treated cells even though the dose of sodium arsenite used was more toxic than irradiation with 2 Gy. This strongly suggests that sodium arsenite at 1000 $\mu\text{g/L}$ is a poor inducer of DNA double-strand breaks.

3. p53 protein levels and phosphorylation in response to sodium arsenite

Unlike normal cells, AT2BE and AT5BI cells failed to display any discernible accumulation of p53 after increasing exposure to arsenite. This confirms an observation made by others using a different AT cell line, GM3395. Further analysis of p53 in the repair-proficient cell line A549 revealed that exposure to As(III), unlike UV and γ -radiation, did not lead to phosphorylation of serine 15. Phosphorylation of this residue has been associated with DNA double-strand break induction.

4. Influence of sodium arsenite on the cell cycle

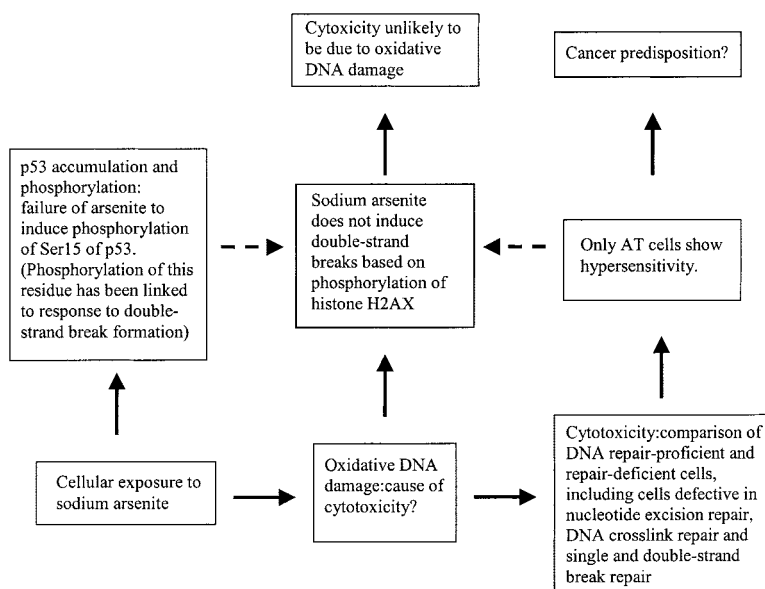
GM43 cells and AT5BI cells were examined for alterations to the percentage of cells in each phase of the cell cycle after 24 h exposure to sodium arsenite at 300 and 1000 $\mu\text{g/L}$. The results indicated that As(III) caused a significant increase in the percentage of GM43 cells in S-phase and a modest increase in cells in G2/M

TABLE 1. Fluorescence intensity of phosphorylated histone H2AX^a

Cell treatment	Fluorescence intensity \pm SE (arbitrary units)	2-tailed <i>t</i> test <i>P</i> -value
Untreated (<i>n</i> = 6)	681 \pm 186	
1 Gy (<i>n</i> = 6)	3162 \pm 690	0.012
2 Gy (<i>n</i> = 5)	6708 \pm 1504	0.0018
1000 $\mu\text{g/L}$ As(III) (<i>n</i> = 5)	717 \pm 322	0.92

^a Used with permission from X. C. Le, and M. Weinfeld (2003) *Cellular Responses to Arsenic: DNA Damage and Defense Mechanisms*, Awwa Research Foundation, Denver, CO.

Figure 2. Schematic representation of the experiments and conclusions. Treatment of cells with sodium arsenite generates oxygen free radicals and oxidative DNA damage, but is this responsible for cytotoxicity? Of a panel of DNA repair-deficient cell lines, only AT cells show significant hypersensitivity. However, sodium arsenite does not generate DNA double-strand breaks nor does it lead to phosphorylation of serine-15 of p53. Consequently, we suggest that DNA damage is unlikely to be responsible for sodium arsenite-induced cytotoxicity.



phase. By comparison, cell cycle distribution of the AT cells appeared to be unaffected by arsenic exposure.

CONCLUSIONS AND SIGNIFICANCE

Cumulatively, our observations with DNA repair-deficient cell lines, as well as direct measurement of double-strand breaks and other cellular responses that frequently accompany double-strand breaks, call into question whether DNA damage is the primary cause of death when cells are exposed to arsenic. Bulky DNA adducts requiring the nucleotide excision repair pathway can be ruled out because of the lack of abnormal sensitivity shown by the XP cells. Similarly, the cell survival curves for EM9, BS, MO59J and *NBS1*, *XRCC2*, and *XRCC3* mutated cells argue against the importance of oxidative base damage, single-strand breaks, or double-strand breaks. Fanconi anemia cells and *XRCC2* and *XRCC3* mutated cells are also known to be hypersensitive to cross-linking agents that cause DNA interstrand

cross-links, such as mitomycin C. Their normal response to arsenite suggests that DNA interstrand cross-links do not contribute to arsenic toxicity. DNA-protein cross-links are the only remaining major class of DNA lesions that cannot be ruled out by our experiments. The response of AT cells to DNA-protein cross-linking agents is unknown, but it is more likely that the hypersensitivity of AT cells to arsenite is related to faulty cell cycle regulation.

Although we have used cell survival as our major end point, this study has implications for arsenic carcinogenesis because AT is a cancer-prone disorder. The question of the cancer predisposition of AT heterozygotes, which constitute ~1% of the general population, remains controversial. However, if it is confirmed that AT heterozygotes are over-represented in cancer patient populations, it will be important to identify potential carcinogens to which AT heterozygotes are more susceptible. The results of this and other studies suggest that arsenic, because of its prevalence, could belong to this group of agents. **[F]**