Role of Oxyradicals in Mutagenicity and DNA Damage Induced by Crocidolite Asbestos in Mammalian Cells

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ABSTRACT

Crocidolite, one of the most carcinogenic forms of asbestos, is mutagenic in cultured mammalian cells when assayed using a system that can detect multilocus deletions. In the present study, we examined the effect of buthionine sulfoximine (BSO) on mutation frequency and the formation of 8-hydroxydeoxyguanosine (8-OHdG) in human-hamster hybrid (A1) cells induced by crocidolite fibers in an attempt to determine the role of oxyradicals in mediating fiber mutagenesis. BSO, a competitive inhibitor of the enzyme γ-glutamyl cysteine synthetase, depleted nonprotein sulfhydryls to <5% of control within 24 h at a nonmutagenic dose of 25 μM. In cells pretreated with BSO for 24 h, the mutation yield at the CD59 locus induced by a 4 μg/cm² dose of crocidolite fibers was increased by more than 3-fold (P < 0.05). Using immunoperoxidase staining with a monoclonal antibody specific for 8-OHdG, we demonstrated that crocidolite fibers induced a dose-dependent increase in oxidative DNA damage in A1 cells. Furthermore, addition of DMSO, a well-established hydroxyl radical (OH−) scavenger, dramatically suppressed 8-OHdG induction (P < 0.005). Our results definitely demonstrate that reactive oxygen species mediate fiber-induced DNA damage mutagenesis in A1 cells in a concentration-dependent manner.

INTRODUCTION

Asbestos, a fibrous mineral silicate, is known to cause pulmonary fibrosis, bronchogenic carcinoma, and malignant mesotheliomas of the pleura and peritoneum (1, 2). Although various in vitro and in vivo studies have shown that asbestos is cytotoxic, mutagenic, and induces a persistent increase in expression of the proto-oncogenes c-jun and c-fos in mammalian cells, the molecular mechanisms responsible for these biological effects are not yet understood (3–5).

There is evidence to suggest that ROS play an important role in asbestos-induced cytotoxicity and mutagenicity (6–8). In vitro studies with asbestos and concurrent exposure to antioxidant enzymes such as superoxide dismutase, catalase, and the radical scavenger, Tempol, have been carried out in different cell systems (7, 9–11). The protective effects of antioxidants vary in different experiments and may be the result of different experimental conditions, type or dose of fibers used, or the nature of the assays. Nevertheless, these studies indicate a close relationship between the induction of ROS and asbestos-mediated toxicity. Among the most biologically active ROS such as superoxide anions (O2−), hydrogen peroxide (H2O2), hydroxyl radical (OH•), singlet oxygen (1O2), and hydroperoxy radical (HO2•), OH• is thought to be the most likely mediator for asbestos-induced carcinogenesis (12). Recent evidence has shown that these ROS, especially OH•, when formed in the vicinity of DNA can react with DNA, leading to oxidative damage such as strand breaks and base modifications (13). Among oxidative DNA damage products, 8-OHdG is one of the most specific and representative of base modification (14). There is evidence to suggest that 8-OHdG is a major mutagenic lesion, producing predominately G→T transversion mutations (15). Recent studies have demonstrated that 8-OHdG is highly mutagenic in NIH 3T3 cells, HL60, and Escherichia coli (16–18). However, 8-OHdG levels differ considerably from study to study. The major factors accounting for the discrepancy in results could be DNA extraction procedures and the conditions of DNA digestion (19). Thus, a comprehensive study is clearly needed for understanding the multifunctional effects of ROS.

Crocidolite (Na2O·Fe2O3·8SiO2·H2O), containing up to 27% iron by weight, is one of the most potent carcinogenic asbestos fibers. Previous studies from this laboratory have shown that the human-hamster hybrid (A1) cells assay is sensitive in detecting mutagens such as ionizing radiation, asbestos, and certain heavy metals that induce mostly large, multilocus deletions (3, 20, 21). Furthermore, there is a 50-fold increase in mutations at the CD59 locus when compared with the HPRT locus in crocidolite-treated A1 cells (22). Our present studies focused on understanding the role of ROS in mediating the mutagenicity of crocidolite fibers. Therefore, we investigated the role of BSO and the effect of an increase in intracellular oxidative stress on fiber mutagenesis in A1 cells. BSO inhibits the synthesis of NPSHs, of which 90% is glutathione in mammalian cells (23). If oxyradicals mediate the mutagenicity of asbestos, one would expect the mutagenic incidence to increase in fiber-exposed A1 cells containing little or no intracellular glutathione. Furthermore, the formation of 8-OHdG, in the presence of the radical scavenger DMSO was determined by an immunoperoxidase procedure using a monoclonal antibody specific for recognizing 8-OHdG in single cells. We show here that BSO pretreatment enhanced crocidolite-induced CD59 mutation frequency. There was a dose-dependent increase in the formation of 8-OHdG and oxyradicals, particularly OH•, highly suggestive that this species is responsible for the genotoxicity of asbestos fibers.

MATERIALS AND METHODS

Cell Culture. The human-hamster hybrid (A1) cell line, formed by fusion of human fibroblasts and the γ−mutant of CHO cells (20), was used in these studies. In addition to a standard set of CHO-K1 chromosomes, these hybrid cells contain a single copy of human chromosome 11. Chromosome 11 encodes several cell-surface antigenic markers that render the cells sensitive to killing by specific monoclonal antibodies in the presence of rabbit serum complement (HPR, Denver, PA). Cells were cultured in Ham’s F-12 medium supplemented with 8% heated inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), 2 × 10−4 M glycine, and 25 μg/ml gentamicin at 37°C in a humidified 5% CO2,95% air incubator and pasaged as described (7).

Preparation of Crocidolite Fibers. International Union Against Cancer standard reference crocidolite fibers (average length, 3.2 ± 1.0 μm; average diameter, 0.22 ± 0.01 μm) were used in these studies. The fiber preparation was described previously (7, 22). Briefly, samples of fibers were weighed out and suspended in distilled water. A stock solution of the fibers was prepared at a concentration of 2.5 mg/ml. The fiber suspension was passed through a 18-gauge needle fitted with a 20-ml syringe six to eight times to disperse the fibers. Fibers were sterilized by autoclaving and used at the concentrations indicated.

NPSH Depletion and Assay. BSO (Chemalog, S. Plainfield, NJ) at concentrations ranging from 25 to 250 μM in complete medium was prepared fresh...
each time and added to A₅ cultures for various treatment periods under aerobic conditions. After treatment, the NPSH levels in cells were determined using a modified Tietze assay with 10% cold sulfosalicylic acid (Sigma Chemical Company, St. Louis, MO) and 0.05% 5,5-dithio-bis-2-nitrobenzoic acid as described previously (24, 25). The absorbance of the sample was then determined spectrophotometrically at 412 nm, and the NPSH level was determined based on a standardized absorbance curve.

Crocidolite Fibers and BSO Cytotoxicity. Exponentially growing A₅ cells were trypsinized and repleted in T-25 tissue flasks at 1 × 10⁶ cells/flask. After incubation with BSO for 24 h, the cells were treated with a 4 µg/cm² dose of fibers for 24 h in either the presence or absence of BSO. After treatment, the cultures were washed with balanced salt solution, trypsinized, and repleted into 100-mm diameter Petri dishes for colony formation. Cultures were incubated for 7 days, at which time they were fixed with formaldehyde, stained with Giemsa, and the number of colonies counted to determine the surviving fraction (4, 7).

Mutations Assay. After treatment, cultures were repleted in T-75 flasks and cultured for 5–7 days. This expression period is needed to permit surviving cells to recover from the temporary growth lag from fibers treatment and to multiply such that the progeny of the mutated cells no longer express lethal amounts of the CD59 surface antigen. To determine mutant fractions, 5 × 10⁴ cells were plated into each of six 60-mm dishes in a total of 2 ml of growth medium as described (3, 7, 21). The cultures were incubated for 2 h to allow for cell attachment, after which 0.2% CD59 antisera and 1.5% (v/v) freshly thawed complement were added to each dish. The cultures were incubated an additional 7–8 days, at which time they were fixed, stained, and the number of CD59− mutants scored. Controls included identical sets of dishes containing antiserum alone, complement alone, or neither agent. The cultures derived from each well were tested for mutant yield for 2 consecutive weeks to ensure full expression of the mutations. Mutant fractions were calculated as the number of surviving colonies divided by the total number of cells plated after correction for any nonspecific killing due to complement alone.

Immunoperoxidase Staining for 8-OHdG. Exponentially growing cells were inoculated into Lab-Tek glass chambered slides (Nunc Inc., Naperville, IL) at 5 × 10⁴ and cultured for 24 h. The cultures were exposed to graded doses of fibers for 24 h with or without 0.5% DMSO. The dose of DMSO was nontoxic, nonmutagenic, and had been shown to be an effective free radical scavenger (21, 26). After treatment, cultures were rinsed twice with PBS and fixed with 5% acid-alcohol at −20°C. Immunoperoxidase staining for 8-OHdG was performed as described previously (27, 28). Briefly, fixed cultures were treated with RNase (100 µg/ml) for 1 h at 37°C and proteinase K (10 µg/ml) for 10 min at room temperature. DNA was denatured with 4 N HCl for 10 min at room temperature, and 10% normal horse serum was used to block nonspecific antibody binding sites. The cultures were then incubated with the primary antibody 1F7 (1:50 dilution) at 4°C overnight followed by goat antimouse IgG conjugated to biotin at 37°C for 30 min. Endogenous peroxidase was blocked by treating the cultures with 3% H₂O₂ in methanol for 30 min at room temperature. ABC reagent, avidin conjugated to horseradish peroxidase, was added, and the slides were incubated for 30 min at 37°C, followed by extensive washing. Cells were treated with diaminobenzidine to localize peroxidase, cleaned with xylene, and mounted on a coverglass, using Permount. A Cell Analysis System CAS 200 microscope and a Cell Measurement Software package (Becton Dickinson, San Jose, CA) were used to quantify the relative staining intensity from 50 randomly selected cells per group per experiment. Staining data represent the average absorbance multiplied by 1000.

Statistics. Statistical analysis of data was carried out using Student’s t test. Differences between means are regarded as significant at P < 0.05.

RESULTS

Effects of BSO on the Depletion of NPSHs in A₅ Cells. It is now generally accepted that sulfhydryl compounds such as glutathione, cysteine, and cysteamine can protect mammalian cells from oxidative damage (29). To investigate the effect of BSO on the NPSH level in A₅ cells, we examined both the BSO concentration level and length of incubation time on the NPSH content of A₅ cells. There was a dose-dependent decrease in NPSH levels after a 24-h treatment with BSO at concentrations ranging from 25 to 250 µM. Less than 2 n mole of NPSH per 10⁷ A₅ cells were detected after treatment with a 25-µM dose of BSO, a level that was <5% of the normal level (Fig. 1). A clear dose-treatment period-dependent reduction of NPSH levels by BSO was observed when exponentially growing A₅ cells were subject to BSO treatment at doses of either 25 or 250 µM (Fig. 2). These results showed that depletion of NPSH levels was dependent on the concentration and time of incubation with BSO, consistent with previous studies in C₅H₁₀T¹/₂ cells (25). All our subsequent studies were carried out using 25 µM, the lowest effective dose of BSO that resulted in a surviving fraction of ~0.67.

Role of BSO in Mutagenicity of Crocidolite Fibers. Considerable evidence points to the fact that depletion of NPSHs with BSO can increase the sensitivity of mammalian cells to the cytotoxic/genotoxic effects of radiation and chemotherapy drugs (30, 31). Fig. 3 shows the CD59 mutant fraction induced by a 4 µg/cm² dose of crocidolite with or without pretreatment with 25 µM BSO. The average number of spontaneous CD59− mutants per 10⁶ survivors in A₅ cells used for these experiments was 43 ± 18. The induced mutant fraction in fiber-treated A₅ cells was ~2-fold higher than background. BSO treatment by itself induced a low and nonsignificant increase in the background CD59− mutant yield. In contrast, BSO pretreatment enhanced the mutagenic potential of crocidolite fibers such that there was a 3-fold increase in CD59− mutant yield in cells treated with both BSO and fibers compared with those treated with fibers alone (P < 0.05). Furthermore, pretreatment of A₅ cells with BSO also enhanced their sensitivity to the cytotoxicity of fibers (Fig. 3). These results indicate that intracellular antioxidant status has a profound effect on the mutagenic response of A₅ cells to asbestos treatment and suggest that ROS play a significant role in mediating the mutagenic process.
Effects of Crocidolite on the Formation of 8-OHdG. Crocidolite is reported to increase the formation of 8-OHdG with or without H\textsubscript{2}O\textsubscript{2} in calf thymus DNA (32). Fig. 4 shows a representative immunoperoxidase staining for 8-OHdG in A\textsubscript{L} cells treated with a 6 mg/cm\textsuperscript{2} dose of fibers for 24 h. 8-OHdG was localized mainly in the nucleus of both control and fiber-treated cells. Although a faint background staining was evident in the control cultures, treatment of A\textsubscript{L} cells with crocidolite fibers resulted in a dose-dependent induction of 8-OHdG (Fig. 5). A significant increase in 8-OHdG induction was observed at fiber concentrations >4 mg/cm\textsuperscript{2} (\(P < 0.05\)). Quantification of staining in 150 randomly selected cells indicated that treatment of cells with a 6 mg/cm\textsuperscript{2} dose of fibers induced a 2-fold increase in the 8-OHdG level.

Effect of DMSO on the Formation of Crocidolite-induced 8-OHdG. Fig. 6 shows the suppressive effect of 0.5% DMSO on the formation of 8-OHdG induced by a 6 mg/cm\textsuperscript{2} dose of fibers in A\textsubscript{L} cells. The relative staining intensity decreased from 290 ± 15 to 170 ± 6 in the presence of DMSO (\(P < 0.005\)). DMSO by itself, however, had little or no effect on the formation of 8-OHdG among control A\textsubscript{L} cells.

DISCUSSION

Accumulating evidence has indicated that asbestos induces the generation of ROS in many rodent and human cell systems (12). It has been demonstrated that ROS are generated not only from fiber-treated cells, but may also be generated by the fibers themselves in the presence of iron (26–30% in crocidolites and 1–3% in chrysotiles), which subsequently lead to hydroxyl radical formation, lipid peroxidation, and DNA single-strand breaks (13). The involvement of superoxide anions and hydroxyl radicals in asbestos-induced cytotoxicity has been suggested (4, 9) and the radical scavenging enzymes, superoxide dismutase and catalase, have been shown to protect against the toxicity of fibers, both in vitro and in vivo (10, 12). The observations that mesothelioma induction in rats and humans can be correlated with fiber-induced hydroxyl radicals provide further support for the possible role of ROS in fiber carcinogenesis (33). These data are consistent with our present finding that a reduced intracellular antioxidant level enhanced crocidolite-induced CD59 mutation frequency. Furthermore, there was a dose-dependent increase in the formation of 8-OHdG among A\textsubscript{L} cells treated with asbestos.

Cellular NPSHs consist essentially of glutathione (~95%) and other low molecular weight aminothiols such as cysteine and cysteamine. They are considered to have significant free radical scavenging abilities that contribute to the maintenance of cell integrity (25). Although a decrease in cellular NPSHs is not necessarily lethal to cells, sulphydryl depletion has been shown to enhance the cytotoxicity of a variety of agents, including ionizing radiation, heavy metals, and some chemotherapeutic drugs (28, 30). There is also sufficient evidence to suggest that cellular thiols such as glutathione and cysteine protect mammalian cells against the toxicity and mutagenicity of ionizing radiation and H\textsubscript{2}O\textsubscript{2} (34, 35). BSO, a competitive inhibitor of the enzyme \(\gamma\)-glutamyl cysteine synthetase, functions to deplete the intracellular level of NPSHs and leads to enhancement in the frequency of mutations (31, 36). Our data demonstrated that the intracellular NPSH level was greatly reduced by BSO treatment in A\textsubscript{L} cells.
and that the mutagenic potential of asbestos increased by >3-fold. These findings provide strong corroborating evidence that the CD59− mutations induced by fibers are mediated by ROS.

DNA damage induced by ROS is important in mutagenesis and carcinogenesis (37). 8-OHdG is one of the most abundant oxidized DNA bases and has been shown to be a mutagenic DNA lesion (38). There is evidence that E. coli or other bacterial strains defective in the 8-OHdG repair systems show higher mutant frequencies than their wild-type counterparts (18, 39). Crocidolite, which is more carcinogenic to humans than other types of mineral fibers, has been reported to increase the formation of 8-OHdG in extracted calf thymus DNA samples and in cellular DNA from fiber-treated mammalian cells (17, 32, 40). In this study, we used 8-OHdG as a reliable biomarker for oxidative DNA damage as shown previously (27, 28).

Although there are other methods available for the quantification of 8-OHdG in mammalian DNA, including HPLC-electrochemical detection, gas chromatography-mass spectroscopy, and 32P postlabeling, the immunoperoxidase assay as used in the present study offers several advantages. This assay, based on a monoclonal antibody specific for 8-OHdG (1F7), was previously developed by one of the coauthors (27, 41, 42). The assay allows the detection of 8-OHdG in single cells and in a highly reproducible manner. The specificity of 1F7 for 8-OHdG has been demonstrated previously by competitive ELISA assay (41). Furthermore, using DNA extracted from mammalian cells exposed to inducers of oxidative damages (e.g., hydrogen peroxide), the quantification of 8-OHdG formation correlated well between the immunoperoxidase assay and the more conventional HPLC-electrochemical detection method (41). Because the immunoperoxidase method does not require isolation and processing of DNA, the potential for artificial generation of oxidative damage is eliminated. Crocidolite exposure induced a dose-dependent increase in the formation of 8-OHdG in A549 cells. Our results are similar to previous studies of Takeuchi and Morimoto (17), who quantified the formation of 8-OHdG in crocidolite treated HL60 cells using HPLC analysis. Furthermore, the induction of 8-OHdG in crocidolite-treated A549 cells was consistent with our previous findings on mutation induction by the fibers (22).

It is of interest to consider what types of oxyradicals are involved in mutation induced by asbestos. DMSO, a well-established free radical scavenger in mammalian cells, is effective in protecting against mutation, chromosomal aberrations, and oxidative DNA damage (26). Here, we demonstrate that addition of DMSO in the medium suppresses the formation of 8-OHdG (P < 0.005). The level of 8-OHdG induced by asbestos was reduced to close to background in the presence of DMSO. These data highlight the involvement of ROS, particularly OH•, in asbestos-mediated oxidative stress. However, OH• is short-lived and can diffuse only a few nm (43). Thus, mutation induction by asbestos may include more than one type of ROS. In addition to OH•, O[ bardot;2] and H2O2 may also be involved in this process. Several studies have indicated that asbestos catalyzes the formation of O[ bardot;2] or OH• from H2O2 via the Fenton reaction or the Haber-Weiss reaction and that the perpetuated cascading events may involve lipid peroxidation (12, 44), which can damage DNA, leading to mutational events. Several mechanisms have been proposed for the generation of ROS by asbestos. First, fibers per se trigger ROS induction in iron-catalyzed reactions as discussed above; and second, fibers may activate phagocytosis to enhance the production of ROS as described above. It has been postulated that “frustrated phagocytosis” of long fibers is a potent stimulus for ROS production in macrophages and leukocytes (45). The observations that non-iron-containing fibers such as erionites are carcinogenic and mutagenic (7, 46) suggests that the mobilization of iron from fibers may not be the only pathway for ROS induction. The observations that asbestos fibers are commonly found internalized in A549 cells that undergo mutational changes (22) and that inhibition of cellular phagocytosis by cytochalasin B significantly reduced fiber uptake in alveolar macrophages (47) suggest the importance of fiber-cell interaction in asbestos-mediated mutagenicity.

If oxyradicals do indeed mediate the genotoxicity of asbestos, why has the fiber not been shown to induce the point mutations that are frequently detected in bacterial assays and among some HPRT− mutants in mammalian cells exposed to ROS (48)? First, the types of mutants recovered depend on several factors, including the mutagen used, the conditions for mutant selection, DNA repair rate and fidelity, and, more importantly, the proficiency of the assay for selecting a full range of mutants, including multilocus deletions. Most mutagenesis studies using bacterial assays have reported that oxyradicals induce either base-substitution or frame-shift mutations (49). In mammalian cells, it has been reported that ~64% of the HPRT− mutants induced by ROS (48) have no discernable alterations in the gene (i.e., changes <30 bp, which can either be a point mutation or no change) and the remaining 36% of the mutants have either partial or total deletions. In contrast, using the AS52 system, which uses a bacterial gpt gene functionally integrated in the CHO genome, Hsie et al. (50) have shown that assays that allow the recovery of multilocus deletions will score predominately deletion mutations when challenged with ROS. These findings are consistent with the induction of dicentric and chromosomal deletions in H2O2-treated human fibroblasts (51). Similarly, a significantly higher mutant yield have been demonstrated with the L5178TK−/− mouse lymphoma cell line, which is hemizygous at the TK locus when...
exposed to either ROS (52, 53) or to agents whose biological effects are known to be mediated by ROS, including ionizing radiation and mitomycin C (54). The enhanced recovery of large deletions in the L5178 assay system is due to the presence of active copies of the linked essential genes on the homologous chromosome 11 (53). These findings are consistent with several recent reports demonstrating that asbestos is indeed mutagenic in mammalian cells when assayed using systems that favor either recovery of deletion mutants or homologous recombinations (6, 55, 56).

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