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Low dose exposure to sodium arsenite synergistically interacts with UV radiation to induce mutations and alter DNA repair in human cells

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Inorganic arsenic is a known human carcinogen, yet its mechanism of action remains poorly understood. Epidemiological data suggest that arsenic exposure interacts with UV radiation exposure to increase the risk of skin cancer. Studies have suggested that arsenic is able to impair DNA repair enzymes and alter the repair of UV-induced DNA damage. Here we have tested the hypothesis that arsenite [As(III)] and UV interact synergistically to enhance mutagenesis. TK6 human lymphoblastoid cells that are functionally heterozygous at the thymidine kinase (*TK*) locus were pre-exposed to As(III) alone and in combination with UV. Our data suggest that As(III) is mutagenic only at high doses at the *TK* locus. As(III) enhanced UV mutagenesis in a more than additive fashion. To investigate the mechanism underlying this synergy we assessed the removal of UV-induced dimers in TK6 cells using the T4 endonuclease-incorporated Comet assay. Pre-treatment with As(III) specifically inhibited the repair of UV-induced pyrimidine dimer-related DNA damage. Taken together, these data suggest that pre-treatment of human cells with arsenic impairs the nucleotide excision repair pathway and leads to enhanced UV mutagenesis.

Introduction

Arsenic is a naturally occurring element that is present ubiquitously in the environment. Humans can be exposed to arsenic through food and, in some regions, through drinking water (Karagas *et al*., 1998). Epidemiological studies have shown that chronic exposure to inorganic arsenic is associated with cancers of the skin, lung and bladder (IARC, 1980). This increased risk has been mainly attributed to the presence of inorganic trivalent arsenic (arsenite) [As(III)], although pentavalent inorganic arsenic is readily converted to the trivalent form *in vivo* (Rossman *et al*., 2001). An understanding of the mechanism of action of arsenic in the carcinogenic process is still lacking. To date no animal model has shown arsenic to be directly carcinogenic.

The lack of an established animal model for arsenic-induced cancers has made studies that assess the possible mechanisms of cellular damage attributable to arsenic exposure important. Mutation assays using bacterial or mammalian cells have shown that As(III) is not a potent point mutagen (Rossman *et al*., 1980). More recently, it has been shown that low dose chronic exposure to As(III) in human osteosarcoma cells does cause mutations at the *HPRT* gene locus (Mure *et al*., 2003). Hei *et al*. (1998) demonstrated that arsenic induces large deletion mutations in a human–hamster hybrid cell. This is consistent with work in mouse lymphoma cells, where large deletions were induced at the thymidine kinase (*TK*) locus (Moore *et al*., 1997). Genotoxic damage, including chromosomal abnormalities, sister chromatid exchange, micronuclei and unscheduled DNA synthesis, as well as DNA–protein crosslinks have been shown to occur in human cells exposed to arsenic (Dong and Luo, 1993; Gonsebatt *et al*., 1997; Ramirez *et al*., 2000). Arsenic has also been found to induce the neoplastic transformation of Syrian hamster embryo cells (Takahashi *et al*., 2002). Alterations of DNA repair enzymes have also been hypothesized to be a mode of action of arsenic *in vivo* and *in vitro* (Kitchin, 2001; Vogt and Rossman, 2001).

UV damage to DNA is repaired primarily by nucleotide excision repair (NER), a major pathway for the removal of damage caused by various environmental mutagens (Hartwig *et al*., 1997; Bau *et al*., 2001). The interaction of arsenic with DNA repair enzymes has been suggested to be an important mode of action since arsenic has a high affinity for sulfhydryl groups and these enzymes are known to have sulfhydryls that could bind arsenic (Wiencke and Yager, 1992). This mechanism is consistent with arsenic acting as a co-mutagen in carcinogenesis by compromising the repair of DNA damage induced by other genotoxic agents (Li and Rossman, 1989b). Epidemiological evidence showing an increased risk of lung cancer in individuals exposed to both tobacco smoke and arsenic is also consistent with this model (Hertz-Picciotto *et al*., 1992; Tsuda *et al*., 1995). Several studies have shown As(III) to be co-mutagenic with UV light in both *Escherichia coli* (Rossman, 1981) and Chinese hamster V79 cells (Okui and Fujiwara, 1986; Li and Rossman, 1991). A synergistic interaction of arsenic and UV in the induction of mutation would be consistent with studies showing an arsenic-related impairment of DNA ligase (Li and Rossman, 1989a; Lee-Chen *et al*., 1993), poly(ADP-ribose) polymerase (PARP) (Yager and Wiencke, 1997) and inhibition of the NER pathway (Okui and Fujiwara, 1986; Hartwig *et al*., 1997). Data showing the co-mutagenic capability of arsenic and UV in human cells is lacking.

We have used a human lymphoblastoid cell line (TK6) that is functionally heterozygous at the *TK* locus (Liber and Thilly, 1982) to study As(III)- and UV-induced mutations at the *TK* locus. We have also examined the DNA damage associated with UV-induced dimers in arsenic-exposed and non-exposed cells using the T4 endonuclease V (endo V)-incorporated Comet assay.

Materials and methods

Cell culture and exposures

TK6 cells were maintained in exponential growth in RPMI 1640 medium (Mediatech Inc.) supplemented with 10% horse serum (Sigma Chemical Co.), which had been heat inactivated at 55°C for 2 h, 100 U/ml penicillin and

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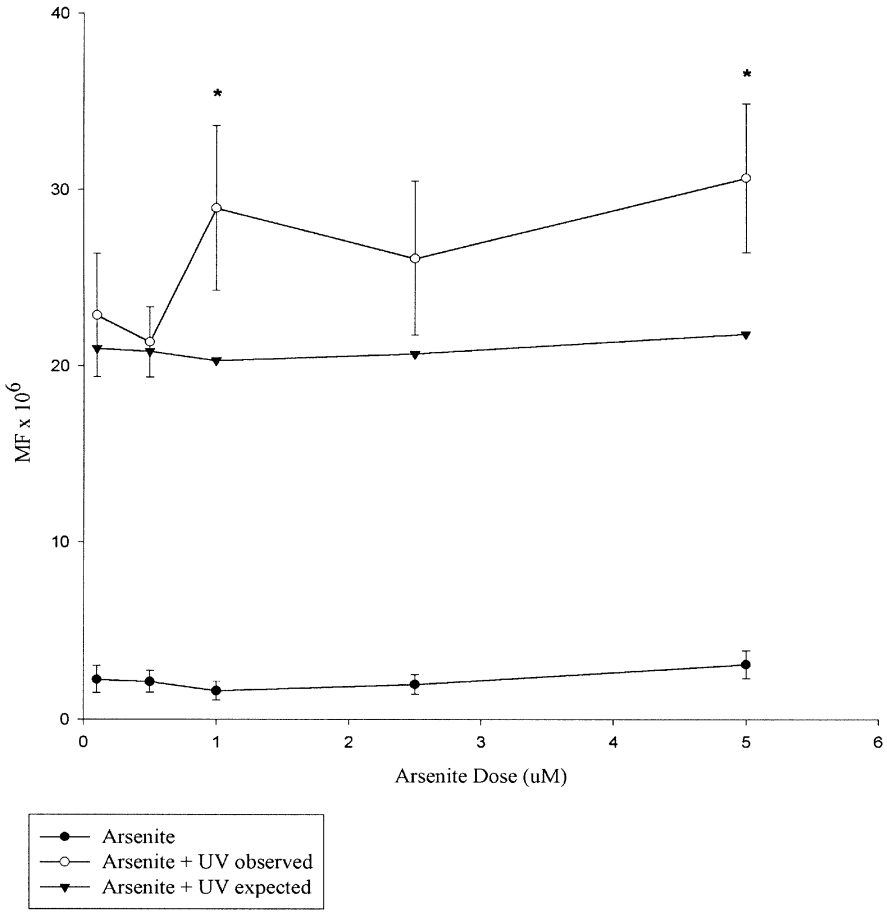

Fig. 1. As(III)-, UV- and UV + As(III)-induced mutations at the *TK* locus in TK6 cells.

100 μg/ml streptomycin (Gibco). Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. A stock solution of sodium arsenite at 1 mM (Sigma Chemical Co.) was prepared for each experiment in distilled water and filter sterilized by passage through a 0.22 μM syringe filter (Corning Corp.).

Arsenic and UV exposures

All As(III) exposures were done for 24 h at 37°C. For the mutation assay, a total of 4×10^7 TK6 cells at a density of 4×10^5 cells/ml were exposed to As(III) at doses ranging from 0 to 5 μM in T-162 flasks (Corning Corp.). Twenty-four hours after As(III) exposure the cells were harvested and the As(III) removed by washing in buffer. TK6 cells used for the Comet assay were exposed to As(III) as mentioned above, at a dose of 0 or 1 μM.

UV exposure was carried out at a dose of 5 J/m^2 using the method of DeLuca *et al*. (1983). Briefly, 3×10^6 TK6 cells were washed with serum-free phosphate-buffered saline (PBS). A 1 ml volume containing 3×10^6 cells was dispensed into the center of a 100 mm dish and the level dish was exposed to UV using a chamber containing 5 GE G8T5 germicidal lamps that deliver predominantly 254 nm light at 0.385 J/m^2/s. All UV treatments were done following 24 h of control or As(III) pre-incubation.

Mutation assay

Selection of *TK*$^-$ mutants was done using the method of Liber and Thilly (1982). Briefly, 3 days after treatments selection was performed in 96-well plates by seeding 2×10^4 cells/well in normal RPMI 1640 medium supplemented with 2 μg/ml trifluorothymidine (TFT) (Sigma Chemical Co.). Colonies were counted following 13 days growth, the plates were then re-fed with TFT and a final count was done on day 20 in order to account for slow growing mutants. Plating efficiencies of the cells were determined by plating at 1 cell/well in normal medium. Mutant frequencies were determined using the Poisson distribution and calculated using the formula:

mutant colonies observed/(plating efficiency) × (number of cells plated).

Flow cytometry analysis

TK6 cells were exposed to As(III) for 24 h at doses ranging from 0–5 μM. Following this exposure, the cells were fixed in 70% ethanol overnight and then washed in PBS containing 1.05% bovine serum albumin (BSA). A total of 5×10^5–10^6 cells were re-suspended in staining solution comprising PBS, RNase A (100 μg/ml) (Sigma) and propidium iodide (50 μg/ml) (Sigma) and incubated for 1 h in the dark. The samples were then analyzed on a Coulter Epics Altra Flow Cytometer (Beckman Coulter).

Single cell alkaline electrophoresis (Comet assay)

The alkaline Comet assay was performed according to the method of Singh *et al.* with some minor modifications (Singh *et al*., 1988). A total of 75 μl of low melting point agarose (0.6%) in PBS at 37°C was added to a 25 μl cell suspension (10^5 cells). The mixture was then dropped onto a microscope slide pre-coated with 1% agarose and a coverslip was placed on top. Once the agarose had solidified the coverslips were removed and the slides were immersed in ice-cold lysis buffer (2.5 mM NaCl, 85 mM EDTA, 10 mM Trizma base, 1% Triton X-100, 10% DMSO and 1% sodium lauryl sarcosinate, adjusted to pH 10) for 1 h at 4°C.

After lysis the slides were washed three times in PBS at room temperature. Next, 50 μl of buffer (control) or T4 endo V (Epicentre) (4 U/slide) in buffer was transferred to the slides. Coverslips were put on and the slides were incubated at 37°C for 45 min. The coverslips were then removed and the slides were washed in water twice more to remove any excess salt. Slides were then placed in a submarine gel electrophoresis chamber filled with alkaline electrophoresis buffer (0.3 M NaOH and 1 mM EDTA) for 25 min. Following this incubation, electrophoresis was performed for 30–40 min at 25 V and the current was adjusted to 300 mA by raising the buffer level. Slides were then neutralized with PBS and stained with a solution of 10 μg/ml ethidium bromide for 3–4 min. Excess stain was removed by washing in water. Slides were read using a fluorescence microscope, with the VisComet (Impuls Bildanalyse) software. A total of 100 comets/slide were read for each experiment.

Statistical analysis

Statistical tests were done using the standard *t*-test and graphed using the standard error. The mean of 3–7 experiments was used for the determination of mutation frequency. Error bars are the standard error of the mean of serial

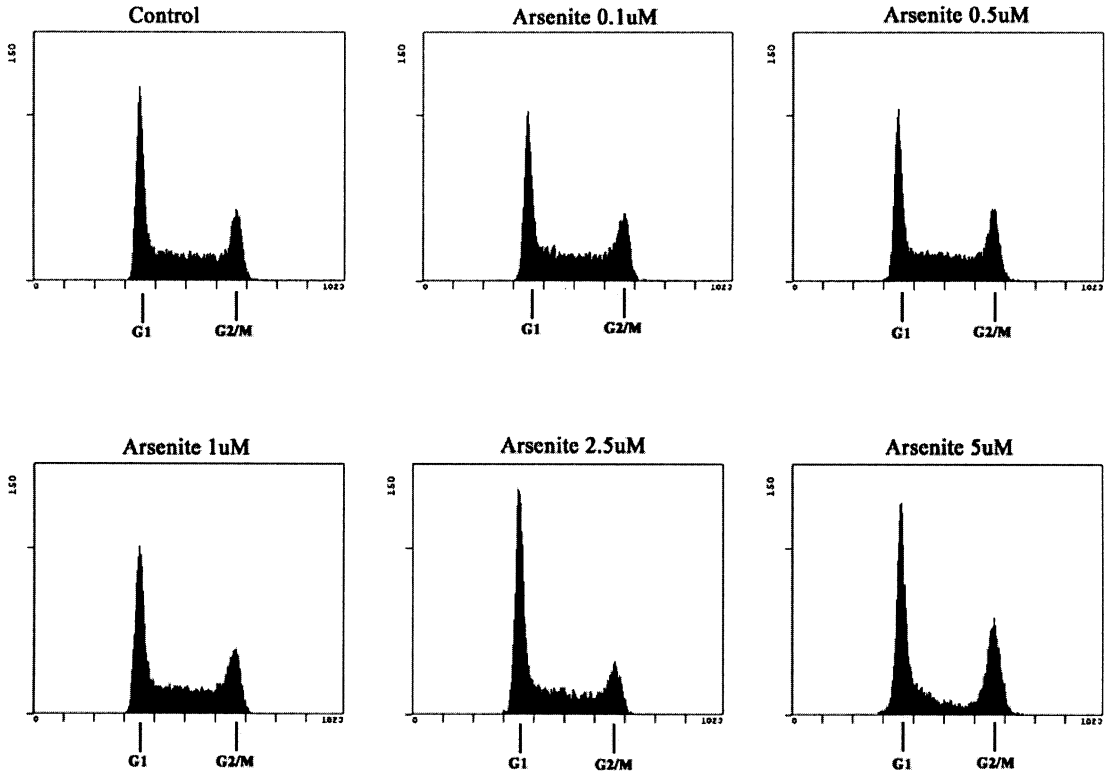

Fig. 2. Flow cytometric profile of TK6 cells following 24 h treatment with As(III) at doses of 0–5 μM. The lack of synchronization by As(III) is demonstrated in the cell cycle profile showing G_1 and G_2/M peaks for each treatment condition. No statistical difference was seen between each treatment.

experiments [* indicates $P < 0.05$ when comparing UV + As(III) expected versus observed]. The *t*-test was performed when comparing control with treated cells and UV- with As(III) + UV-treated cells. Comet data was analyzed by looking at the mean, median and the 90th percentile of the data. The mean was chosen as an appropriate measure since there were no appreciable differences in the distribution of the data. Here, the mean of 6–16 experiments was used, and 100 comets were read for each treatment. Error bars are the standard error of the mean. A linear mixed effects regression model was used for analysis of the comet data. The fixed variables in the model were time and treatment [UV, As(III) or UV + As(III)]. Experiment number was chosen as a random effect in order to control for experimental variability and in order to compare the different treatment groups. The tests for overall group differences in the model were performed using the *F*-test, and the corresponding *P* value is reported. All the data were analyzed using SAS and StatView software.

Results

Co-mutagenicity of arsenite and UV

To better understand the mutagenicity of UV and As(III), we first studied mutations induced at the *TK* locus in TK6 cells by either of these agents alone. Neither UV exposure nor As(III) treatment induced appreciable cell death at the doses tested (data not shown). We measured the mutant frequency (MF) induced by combined UV and As(III) exposure and compared this with the expected values in these groups if the effect was only additive. The expected values were calculated by adding the mean MF of As(III) at each dose to the mean MF of UV at 5 J/m^2 (18.7 ± 3.5) (data not shown). At all the doses of As(III) tested in combination with UV, the MFs were higher than expected if their effects were additive (Figure 1). In particular, the UV + As(III) groups at As(III) doses of 1 and 5 μM were significantly different from that expected for UV and As(III) treatment (Figure 1; $P < 0.04$ and $P < 0.05$, respectively). These data demonstrate that there is a greater than additive induction of mutation at the *TK* locus when cells are treated with the combination of As(III) and UV.

Arsenite exposure and cell cycle alterations

Pre-exposure to As(III) could lead to partial cell cycle synchrony, resulting in altered susceptibility to the effects of UV, in a cell cycle-dependent manner, and an increased frequency of mutations. In order to address this question, TK6 cells were exposed to As(III) at various doses ranging from 0 to 5 μM for 24 h and flow cytometry was performed to assess whether As(III) caused cell synchronization. Figure 2 shows that only at the highest dose of 5 μM was there an apparent change in the cell cycle profile, with a decrease in cells in mid to late S phase and an increase in G_2/M (although not significant). Because this change was not seen at the lower dose of 1.0 μM, where the mutagenic effect of As(III) was essentially the same as at 5 μM, it does not seem likely that partial synchrony is a factor in the response to As(III).

Arsenite delays UV-damaged DNA repair

We next sought to determine whether the enhanced mutagenesis we observed when TK6 cells were treated with As(III) prior to UV exposure was attributable to altered DNA repair as assessed by the Comet assay. At the dose of 5 J/m^2 we saw no substantial comet formation. When we incorporated endo V exposure following cell lysis, a method that specifically detects UV damage, substantial comet formation was seen (Figures 3 and 4). To evaluate changes in NER repair with co-exposure, control and As(III)-treated cells were irradiated with 5 J/m^2 UV, lysed and then treated with endo V. The amount of damage present immediately after irradiation and at 15, 60, 90 and 120 min post-irradiation was assessed using the Comet assay. Figure 3 shows representative comets from control (Figure 3A)

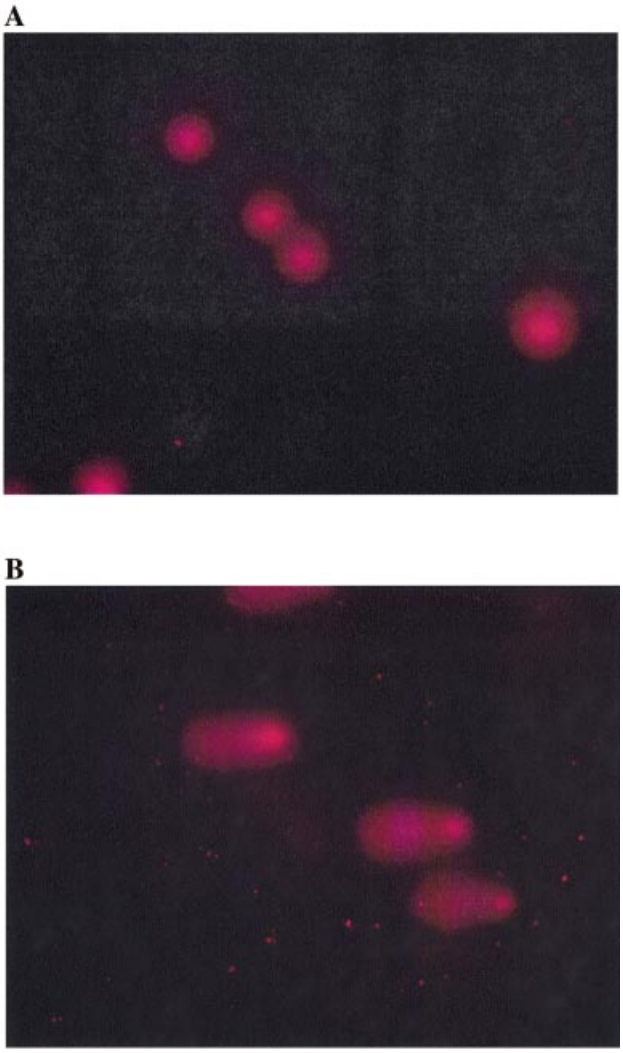

Fig. 3. Comet formation in TK6 lymphoblasts following control (**A**) or UV (5 J/m^2) treatment (**B**). Following exposures cells were lysed, treated with T4 endonuclease V, electrophoresed and stained with ethidium bromide.

and 5 J/m^2 UV-treated (Figure 3B) TK6 cells. The data were analyzed to assess differences in detectable DNA damage and repair between UV alone and UV + As(III) treatment. Comet formation was analyzed to detect differences between treatment groups.

The data for the mean of the comet distributed moment as a function of time is shown in Figure 4. There was a rapid component (15–60 min; Figure 4) of repair of UV-induced DNA damage. This rapid response was not present in the As(III) + UV-treated cells (Figure 4). In fact, we saw an initial increase (by 15 min) within As(III) pre-treated cells. The data were further analyzed by regression analysis using two mixed models (the terms are shown in Table I). This method allowed us to analyze the repair of pyrimidine dimers over time while taking into consideration random (i.e. inter-experimental) and fixed (i.e. treatment group) effects. Within these models the outcome was the comet parameter and an interaction term of UV plus As(III) was added in order to assess synergy. Two models were created taking into account first all treatment groups and time (model 1) and, second, in a more robust way looking at the interaction of time with each treatment group (model 2). We found a significant association of comet formation and UV treatment of cells (compared with control) in both models that were analyzed (Table I; $P < 0.0001$). Model 1 shows the effects of time, As(III), UV and UV + As(III). There was a significant interaction of UV + As(III) ($P = 0.04$). The more robust model taking into effect the interaction of time and the different treatments also showed similar results (model 2). Again, the same trend was seen in this model with a significant interaction between UV and As(III) ($P = 0.04$).

Discussion

Many studies examining the cellular effects of arsenic are limited or lack physiological relevance because they have been performed using high concentrations of arsenic (Hamadeh *et al*., 2002). Although arsenic has been associated with mutagenesis, these reports have utilized doses of As(III) that either affect cell survival or cell growth (Wiencke *et al*., 1997; Hei *et al*., 1998). Here we have demonstrated that by itself As(III) was slightly mutagenic at 5 μM, a dose that did not affect cell survival or substantially alter cell cycle kinetics.

We have shown that pre-treatment of human cells with As(III) for 24 h enhanced induction of mutations at the *TK* locus by UV exposure. Other groups have shown that arsenic interacts with DNA-damaging agents such as diepoxybutane, X-rays and UV to increase chromosomal aberrations (Jha *et al*., 1992; Wiencke and Yager, 1992). However, to our knowledge, this is the first report showing arsenic to be co-mutagenic with UV light in human cells.

Exposure to As(III) did not cause mutations in a classic linear dose–response fashion. In fact, it appeared that the response we saw with As(III) and UV was more of a threshold and only at As(III) doses of 1 μM and above was there a substantial effect on mutant frequency. This response to As(III) is consistent with what others have seen with studies looking at As(III) exposure (reviewed in Rudel *et al*., 1996). The idea that arsenic may act on DNA indirectly by altering enzymes involved with DNA repair may explain why arsenic has been shown to be an effective co-clastogen (Li and Rossman, 1991; Yager and Wiencke, 1993). Typically, enzyme-mediated effects would be expected to require exposure to multiple molecules of chemicals before a significant biological effect is seen (Rudel *et al*., 1996). Two of the three doses above 1 μM showed significant enhanced mutant frequency, with the third point estimate essentially identical to the other two. This is consistent with the major effect of arsenic being related not to damage induction, but to repair; in fact, measuring mutation induction as we have done at one time point after single doses might poorly reflect an alteration in the rate of repair.

The mutagenicity of UV varies by cell cycle phase (Riddle and Hsie, 1978; Enninga *et al*., 1985). If As(III) treatment synchronized the cell cycle prior to UV treatment our results would not reflect true synergy, but would rather represent an experimental artifact. However, flow cytometry data demonstrated that the cells were not significantly synchronized by As(III) treatment, thus supporting a true mechanistic synergy between arsenic and UV exposure in mutagenesis.

The removal of UV-induced DNA damage occurs through a rapid mechanism as well as a slower component (Reusch *et al*., 1988; Henriksen *et al*., 1996; Yamauchi *et al*., 2002a,b). Tail moments measured using the Comet assay for UV-exposed lymphocytes seem to reach a maximum at 1 h and decrease thereafter, suggesting that the damaged DNA is rapidly

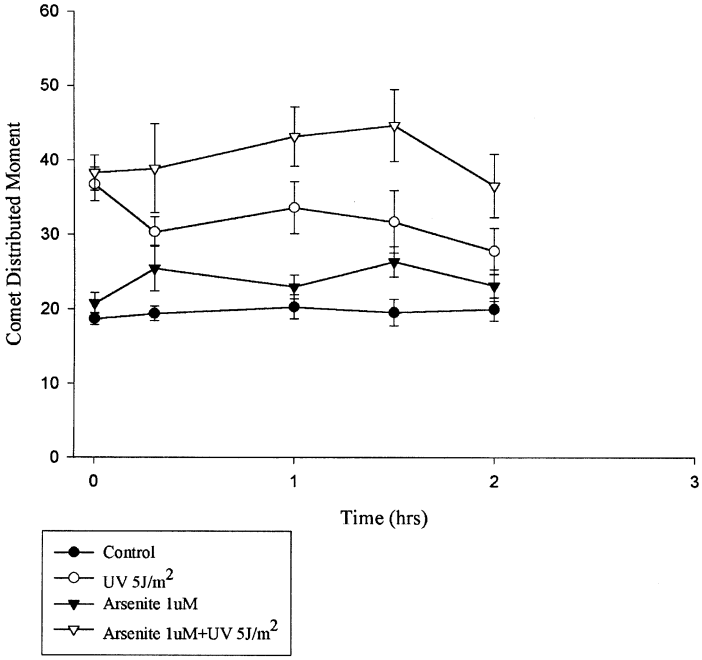

Fig. 4. Effect of a 24 h 1 μM As(III) pre-treatment on the repair of UV-induced pyrimidine dimers. The comet distributed moment was scored for all groups and was followed over time.

Table I. Comet distributed moment versus different treatment groups and time

Effects	Degrees of freedom	*F* value	*P*
Model 1			
Time	4	1.3	0.27
As(III)	1	0.2	0.65
UV	1	187.8	<0.0001
UV × As(III)	1	4.4	0.04
Model 2			
Time	4	0.1	0.99
As(III) × time	4	1.5	0.2
UV × time	4	3.9	0.005
As(III)	1	1.2	0.28
UV	1	180.7	<0.0001
UV × As(III)	1	4.6	0.03

Mixed linear regression models using data from the 16 pooled Comet assay experiments. Each fixed effect listed was included in the corresponding model for analysis; within both models experiment was classified as a random effect. Model 1: variables included all treatment groups and time. Model 2: the interaction of treatment and time is included.

repaired and rejoined by ligation (Yamauchi *et al*., 2002a,b). To further study the mechanism of As(III)–UV synergy at low dose, we used the T4 endo V-incorporated Comet assay. Within the first 15 min post-UV exposure there was rapid DNA repair, as evidenced by reduced comet scores. With As(III) pre-treatment, not only was this rapid repair of UV damage ablated, there was a persistent increase in DNA fragment migration. Longer time-course experiments (up to 6 h) indicated that the As(III) pre-treatment effect dissipated after 2 h (data not shown), i.e. the initial decline in the distributed moment that we observed is the major alteration in repair observed in these experiments. Whether the arsenic pre-treatment resulted in increased DNA damage is unclear, however, the data demonstrate that the rapid repair phase of NER is affected by arsenic pre-treatment.

The co-carcinogenic mechanism of arsenic and DNA-damaging agents has been postulated to occur at the DNA repair level. This hypothesis is particularly attractive because arsenic species bind strongly to dithiols as well as free sulfhydryl groups commonly found within repair enzymes (Kitchin, 2001). Repression of DNA repair by arsenic may also occur as a result of the down-regulation of repair enzymes at the transcriptional level (Hartwig *et al*., 1997; Bau *et al*., 2001; Hamadeh *et al*., 2002; Andrew *et al*., 2003). These authors have shown that cells treated with arsenic cannot efficiently repair UV-induced DNA damage. Additional evidence implicates inhibition of the NER pathway as the underlying mechanism for this effect (Rossman *et al*., 2001). All of these mechanisms may be interrelated, as decreased transcription and direct inhibition may be indistinguishable in our work.

We hypothesize that inhibition of UV-induced DNA damage repair is responsible for the increase in mutations seen when cells are exposed to UV light and low levels of As(III) comparable with those found in the environment. This alteration of repair may also be a primary mechanism responsible for the increased incidence of skin cancer within individuals chronically exposed to arsenic in drinking water.

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