Formamidopyrimidine-DNA Glycosylase Enhances Arsenic-Induced DNA Strand Breaks in PHA-Stimulated and Unstimulated Human Lymphocytes

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To confirm that arsenic (As) induces oxidative DNA damage in phytohemagglutinin (PHA)-stimulated and unstimulated human lymphocytes, we used the alkaline comet assay combined with specific enzyme [formamidopyrimidine-DNA glycosylase (FPG)] digestion to measure As-induced base damage. The results showed that the enzyme-sensitive sites were readily detected with the alkaline comet assay after the cells were treated with 10 μM As for 2 hr. The repair patterns observed for FPG-created DNA single strand breaks (SSBs) in As-treated cells were comparable to those in hydrogen peroxide (H_2O_2)-treated cells. The enzyme-created SSBs, As-induced base damage, were more significant in PHA-stimulated lymphocytes. About 63% and 68% of SSBs induced by As and H_2O_2, respectively, were repaired in PHA-stimulated lymphocytes by 2-hr repair incubation, but about 34% and 43%, respectively, were repaired in unstimulated cells. About 40% and 49% of base damage induced by As and H_2O_2, respectively, were repaired in PHA-stimulated lymphocytes, but about 19% and 21%, respectively, were repaired in unstimulated cells. These results indicated that As induced oxidative DNA damage in human lymphocytes at micromolar concentrations. The damaged bases could be chiefly purines or formamidopyrimidines. Like the damage induced by H_2O_2, As-induced DNA damage was repaired more slowly in unstimulated lymphocytes. *Key words*: arsenic, comet assay, formamidopyrimidine–DNA glycosylase (FPG), human lymphocytes, oxidative DNA damage. *Environ Health Perspect* 109:523–526 (2001). [Online 11 May 2001]
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An early study indicated that superoxide dismutase (SOD) and catalase (CAT) could prevent arsenic (As)-induced sister chromatid exchanges (SCEs) in cultured human lymphocytes (*1*). Recent studies showed similar results using reactive oxygen species (ROS) scavengers such as SOD, CAT, and dimethyl sulfoxide (DMSO) to counteract mutagenicity, DNA fragmentation, and micronuclei (MN) caused by As (*2–4*). We observed that DMSO and CAT basically abolished most of the As-induced DNA single strand breaks (SSBs) in human cells with single-cell gel electrophoresis (SCGE) assay (*5*). These observations have provided indirect but powerful evidence indicating that hydrogen peroxide (H_2O_2) and superoxide anion (O_2-) are chiefly involved in the genotoxicity of As.

However, it is known that ROS-induced DNA damage consists primarily of single strand breaks (SSBs), double strand breaks (DSBs), sites of base loss [apurinic/apyrimidinic (AP) sites], and base lesions (*6*). Great attention has been paid to ROS-related base damage. SSBs are repaired quickly by cells and of no biologic importance, and the damage of DSBs is important for understanding the lethal effects on cells, but ROS-induced base damage is believed to be one of the main contributors to carcinogenesis. More significantly, base modifications are potential biomarkers of oxidative DNA damage (*7*).

DNA damage-specific endonucleases from *Micrococcus luteus* have been used previously in combination with sucrose gradient sedimentation (*8*), alkaline elution (*9*), and alkaline unwinding (*10*) to reveal base damage inflicted by ionizing radiation. Now the gene for *E. coli* formamidopyridine-DNA (FPG) has been cloned and the protein purified to available homogeneity. This makes it possible to combine enzyme digestion with the newly developed sensitive alkaline comet assay to measure ROS-related base damage. FPG cleaves purines including 7,8-dihydro-8-oxoguanine (8-oxoG), formamidopyrimidines, and AP sites (*11*).

Using lesion-specific enzymes in the comet assay to reveal DNA base damage has been described in detail by Collins et al. (*12*). Observations have been made on As-related alterations in base damage with FPG digestion in bovine aortic endothelial cells (*13*) and in human vascular smooth muscle cells (*14*). Detection of 8-hydroxydeoxyguanosine, a biomarker of oxidative DNA damage, was associated with As-related Bowen's disease (*15*). To clarify further that As induces oxidative DNA damage in human phytohemagglutinin (PHA)-stimulated and unstimulated lymphocytes, we applied the sensitive alkaline comet assay combined with FPG digestion to measure As-induced base lesions. We postulated that if As produces oxidative DNA damage, this specific enzyme, having both DNA glycosylase and endonuclease activities, will recognize and cleave the damaged bases, and that consequently DNA strand breaks will be produced at enzyme-sensitive sites. The breaks, including AP sites, will be converted into comet tail by the alkaline comet assay (SCGE). As a result, the specific enzyme will increase the frequency and quantity of the tail moment induced by treatment of As.

Materials and Methods

Cell treatment. We separated lymphocytes from peripheral blood of a healthy man. Briefly, we mixed 5 mL blood with 5 mL RPMI 1640 medium (Nikken, Osaka, Japan) and kept the mixture on ice for 15 min. We separated lymphocytes by centrifugation over 10 mL Ficoll-Hypaque solution (Amersham, Uppsala, Sweden), at 200 × *g* for 30 min at 4°C. Lymphocytes above the Ficoll-Hypaque layer were washed two times at 200 × *g* for 5 min at 4°C and suspended in RPMI 1640 complete culture medium containing 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL, Gaithersburg, MD, USA) with a concentration of 2.5×10^6 cells/mL in a 60-mm culture dish. The cells were immediately treated with 10 μM arsenic [As_2O_3, prepared in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS); Wako Pure Chemical Industries, Ltd., Osaka, Japan] under the conditions of 37°C, 5% CO_2, and 95% air for 2 hr. For PHA stimulation, we cultured the separated lymphocytes (2.5×10^6 cells/60-mm dish) in RPMI 1640 complete culture medium containing 2% PHA M (DIFCO, Detroit, MI, USA) for 70 hr in complete darkness and incubated them further with the same concentration of arsenic under the same conditions as in unstimulated cells. To compare the As- and H_2O_2-induced DNA damage, we treated the cells (10^5 cells/mL) with 50 μM H_2O_2 in PBS on ice for 5 min and then allowed to repair under the same conditions as above for As-treated cells.

After treatment, the cells were washed twice and suspended in RPMI 1640 complete

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medium warmed at 37°C at concentrations between 5×10^5 and 1×10^6 cells/mL for the comet assay.

For the enzyme digestion experiment, the cells with or without As and H_2O_2 treatment were washed twice in PBS and then incubated for another 2 hr under the conditions of 37°C, 5% CO_2, and 95% air in RPMI 1640 containing 20% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin to allow the cells to repair arsenic-induced DNA damage. During hourly intervals, samples of cells were processed for the comet assay combined with specific enzyme digestion.

Comet assay (SCGE assay). We used an adaptation of the method introduced by Singh et al. (*16*). Briefly, cells with or without arsenic treatment were suspended in 0.75% low melting-point agarose (Nusieve GTG, FMC BioProducts, Rockland, NY, USA) in phosphate-buffered saline, pH 7.4, at 37°C and pipetted onto a frosted glass microscope slide (Matsunami Glass Ind., Ltd., Kishiwata, Japan) precoated with a layer of 0.5% normal melting-point agarose (Sigma, St. Louis, MO, USA). Then the slide was subjected to a temperature of 4°C for 10 min to consolidate the gel with a coverslip. After a third layer of 0.75% low melting-point agarose was applied and consolidated at 4°C as described above, the slides were immersed in alkaline lysis solution (100 mM Na_2-EDTA, 2.5 mM NaCl, 1% Na sarcosinate, 10 mM Tris, pH 10, 1% Triton X-100 added fresh) at 4°C for 1 hr to remove cellular proteins. After lysis, the slides were placed in a horizontal gel electrophoresis tank filled with fresh electrophoresis buffer (300 mM NaOH and 1 mM Na_2-EDTA, pH 13) to a level of 0.25–0.5 cm above the slides to allow DNA to unwind for 20 min. Electrophoresis was conducted for next 20 min at 25 V and 300 mA using an electrophoresis compact power supply (ATTO Corporation, Tokyo, Japan). Electrophoresis was performed in darkness and ice-cold surroundings to avoid further damage to DNA. After electrophoresis, slides were drained and flooded with three changes of neutralization buffer (400 mM Tris-HCl, pH 7.5) for 5 min each. The slides were then stained with 50 µL ethidium bromide (20µg/mL; Sigma) and covered with a coverslip for image analysis.

For specific enzyme digestion, we adapted the procedures introduced by Collins et al. (*6*). Briefly, cells were embedded in 0.75% low melting-point agarose at 37°C and immediately processed for lysis after consolidation of the gel at 4°C for 10 min without the third layer of agarose. After lysis, the slides were washed three times for 5 min each in enzyme buffer (40 mM Hepes-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL bovine serum albumin fraction V, pH 8.0) and drained. The agarose was then covered with 50 µL of either enzyme buffer or FPG (Trevigen, Gaithersburg, MD, USA, stored at –20°C before use) in buffer (0.5 U/µL), sealed with a coverslip, and incubated at 37°C for 30 min. A third layer of agarose was applied as before, with 37°C-warmed 0.75% low melting-point agarose after a swift wash with PBS. The next steps (alkaline unwinding, electrophoresis, neutralization, and staining) were as described above.

Observation and DNA migration analysis. We made observations at a magnification of 200× under a fluorescence microscope (Olympus BX50, Tokyo, Japan) connected with an HCC-600 color camera system and SCG image analysis software (DHS-SCG, version 1.0, 1998, KEIO Electronic Ind., Co., Ltd., Ibaraki, Japan). We measured 100 cellular nuclei at random for tail moment, a parameter of the percentage of DNA in the tail (tail length × tail intensity). To show the number of As-induced DNA SSBs, we subtracted the tail moment for background damage (control without FPG digestion). To show the number of FPG-created SSBs at sensitive sites or arsenic-induced base damage converted into SSBs by FPG, we subtracted the tail moment for As-induced SSBs from the tail moment for As-induced SSBs plus enzyme-sensitive sites. The background damage (control with FPG digestion) was also subtracted.

Statistics. The results were from three independent experiments. Each experiment was performed in duplicate. The SCGE data were transferred into Microsoft Excel (Japanese Windows 98 version; Microsoft Corporation, Tokyo, Japan) and pooled from the repeated experiments. Tukey multiple analysis of variance comparisons were done with SPSS (Japanese Windows version 7.5). The results are expressed as means ± SD.

Results

The tail moment for As-induced SSBs and FPG-created SSBs in PHA-stimulated and unstimulated lymphocytes are shown in Figure 1. It is clearly seen from Figure 1 that FPG created a significant number of SSBs at sensitive sites at each sampling interval of repair incubation in both PHA-stimulated and unstimulated lymphocytes compared with the background levels ($p < 0.001$).

As-induced SSBs in PHA-stimulated lymphocytes at the repair incubation time of 1 and 2 hr were significantly lower compared with the incubation time of zero ($p < 0.001$). The damage induced in unstimulated cells showed no difference between hourly sampling intervals ($p > 0.05$). Similarly, FPG-created SSBs did not show significant reduction in unstimulated lymphocytes over the 2-hr repair incubation, but significantly decreased at the end of 2-hr incubation in PHA-stimulated cells ($p < 0.01$).

To demonstrate further evidence, we show the curves of SSBs and base damage caused by H_2O_2 in PHA-stimulated and unstimulated lymphocytes in Figure 2. The kinetics shown were similar to the curves from the cells treated with arsenic shown in Figure 1. FPG-created SSBs for H_2O_2-induced base damage were significant at each sampling interval of repair incubation ($p < 0.001$). The tail moment for both SSBs and base damage in PHA-stimulated lymphocytes descended more significantly than those in unstimulated cells ($p < 0.001$ compared at the incubation time of zero).

Both SSBs and base damage induced by the two agents in PHA-stimulated and unstimulated lymphocytes did not recover to the background levels at the end of 2-hr repair incubation, indicating that both SSBs and base damage were not completely repaired over 2-hr incubation. Statistical analyses of the FPG-created SSBs induced by the two agents also showed significant differences between PHA-stimulated and unstimulated cells at sampling interval of zero ($p < 0.001$).

To show clearly the different cellular repair capacities of both SSBs and base damage induced by the two agents in PHA-stimulated and unstimulated lymphocytes,

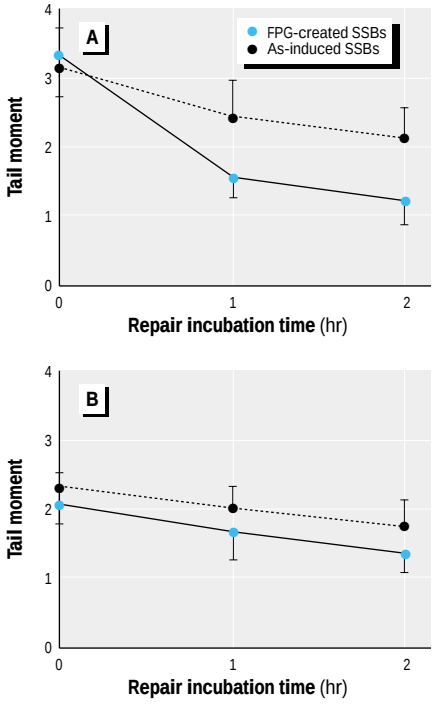

Figure 1. FPG-created DSBs at sensitive sites in (*A*) PHA-stimulated and (*B*) unstimulated lymphocytes. Data are expressed as mean ± SD.

we calculated the percent repair using the equation

$$100\ [(D_0 - D_t)/D_0],$$

after background damage was subtracted. D_0 is defined as DNA damage at the incubation time of zero, and D_t as that at the sampling interval of 1- or 2-hr incubation. The results are plotted in Figure 3.

Figure 3 clearly shows that the repair capacities of both SSBs and base damage induced by the two agents are also comparable in stimulated and unstimulated lymphocytes. In PHA-stimulated lymphocytes, 63.3% and 68.3% of the SSBs induced by As and H_2O_2, respectively, were repaired by 2-hr incubation, but about 34.2% and 43.6%, respectively, were repaired in unstimulated cells. In PHA-stimulated lymphocytes, 40.6% and 49.4% of base damage induced by both agents were repaired by 2-hr incubation, and 19% and 20.8% were repaired in unstimulated cells.

Discussion

Application of FPG protein significantly increased the arsenic-induced tail moment in the present study. The enzyme-sensitive sites were easily detected with the alkaline comet assay after the cells were treated with 10 µM arsenic for 2 hr. In addition, the repair patterns derived from FPG-created SSBs for arsenic were comparable to those for H_2O_2. It is known that FPG specifically recognizes and excises the oxidative bases (*17*). Thus, our results indicate that arsenic induces oxidative DNA damage in human cells.

The most common primary oxidative base lesion is a ring-saturated base derivative (*11*). The key purine lesion is 8-hydroxyguanine (8-OHgua) or 8-oxoG. The 8-OHgua lesion has attracted special attention because it is a premutagenic lesion and forms an 8-oxoG: A mispair during DNA replication, which induces G: C to T: A transversions because of its mispairing properties in bacterial and mammalian cells. The induction of mutations is one of the critical events in carcinogenic transformation, and it has been suggested that ROS-induced 8-OHgua is directly involved in the process of carcinogenesis. FPG is a distinct repair enzyme for formamidopyrimidine and 8-oxoG (*18*). In the present study, we showed that FPG digestion significantly enhanced arsenic-induced DNA tail moment, suggesting that arsenic induced modifications of purines and formamidopyrimidines, and that possibly 8-oxoG was incurred.

Generally, SSBs are repaired rapidly in normal human cells with a half-time of a few minutes. DSB rejoining is relatively slow, with a half-time of more than 1 hr (*19*). Repair of oxidized DNA base is generally slow, with a half-time of more than 90 min (*20*). Therefore, theoretically, after strand breaks are rejoined, base damage would become evident. We have shown that as arsenic-induced tail moment decreased with repair incubation, enzyme-created SSBs at enzyme-sensitive sites were more obviously revealed, persisting over 2-hr incubation, providing further evidence indicating that arsenic induced base damage.

We have confirmed that arsenic induces oxidative DNA damage in human lymphocytes. However, the exact mechanisms by which arsenic causes oxidative DNA damage are still not clear. Studies indicate that arsenic might inhibit the activities of CAT and glutathione peroxidase, leading to accumulation of H_2O_2 (*21*,*22*). As may stimulate SOD to produce O_2- and increase the activity of heme oxygenase to release reactive ions (*22*). It was reported that arsenic could stimulate cell signaling and activate transcription factors to enhance production of H_2O_2 and O_2^- (*23*). Other recent studies have suggested that arsenite activates NADH oxidase to produce O_2-, which then causes oxidative DNA damage (*13*,*24*). These studies suggest that arsenic may trigger oxidative stress through multiple pathways, but H_2O_2 and O_2^- are the main ROS involved in arsenic-induced DNA damage, as shown in our previous study (*5*).

ROS not only cause oxidative DNA damage but also influence DNA repair (*25*). Oxidative DNA damage is one of the main mechanisms of carcinogenesis (*26*,*27*). It is postulated that oxidative DNA damage is one of the mechanisms by which arsenic causes multiple cancers in humans.

We have shown that enzyme-created SSBs are more evidently revealed by FPG digestion in PHA-stimulated lymphocytes, indicating that stimulated proliferating cells are more susceptible to arsenic-induced DNA damage. We observed a similar response to H_2O_2-induced base damage. Different amount or activities of DNA repair enzymes in proliferating and nonproliferating cells (*28*) could be the explanation for the diversity in DNA repair capacities between stimulated and unstimulated lymphocytes observed in the present study.

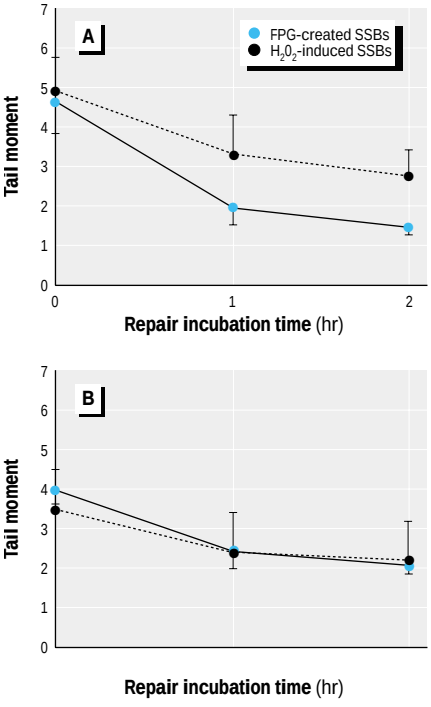

Figure 2. FPG-created DSBs in (*A*) PHA-stimulated and (*B*) unstimulated lymphocytes treated with H_2O_2. Data are expressed as mean ± SD.

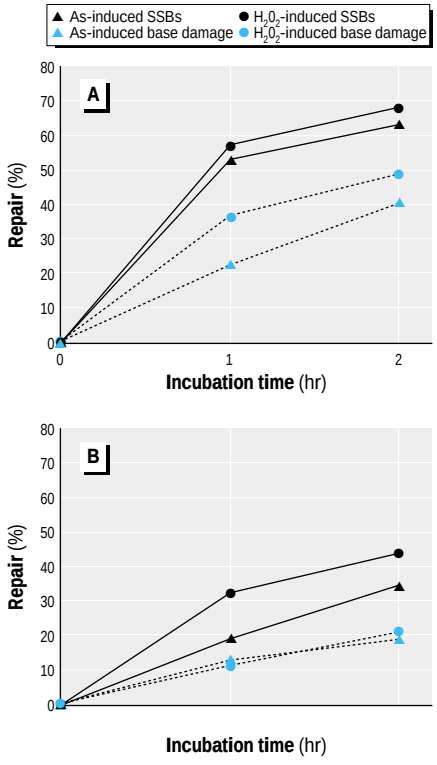

Figure 3. DNA repair capacities for As- and H_2O_2-induced SSBs and base damage in (*A*) PHA-stimulated and (*B*) unstimulated human lymphocytes. Black triangles: As-induced SSBs.

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