Since arsenite is known to induce oxidative DNA damage in human cells, we asked if it induces other types of DNA damage and how the DNA damage is repaired. Treatment of human promyelocytic leukemia NB4 cells with 0.5μM As₂O₃ for 30 min induced no DNA breaks, as analyzed by a standard comet assay. However, breaks were detected if these cells were then digested with endonuclease III (EnIII), formamidopyrimidine-DNA glycosylase (Fpg), or a nuclear extract (NE) of NB4 cells. Using either H₂O₂-Fe³⁺-treated nuclei or As₂O₃-treated cells, digestion with either NE or EnIII + Fpg generated the same amount of breaks, and subsequent treatment with EnIII + Fpg resulted in no increase in breaks in NE-digested cells and vice versa. The human cell lines, defective in nucleotide excision protein, such as xeroderma pigmentosum (XP) A, XPD, and XPG, excised Ultraviolet C-induced adducts less rapidly than normal fibroblasts, but excised As₂O₃ adducts at the same rate as the normal cells. Immunodepletion of the NE with antibody against 8-oxoguanine DNA glycosylase (OGG1) or MutY homolog (MYH) decreased the incision of As₂O₃-induced adducts, while antibodies against XPA, XBP, XPD, XPF, or XPG, did not. These results suggest that As₂O₃ induces the formation of only oxidative DNA adducts and that OGG1 and MYH are involved in this incision process.

Key Words: arsenic trioxide; oxidative damage; monomethylarsonic acid; dimethylarsinic acid; sodium arsenite.

Arsenic, an environmental toxicant, is associated with several human diseases, including Blackfoot disease (Tsai et al., 2005), diabetes (Walton et al., 2004), hypertension (Rahman, 2002), and cancers of the skin (Rossman et al., 2004), lung, bladder, kidney, and liver (Chen et al., 1992). Although several hypotheses have been proposed, the mechanism of arsenic toxicity has not been clearly established. However, several independent studies have demonstrated increased numbers of micronucleated cells in exfoliated bladder cells of humans exposed to arsenic in drinking water (Basu et al., 2001; Vega et al., 1995; Warner et al., 1994). Another study demonstrated that the number of micronucleated cells was decreased if drinking water highly contaminated with arsenic was replaced with water containing low concentrations of arsenic (Moore et al., 1997b). These data provide strong evidence that DNA damage is involved in arsenic-induced carcinogenesis in the human bladder. This is consistent with reports that 8-hydroxy-2′-deoxyguanosine occurs at a higher frequency in arsenic-related skin neoplasms (Matsui et al., 1999) and in lymphocytes from arsenic-exposed persons (Basu et al., 2005) and that trivalent arsenicals induce oxidative DNA damage in cultured human cells at pathologically meaningful concentrations (Schwerdtle et al., 2003; Wang et al., 2001, 2002).

8-Hydroxy-2′-deoxyguanosine causes miscoding by DNA polymerase in vitro, as well as the induction of G to T transversions (Tajiri et al., 1995). Such mutations are seen in carcinoma of the liver, lung, and prostate following oxidative DNA damage (Feig et al., 1994). However, mutation assays using bacterial or mammalian cells have shown that arsenite is not a potent point mutagen (Lee et al., 1985; Rossman et al., 1980). More recently, it was shown that low-dose chronic exposure of human osteosarcoma cells to arsenite causes mutations at the HPRT gene locus (Mure et al., 2003). Hei et al. (1998) demonstrated that arsenic induces large-deletion mutations in human-hamster hybrid cells through a reactive oxygen species–mediated mechanism. However, 8-oxo G/C pairs are predominantly repaired by short-patch base excision repair, although mismatch repair, long-patch base excision repair, and nucleotide excision repair may compliment the repair system (Evans et al., 2004).

The aims of this study were to investigate whether arsenite induces any other type of DNA damage in addition to oxidative DNA damage and to determine how arsenite-induced DNA damage is repaired.

MATERIALS AND METHODS

Cells. Detroit 515 is a human embryonic skin cell line from the Food Industry Research and Development Institute (http://www.bcrc.firdi.org.tw/wwwbcr/c02_profile.do). HFW cells are normal Human Fibroblast cells, derived from human newborn foreskin, were kindly provided by Dr W. N. Wen
(Biochemistry Department, National Taiwan University, Taipei). The xero-
derma pigmentosum (XP) complementation group mutant cell lines, XPA (GM0431C), XPD (GM00434A), and XPG (GM13370), were purchased from the National Institute of General Medical Sciences Human Genetic Cell Repository, Coriell Institute for Medical Research (Camden, NJ). NB4 cells, a human promyelocytic leukemia cell line, were kindly provided by Dr C. Y. Liu (Veterans Hospital, Taipei, ROC).

Detroit 551 and HFW cells were grown in Dulbecco’s modified Eagle’s medium, XPA and XPD cells in modified Eagle medium, XPG cells in Ham’s F12, and NB4 cells in RPMI 1640. The media were supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 0.03% glutamine, and 10% fetal calf serum. The cultures were incubated at 37°C in a water-saturated atmosphere containing 5% CO₂.

Chemicals. Endonuclease III (EnIII) and formamidopyrimidine-DNA glycosylase (Fpg) were purchased from Trevigen (Gaithersburg, MD). T4 UV endonuclease V (Den V) was from Epicentre Technologies (Madison, WI). Sodium nitrosoprusside (SNP), 3-morpholinosydnonimine (SIN-1), and benzo(a) pyrene diolepoxide (BPDE) were purchased from Sigma-Aldrich (St Louis, MO). Arsenic trioxide (As₂O₃), sodium arsenite (NaAsO₂), hydrogen peroxide (H₂O₂), ferrous sulfate (FeSO₄·7H₂O), and cis-platinum were purchased from Merck (Darmstadt, Germany). The source of methylarsonous acid (MMA III) was the solid oxide (CH₃AsO) and that of dimethylarsinous acid (DMA III) was (H₂O₂), ferrous sulfate (FeSO₄·7H₂O), and cis-platinum were purchased from

The iodide ((CH₃)₂AsI). The precursors were prepared following previously described procedures (Cullen et al., 1989; Goddard, 1930) and were kept at –20°C. CH₃AsO and (CH₃)₂As were more than 99% pure when analyzed using a nuclear magnetic resonance spectrometer (AMX400) and a Heraeus CHN-OS rapid element analyzer. Dilute solutions of the precursors were freshly prepared in deionized water to form CH₃As(OH)₂ (MMA III) and (CH₃)₂AsOH (DMA III).

Comet assay. The standard comet assay without enzyme digestion was performed as described previously (Wang et al., 2001). For enzyme digestion, the slides after lysis were washed with distilled water, then incubated at 37°C for 30 min in 10mM Tris-HCl, pH 7.5, followed by 1 h at 37°C with DenV, EnIII, or Fpg at 2 U per slide in the same Tris buffer.

The method using the comet assay and nuclear extract (NE) incubation was recently described (Wang et al., 2005). To prepare the NE preparation, NB4 cells were treated for 16 h with 2.5mM hydroxyurea and 25μM cytosine-β-D-arabinofuranoside, and then the NE was prepared using the method described by Challberg and Kelly (1979) with some modifications. Briefly, the cells were washed once with hypotonic buffer (20mM N-2-hydroxyethylpiperezine-N’-2-ethanesulfonic acid [HEPES], pH 7.5, 5mM KCl, 0.5mM MgCl₂, 0.5mM dithiothreitol, and 0.2M sucrose) and the pellets resuspended in cold hypotonic buffer without sucrose. The cells were allowed to swell for 10 min on ice and were then lysed with 10 strokes of a Dounce homogenizer. The lysate was centrifuged at 2000 × g for 5 min at 4°C and the nuclear pellet resuspended in resuspension buffer (20mM HEPES, pH 7.5, 10% Sucrose; 5 mM KCl; 0.1mM MgCl₂; 0.5 mM DTT; Protease inhibitor 1×) and the nuclei stored in liquid nitrogen. The nuclei were then thawed on ice and allowed to swell for 1 h in 100mM NaCl on ice, then the mixture was centrifuged at 15,000 × g for 20 min at 4°C. The resulting supernatant (NE) was concentrated on an YM-10 Microcon filter (Millipore, Bedford, MA). The protein concentration was measured using a Bio-Rad Protein Assay Kit (Hercules, CA) with bovine serum albumin as the standard. About 20 μg of nuclear protein was obtained from 2 × 10⁶ NB4 cells. For NE incubation, slides after lysis were washed with

FIG. 1. Detection of DNA damage using the comet assay. NB4 cells were treated with (A) 0.2μM As₂O₃ for 0–24 h or (B) 0–0.2μM As₂O₃ for 1 h. DNA damage was analyzed by a comet assay with or without NE incubation. *p < 0.01 compared with without As₂O₃.

FIG. 2. Incubation of As₂O₃-treated cells or H₂O₂ + Fe-treated nuclei with EnIII + Fpg or with NE increases DNA strand breaks by the same amount. NB4 cells on slides were incubated at room temperature for 30 min without treatment (A) or with 0.5μM As₂O₃ (B), and then lysed. To induce oxidative DNA damage, NB4 cells on slides were lysed, washed, and incubated at room temperature with 100μM H₂O₂ + 1μM FeSO₄ for 15 min (C). Prior to denaturing and electrophoresis, all slides were incubated at 37°C for 1 h with nothing (None), Tris buffer (TB), NE incision buffer (NB), NB then TB (NB → TB), TB then NB (TB → NB), 2 U of EnIII then 2 U of Fpg (E → F), EnIII then Fpg then NE (E → F → NE), NE (NE), or NE then EnIII then Fpg (NE → E → F). *p < 0.01 compared with untreated cells (A).
distilled water and incubated at 37°C for 30 min in NE incision buffer (50mM HEPES-KOH, pH 7.9, 70mM KCl, 5mM MgCl₂, 0.4mM ethylenediaminetetraacetic acid, 2mM adenosine triphosphate, 40mM phosphocreatine, and 50 μg/ml of creatine phosphokinase) and then for 1 h with 0.6 μg of NE/slide in NE incision buffer. Migration of DNA from the nucleus in each cell was measured with Comet Assay III software (http://www.perceptive.co.uk) and expressed as the % tail DNA. Data from 50 individual cells for each treatment were collected.

**Immunodepletion.** Two micrograms of NE protein in 4 × NE buffer mixed with 0.2 μg of antibody in a total volume of 20 μl was gently shaken in a rotator for 1 h at 4°C, and then the sample was subjected to a comet assay. The antibodies used were polyclonal antibodies against XPA (cat. no. sc-853), XPB (cat. no. sc-293), XPF (cat. no. sc-10164), XPG (cat. no. sc-12558), 8-oxoguanine DNA glycosylase (OGG1) (cat. no. sc-12075), or Apurinic/Apyrimidinic Endonuclease (APE) (cat. no. sc-5572) from Santa Cruz Biotechnology (Santa Cruz, CA), monoclonal antibody against XPD (cat. no. x98220-150; BD Transduction Laboratories, San Jose, CA), and polyclonal antibody against the MutY homolog (MYH) (cat. no. PC709; Oncogene Research Products, San Diego, CA).

**RESULTS**

**NE Reveals As₂O₃-Induced DNA Adducts**

Since NE incubation has been shown to increase the amount of DNA strand breaks detected by the comet assay (Wang et al., 2005), we tested whether it could reveal more DNA strand breaks in As₂O₃-treated cells. The results, shown in Figure 1A, demonstrated that incubation with NE significantly increased the amount of DNA strand breaks in cells treated with 0.2 μM As₂O₃; this increase presumably represents DNA adducts that were not excised at the time of cell lysis. The amount of DNA adducts showed a marked increase within the first hour of As₂O₃ treatment, which then slowed down and reached a plateau at around 8 h. This is probably because induction of DNA damage and cellular DNA repair have reached a dynamic level. Using a 1-h As₂O₃ treatment and comet assay with NE incubation, apparent DNA damage was detected at As₂O₃ concentration > 0.025μM (Fig. 1B). The % tail DNA reached a plateau above 0.05μM. This is probably due to the ceiling effect of the % tail DNA parameter.

**Many of the Lesions Induced by As₂O₃ Are Oxidized Bases**

Treatment with 0.5μM As₂O₃ for 30 min did not induce appreciable amounts of DNA strand breaks as analyzed by
a standard comet assay in the absence of NE treatment or other enzyme digestion. However, many more DNA strand breaks were detected if the As$_2$O$_3$-treated cells were digested sequentially with the bacterial enzymes EnIII and Fpg (Figs. 2A and B, E $\rightarrow$ F). These results suggest that As$_2$O$_3$ does not induce DNA strand breaks immediately after treatment, but that many of the lesions are oxidized bases and are transformed into breaks by digestion with EnIII and Fpg.

Treatment with EnIII + Fpg or NE Causes a Similar Increase in DNA Strand Breaks in both H$_2$O$_2$-Fe–Treated Nuclei and As$_2$O$_3$-Treated Cells

A large amount of DNA strand breakage was also revealed when As$_2$O$_3$-treated cells were digested with NE prepared from NB4 cells (Fig. 2B, NE). Subsequent incubation of NE-digested slides with EnIII + Fpg did not cause a further increase in strand breaks nor did subsequent incubation of EnIII + Fpg–digested slides with NE. To learn more about the nature of EnIII, Fpg, and NE digestion, we prepared oxidative DNA adducts by incubating slides of nuclei with H$_2$O$_2$ plus FeSO$_4$, then carried out a digestion with EnIII, Fpg, and NE. The results showed that EnIII, Fpg, and NE increased DNA strand breaks by the same amount in H$_2$O$_2$-Fe–treated nuclei (Fig. 2C) and in As$_2$O$_3$-treated cells (Fig. 2B). These results suggest that most of the lesions induced by As$_2$O$_3$ are oxidative bases.

OGG1, MYH, and APE Proteins Are Required for the Incision of DNA Adducts Induced by As$_2$O$_3$, NaAsO$_2$, MMA$_{III}$, DMA$_{III}$, SNP, SIN-1, and H$_2$O$_2$ Plus Fe

To determine what factors were responsible for the incision activity of NE, we examined the effects of immunodepletion of NE with antibodies directed against proteins known to be involved in DNA excision repair. As shown in Figure 3A,
antibodies against XPA, XPD, XPF, or XPG had no effect, but antibodies against OGG1, MYH, or APE dramatically decreased the incision activity of NE on DNA adducts induced by As$_2$O$_3$. The same results were obtained using slides of cells treated with NaAsO$_2$, MMA$_{III}$, or DMA$_{III}$ (Figs. 3B–D), nuclei treated with H$_2$O$_2$ + FeSO$_4$ (Fig. 4A), or cells treated with SNP (an NO donor) or SIN-1 (a peroxynitrite donor) (Figs. 4C and E). In contrast, antibodies against XPA, XPD, XPF, or XPG dramatically decreased the incision activity of NE on DNA adducts induced by UVC, cisplatin, or BPDE, whereas antibodies against OGG1, MYH, or APE did not (Figs. 4B, D, and F). These results show that OGG1, MYH, and APE are required for the incision of DNA adducts induced by As$_2$O$_3$, NaAsO$_2$, MMA$_{III}$, DMA$_{III}$, SNP, SIN-1, or H$_2$O$_2$ + Fe, while XPA, XPD, XPF, and XPG are not.

To test the notion that nucleotide excision repair was not involved in the repair of arsenite-induced DNA damage, we studied the DNA adduct incision activity of human embryonic skin cells (Detroit 551), normal human fibroblasts (HFW), and the mutant fibroblast lines, XPA, XPD, and XPG, which are deficient in nucleotide excision repair. As shown in Figure 5 (top row), the XPA, XPD, and XPG mutant cells excised UV-induced cyclobutane dimers (DenV digestible) much more slowly than Detroit 551 or HFW cells, but were as efficient as Detroit 551 and HFW cells in excising As$_2$O$_3$- and SIN-1–induced DNA adducts (EnIII- and Fpg-digestible adducts) (middle and bottom rows). In these experiments, the cells were treated with higher concentration of As$_2$O$_3$ but for shorter time (1µM for 30 min) and washed thoroughly and then incubated in drug-free medium to allow cellular DNA repair. These results show that XPA, XPD, and XPG cells are defective in cyclobutane dimer excision, but are as efficient as HFW in incising As$_2$O$_3$- and SIN-1–induced oxidative DNA adducts.

As shown in Figure 6A, using slides of H$_2$O$_2$ + Fe–treated nuclei, Fpg treatment of slides predigested with OGG1-depleted NE markedly increased the amount of DNA strand breakage, whereas EnIII treatment did not, while the opposite results were seen for slides predigested with MYH-depleted NE. Neither EnIII nor Fpg increased the amount of DNA strand breakage on slides predigested with APE-depleted NE. These results are consistent with reports that human OGG1 has functions equivalent to those of the bacterial enzyme, Fpg, and human MYH has functions equivalent to those of the bacterial enzyme, EnIII (Fromme and Verdin, 2003; Girard et al., 1997). The same results were seen using slides of cells treated with SNP (Fig. 6B), SIN-1 (Fig. 6C), As$_2$O$_3$ (Fig. 6D), MMA$_{III}$ (Fig. 6E), or DMA$_{III}$ (Fig. 6F).

**DISCUSSION**

The present results indicate that trivalent arsenicals, such as As$_2$O$_3$, MMA$_{III}$, and DMA$_{III}$, only induce oxidative DNA adducts and that glycosylases, OGG1 and MYH, are involved in the incision of these oxidative DNA adducts. This conclusion is based on the observation that (1) digestion of arsenite-treated cells with NE or with EnIII + Fpg gave the same amount of DNA strand breaks; (2) subsequent incubation of NE-digested slides with EnIII + Fpg did not cause a further increase in strand breakage nor did subsequent incubation of EnIII + Fpg–digested slides with NE; (3) proteins involved in nucleotide excision repair were not required for the incision of arsenite-induced DNA adducts, whereas OGG1, APE, and MYH, which are known to be involved in the repair of oxidative DNA damage, were required; (4) the nucleotide excision–defective cells, XPA, XPD, and XPG, excised UVC-induced adducts more slowly, but excised As$_2$O$_3$-induced adducts at the same rate, as normal fibroblasts; and (5) immunodeletion of NE with antibody of OGG1, MYH, or APE decreased the incision of the...
DNA adducts induced by SNP, SIN-1, and \( \text{H}_2\text{O}_2 + \text{Fe} \) as well as adducts induced by As\(_2\text{O}_3\), MMA\(^{\text{III}}\), and DMA\(^{\text{III}}\). These results suggest that cells or individuals with low antioxidant activity or low OGG1, APE, or MYH activity may be more susceptible to arsenite toxicity and that antioxidants may be effective in reducing arsenite toxicity.

The present results showing that the adducts removed by OGG1 were similar to those removed by Fpg are consistent with the notion that OGG1 is a functional counterpart of the *Escherichia coli* protein, MutM (Fpg) (Bruner *et al.*, 2000), while those showing that the adducts removed by MYH were similar to those removed by EnIII suggest that human MYH is a functional counterpart of the *E. coli* enzyme, EnIII (Boiteux and Radicella, 2000; Bruner *et al.*, 2000). EnIII has been shown to cleave thymine glycol, 5,6-dihydrothymine, 5-hydroxydihydrothymine, 5-hydroxycytosine, 5-hydroxyuracil, and uracil glycol (Sarker *et al.*, 1998), while Fpg has been shown to cleave oxidative bases, such as 8-oxo-7,8-dihydroguanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine, and 4,6-diamino-5-formamidopyrimidine (Perlow-Poehnelt *et al.*, 2004). MYH has also been shown to initiate base excision repair by catalyzing removal of adenine residues mispaired during erroneous replication with either oxidized or nonmodified guanines (Hashimoto *et al.*, 2004). The present results do not provide an answer to whether or not MYH can excise the mispaired adenine and guanine, because in these experiments, cells were treated with arsenite, SIN-1, or SNP for 30 min and then immediately lysed. There was no DNA replication involved.

Both OGG1 and MYH are bifunctional glycosylases with associated lyase activity and excise oxidized bases and then nick the DNA strand 3' to the abasic site by a β-elimination mechanism (Aspinwall *et al.*, 1997; Hilbert *et al.*, 1997; Rosenquist *et al.*, 1997; Zharkov *et al.*, 2000). The resulting 3'-sugar phosphate cannot serve as a primer for DNA synthesis and must be removed by the 3'-diesterase activity of APE (Demple and Harrison, 1994). Digestion of oxidized bases with APE-depleted NE is expected to see breaks in the alkaline comet assay, because alkali-labile sites produced by the glycosylase activity of OGG1 and MYH will be resolved into breaks in alkaline condition without any need of lyase. Similarly digestion of oxidized bases with OGG1-depleted NE is still expected to see breaks, if the MYH remains intact in NE. However, the present results indicated that immunodepletion of NE with antibody either with APE, or with OGG1, or with MYH all decreased the incision activity to the buffer level. One explanation is that the proteins of OGG1 and MYH may have been cointmunoprecipitated by the antibody of APE. The protein of OGG1 may have been cointmunoprecipitated by the antibody of MYH and vice versa. This possibility casts a doubt about the specificity of immunodepletion. However, the results showing that immunodepletion with the antibody of XPA, XPB, XPD, XPF, and XPG did not decrease the incision activity of NE argue that there are some specificities. More experiments are needed to investigate what proteins have been cointmunoprecipitated.

Our results showed that most of the arsenite-induced DNA strand breaks arose from incision of oxidative DNA adducts, and the standard comet assay or alkaline elution without enzyme digestion therefore revealed only a very small proportion of the total DNA damage. Since DNA strand breaks appear transiently during adduct incision and are immediately rejoined, the amount of DNA strand breakage at a given time point will be low. This probably explains why low amounts of DNA strand breaks were detected in many previous studies and why very high arsenite concentrations were required to induce DNA damage. The present results demonstrated that, using NE digestion, DNA damage can be detected after treatment for 1 h with > 0.025μM As\(_2\text{O}_3\) in NB4 cells. In our experimental condition, the concentration required to decrease the NB4 cell viability to 50% as assayed by trypan blue exclusion at the end of a 72-h As\(_2\text{O}_3\) incubation was about 2μM (data not shown). Thus, oxidative DNA damage occurs at very low concentration and at a very early stage in human cells exposed to arsenite. This argues that oxidative DNA damage is an important mechanism of arsenic carcinogenesis.

ACKNOWLEDGMENTS

We thank Dan Chamberlin and Tom Barkas for English editing. This work was supported by a grant from the National Science Council, Republic of China.

REFERENCES


