Asbestos Increases Mammalian AP-Endonuclease Gene Expression, Protein Levels, and Enzyme Activity in Mesothelial Cells

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Abstract

Only two DNA repair enzymes, DNA polymerase β and O6-methylguanine-DNA methyltransferase, have been shown to be inducible in mammalian cells by genotoxic agents. We show here that crocidolite asbestos induces the DNA repair enzyme, apurinic/apyrimidinic (AP)-endonuclease, in isolated mesothelial cells, the progenitor cells of malignant mesothelioma. Asbestos at nontoxic concentrations of 1.25 and 2.5 μg/cm² significantly increased AP-endonuclease mRNA and protein levels as well as enzyme activity (P < 0.05) in a dose-dependent manner in rat pleural mesothelial cells. These increases were persistent from 24 to 72 h after initial exposure to fibers. Changes were not observed with glass beads, a noncarcinogenic particle. Confocal scanning laser microscopy showed that AP-endonuclease was primarily localized in the nucleus but also in mitochondria. Our data are the first to demonstrate the inducibility of AP-endonuclease by a nonfibrous particle.

Introduction

Exposure to asbestos is associated with the development of fibrotic lung disease, malignant pleural mesotheliomas, and carcinomas of the lung (1). The mechanisms of asbestos-induced disease are unclear. However, DNA damage, mutagenesis, and cell proliferation are important features of asbestos-induced lung injury, and recent studies strongly suggest that excessive production of oxidants by asbestos mediates these phenomena (reviewed in Ref. 1). Our previous studies in isolates of RPM² cells (2) have demonstrated that asbestos increases the formation of 8OHdG, one of the most abundant DNA base damages induced by oxidative stress. 8OHdG is mainly removed by DNA base excision repair in mammalian cells (3). In Escherichia coli, 8OHdG is removed by the action of formamidopyrimidine DNA glycosylase, encoded by the MutM gene. This enzyme first breaks the N-glycosidic bond between the 8OHdG and the deoxyribose ring. A lyase activity of the same enzyme catalyzes a 3′β-elimination at the abasic sugar, generating a ring-opened α,β unsaturated aldehyde. An analogous activity has been discovered recently in human cells, and the gene encoding this enzyme has recently been cloned and sequenced and has been designated hOGG1 (4). The resulting α,β unsaturated aldehyde is removed by the action of a 5′ APE (class II).

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2 The abbreviations used are: RPM, rat pleural mesothelial; 8OHdG, 8-hydroxy-2′-deoxyguanosine; AP, apurinic/apyrimidinic; APE, AP-endonuclease; AP-1, activator protein-1; NIEMS, National Institute of Environmental Health Sciences; PBS, fetal bovine serum; MTS, 3-(4,5-dimethylthiazol-2-y1)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; CMF-PBS, calcium- magnesium-free PBS; F. tetrahydrofuranyl abasic site; UDQ, uracil DNA glycosylase; YOYO, oxazole yellow homodimer.
Fig. 1. Crocidolite asbestos, but not glass beads, increases steady-state mRNA levels of APE in RPM cells. Fifteen-μg RNA samples were prepared and loaded in duplicate. Following Northern blot analysis, the level of APE mRNA was quantified by phosphoimaging, and the values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signals (data are the means; bars, SE). *, P < 0.05 in comparison to untreated controls at each time period. Experiments were repeated in duplicate and values are expressed in arbitrary units.

MTS Assay and Trypan Blue Exclusion Technique. Two assays were used to assess the viability of RPM cells after exposure to asbestos. The MTS cell viability assay, based on conversion of MTS to formazan, was performed according to the manufacturer's protocol (Promega Corp., Madison, WI). Cells were grown to confluency in 96-well plates and exposed to particulates in 0.5% FBS-containing medium as described above for up to 72 h. At each time point, 20 μl of MTS stock solution was then added in 100 μl of fresh culture medium, and cells were incubated for 2.5 h at 37°C. After incubation, culture medium was removed and evaluated at 490-nm absorption using an ELISA plate reader (Molecular Devices, Menlo Park, CA). For determination of numbers of viable cells excluding trypan blue, an indication of viability, confluent cells treated identically with asbestos in 12-well plates were trypsinized, pelleted after centrifugation at 500 X g for 5 min at 4°C, and resuspended in complete F12/DMEM medium; an aliquot was counted on a hemocytometer slide in 0.5% trypan blue (Sigma).

Northern Blots. Total RNA was prepared, and Northern blot hybridization performed as described in detail by Shull et al. (15). Purity and concentration were determined by measuring UV absorbance at 260 and 280 nm. A total of 15 μg of total RNA was electrophoresed on a 1% agarose/37% formaldehyde gel, transferred onto nitrocellulose membrane, and hybridized to [α-32P]dCTP-labeled cDNA probes. To ascertain equal loading of RNA on individual lanes, gels were examined after staining with ethidium bromide. Mouse APE cDNA was kindly provided by Dr. Shuji Seki at Okayama University (Okayama, Japan). A cDNA probe for glyceraldehyde-3-phosphate dehydrogenase, kindly obtained from Dr. L. Jeanneur (Paris, France) was also used as a housekeeping gene. cDNA probes were labeled with [α-32P]dCTP by random hexamer priming (Promega). Hybridization signals then were quantified using a phosphoimaging system (GS-250; Bio-Rad, Hercules, CA).

Western Blots. Nuclear extracts were prepared and suspended in the Laemmli loading buffer for 12% SDS-PAGE, as described previously (16). The sample was loaded at 2.5 μg/lane on a mini gel (Bio-Rad). After electrophoresis, proteins were transferred onto nitrocellulose at 100 V for 2 h. Blots were blocked with 2% nonfat dry milk in CMF-PBS before they were incubated with a rabbit polyclonal primary antiserum raised against purified recombinant human APE (a gift from Dr. Samuel H. Wilson, NIEHS), diluted 1:1000 in 2% nonfat dry milk and 0.1% sodium azide in CMF-PBS for 1 h and peroxidase-conjugated goat anti-rabbit secondary antibody (Vector, Burlingame, CA) for another 1 h, and APE protein was visualized using the ECL technique (Amersham International, Buckinghamshire, United Kingdom). The intensity of protein signals was quantified with a phosphoimaging system.

Oligonucleotide Substrate Preparation. APE has the highest cleavage efficiency to a synthetic AP-site analogue, tetrahydrofuranyl (F). Thus, the DNA oligonucleotide, 15-bp F containing (5'-TGAGCAAFAACTAGC-3'), 19-bp uracil (U) containing (5'-CGGTGCGGGCC@CTFOCT-3') and their complementary strands carrying A opposite F, and A opposite U, were used here. The oligonucleotides were 5'-end-labeled with [γ-32P]ATP (Dupont, MA), using T4 polynucleotide kinase (Life Technologies, Inc.), following the manufacturer's instructions. The labeled DNA oligonucleotide strands then were annealed to the complementary strand/s by heating the mixture to 90°C for 3 min and cooling it down gradually to room temperature. The 5'-end-labeled duplex DNA fragments were then diluted in TE buffer and passed through a G-50 Sephadex column (Boehringer Mannheim Corp., Indianapolis, IN) to separate them from free [γ-32P]ATP. Following phenol/chloroform extraction and cold ethanol precipitation, the labeled double-strand oligonucleotides were kept in TE buffer, −20°C.
APE Inducibility Activity Assay. Nuclear extracts were prepared as described above. For determination of APE activity, an aliquot (5 ng) of cellular nuclear extracts was incubated with 100 fmol of 32P-labeled oligonucleotide-F (as described above) in a 10 μl total reaction volume diluted with a buffer solution of 50 mM Hepes-KOH (pH 7.5), 50 mM KCl, 10 mM MgCl2, 0.1 mg/ml BSA, and 0.05% Triton X-100 for 30 min at 37°C. The reactions were stopped by adding an equal volume of 0.05% bromphenol blue and 0.05% xylene cyanole-containing formamide solution and stored at −20°C. For uracil DNA glycosylase, the reactions were carried out in the same way as for APE, but using a 10-ng nuclear extract and 32P-labeled oligonucleotide-containing uracil in a reaction buffer without MgCl2, and subsequently boiling the samples for 30 min. Finally, the DNA reaction products were heated at 65°C for 5 min and separated on a 15% polyacrylamide gel (National Diagnostics, Atlanta, GA) using a sequence gel system. Dried gels were subjected to autoradiography for visualization and quantified using a phosphoimaging system (Bio-Rad).

Dual-Immunofluorescent Labeling and Confocal Scanning Laser Microscopy. All procedures were performed at room temperature. In brief, confluent RPM cells on coverslips were washed two times with CMF-PBS, fixed for 15 min at 3.7% formaldehyde, rinsed one time with CMF-PBS, and permeabilized for 15 min with 1% Triton X-100. After a rinse with CMF-PBS, cells were then blocked in 2% nonfat dry milk in CMF-PBS for 30 min. After washing with CMF-PBS, cells were incubated for 60 min with rabbit anti-human APE polyclonal antibody (1:100 dilution in blocking solution). Coverslips then were rinsed with CMS-PBS and incubated with a mitochondrial marker, mouse anti-human cytochrome oxidase subunit I monoclonal antibody (60 μg/ml; Molecular Probes, Eugene, OR) for another 60 min. Coverslips were rinsed with CMS-PBS and incubated with Cy5-conjugated goat anti-rabbit secondary antibody (20 μg/ml; Jackson Immunoresearch Laboratories, West Grove, PA) and Oregon Green™488-conjugated goat anti-mouse secondary antibody (10 μg/ml; Molecular Probes, Eugene, OR) for 60 min, or with 0.3 μM DNA-staining oxazole yellow homodimer (YOYO; Molecular Probes, Eugene, OR) for 30 min. After coverslips were washed three times for 5 min with CMF-PBS and one time in double-distilled water, they were allowed to air-dry and mounted onto slides with mounting media (Vector-H1000; Vector Laboratories, Burlingame, CA). To examine the specificity of APE immunofluorescence, a group of RPM cells was incubated with anti-APE that had been incubated with excess antigen (200 μg/ml purified recombinant human APE) overnight for comparison. Slides were observed on a Bio-Rad MRC-1000 confocal microscope (Hercules, CA) with a ×60 oil immersion lens. Samples were excited with either a 488-nm (for Oregon Green and YOYO) or a 647-nm (for Cy5) laser wavelength and examined under identical instrument settings including gain (1000 V), confocal iris (3.0 mm), laser intensity (30% of maximum), and Kalman image averaging (nine scans/image). Colocalization analysis was performed with the multiply function using MPL software (Bio-Rad). The multiply command multiplies each pixel in the active display box by the corresponding pixel of a second image. This command is useful for analyzing images for the degree of colocalization of two different dyes. Following the multiplication function, the resulting image will only appear bright in areas that were bright in both images. Conversely, the image will appear dark in areas that were bright in one image and dark in the other.

Statistical Methods. All raw data from individual experiments were analyzed by ANOVA with the use of the Student-Newman-Keuls test for multiple comparisons.

Results

Asbestos Increases APE Gene Expression in RPM Cells. The MTS cell viability assay showed that crocidolite asbestos at the

![Figure 2. Crocidolite asbestos, but not glass beads, increases protein levels of APE in RPM cells. Nucleic extracts (2.5 μg) were prepared and loaded in duplicate. Following Western blot analysis, the level of APE protein was quantified by phosphoimaging.](image-url)
Fig. 3. Crocidolite asbestos, but not glass beads, increases APE incision activity in RPM cells. A, titration study. RPM cell nuclear extracts at indicated concentrations were incubated with 100 fmol of synthetic 32P-labeled duplex oligonucleotide substrate at 37°C for 30 min, and the DNA reaction products were subjected to a 15% polyacrylamide gel. Lanes 1-6, APE incision activity; Lanes 7-11, UDG incision activity. Bands 15-mer and 7-mer represent uncleaved 32P-labeled 15-bp oligonucleotide-F and its 7-bp cleavage products, respectively; bands 19-mer and 12-mer, uncleaved 32P-labeled 19-bp oligonucleotide-U and its 12-bp cleavage products, respectively. B, dose-response study with asbestos at 24 h; C, persistent increases by asbestos over 24-, 48-, and 72-h periods. Five- and 100-ng nuclear extracts were used for APE and UDG studies, respectively, and were prepared and loaded in duplicate. The upper panels of B and C show APE incision activity; lower panels, UDG incision activity. Lane 0, control lane without addition of nuclear extracts. sham, untreated control; Ex, E. coli exonuclease III; croc, crocidolite asbestos in µg/cm²; GB, glass beads in µg/cm². The results were quantified by a phosphoimaging system. APE cleavage products (the 7-mer band) were normalized to UDG cleavage products (the 12-mer band). Data are the means; bars, SE. *P < 0.05 in comparison to untreated controls at each time period. Values are expressed in arbitrary units.

Asbestos Increases APE Incision Activity in RPM Cells. One of the major functions of the APE protein is to cleave the phosphodiester bond on the 5' AP-site. Thus, we used an AP-site incision activity assay to ascertain whether the induction of APE gene expression and protein was accompanied by increases in APE activity. Nuclear extracts from treated and untreated cells were incubated with a radiolabeled synthetic 15-mer duplex oligonucleotide-F, which is specific for testing APE, and the resulting DNA products were resolved on denatured polyacrylamide gels. A ubiquitous and highly conserved DNA repair enzyme, UDG, which removes uracil residues from DNA after dUMP incorporation or deamination of cytosine, was used here for comparison. The samples were loaded in duplicate on the gel for APE and UDG incision detection. As shown in Fig. 3, the lower bands (7- and 12-mer) represent enzyme incision products of APE and UDG, respectively, and the APE band was normalized to the UDG band in quantification of results. Because concentration dependence is important, a titration study was performed. As shown in Fig. 3A, the nuclear protein concentrations used here were in the reaction linear range. Data in Fig. 3, B and C, show that the doses and time periods of asbestos-induced increases in APE mRNA and protein (Figs. 1 and 2) correlated with protracted and cleaved APE activity levels. In con-
D: Inducibility of AP-endonuclease in pleural cells

Fig. 4. Cellular distribution of APE protein in RPM cells using confocal scanning laser microscopy. A–C, D–F, and G–H are the same field of cells. A, an APE antibody imaged by using a 647-nm channel. B, DNA staining by YOYO imaged by using a 488-nm channel. C, the merged image of A and B. D and G, cells labeled with an antibody against subunit I of cytochrome oxidase to demonstrate localization of mitochondria (imaged by using wavelength laser excitation 488-nm). E, normal RPM cells labeled with an APE antibody. H, cells stained with an APE antibody preabsorbed with excess APE antigen and imaged by using 647-nm wavelength laser excitation. F and I, overlap signals from the images of D and E, and G and H, respectively, rendered using a mathematical multiply function as described in "Materials and Methods." The images resulting from the multiply function have been pseudo-colored blue, with the blue representing areas of staining overlap from images D and E, or F and G, respectively. Note the colocalization of APE and subunit I of cytochrome oxidase as represented by the blue staining in F.

Contrast, glass beads had no effects on APE activity at any time period. Because APE and E. coli exonuclease III have class II APE activity, we used exonuclease III here as a positive control. As shown in Fig. 3C, the products generated by APE and exonuclease III are the same size.

APE Is Present in RPM Cell Mitochondria. Using immunofluorescence techniques, we found that APE protein is mainly localized in the nucleus of RPM cells (Fig. 4). We further confirmed its nuclear localization by staining cell nuclei with the DNA dye, YOYO (Fig. 4, A–C). However, APE fluorescence (red) was relatively diffuse in the cytoplasm of RPM cells, suggesting a possibility of mitochondrial distribution (Fig. 4C). Using an antibody against cytochrome oxidase subunit I, which is exclusively present in mitochondria (Fig. 4, D and G), we demonstrated, by using multiple overlap techniques and confocal scanning laser microscopy, that APE protein is present in mitochondria (Fig. 4, E and F). A preabsorption control, using APE antibody in the presence of excess APE, confirmed the specificity of these results (Fig. 4, H and I). Although Tomkinson et al. (17) have found that antibodies raised against APE cross-react with two proteins, 82 kDa and 65 kDa, from mitochondria of the mouse plasma-cytoma cell line (MCP-11), the 65-kDa protein [despite different molecular mass from the major nuclear forms], has similar 5' AP-site cleavage properties compared with the nuclear APE (17).

Discussion

Studies presented here show that asbestos is capable of inducing APE at levels of steady-state mRNA, nuclear protein, and AP-site incision activity in normal mammalian cells of the pleura. These increases are persistent from 24 to 72 h and occur in a dose-dependent manner. By contrast, glass beads, a nonfibrous particle, did not elicit effects. Because APE mRNA levels were unchanged after RPM cells were treated with H2O2 alone at a range of concentrations (10–500 μM) and time frames (data not shown), data indicate that the effects of asbestos are specific in these cell types and may be due to reactive species other than H2O2.

The most frequent target of endogenous DNA damage is probably the AP-site. It has been estimated that 2,000–10,000 AP-sites are generated spontaneously per mammalian cell per day (18). This situation is further aggravated during oxidative stress. Previous attempts have failed to demonstrate that the APE gene is inducible using various oxidative and genotoxic agents, including paraquat and bleo-
mycin in human transformed cell lines (19). However, it has been reported that APE is inducible under hypoxic conditions or during regeneration of the epithelium after physical injury (20, 21). In addition, patterns of inducibility may vary in normal versus transformed cells. Transformed cell lines tend to have high APE basal levels (10–20 times higher than untransformed cells), which may mask or prevent effects of agents inducing APE (19). Using a human SV40 T-antigen transformed mesothelial cell line (Met5A), asbestos at doses and time points used here did not induce APE gene expression (data not shown), supporting this hypothesis.

In comparison to patterns of 8OHdG formation (2), asbestos induced APE gene expression after relatively shorter periods of exposure and at lower nontoxic concentrations of fibers. Recent studies in our laboratory have shown that asbestos persistently induces c-fos and c-jun gene expression and transcription factor AP-1 to DNA-binding activity in RPM cells (22). Mapping of the APE promoter has identified an AP-1 binding site (19). However, phorbol ester increased c-fos mRNA levels, but not APE gene transcription, in this study (19). Thus, it is unclear whether increases in c-fos and c-jun gene expression and AP-1 DNA binding are related directly to increases in APE gene transcription. These observations are noteworthy because APE has redox activity facilitating AP-1 activity (8, 9). Thus, up-regulation of APE could be both a consequence of cellular response to oxidative stress and an important event in this signaling transduction pathway. Although it comprises less than 1% of the total cellular DNA in mammalian cells, mitochondrial DNA is a potential target for oxidative stress because more than 90% of intracellular oxidants are generated in mitochondria. Moreover, mitochondrial DNA lacks a histone structure, which may prevent oxidative DNA damage in the nucleus. Recent studies have revealed that mitochondria have significant DNA repair capacity (10, 11). However, mitochondrial DNA is more sensitive than nuclear DNA to H2O2-induced damage, and protracted exposures lead to persistent mitochondrial DNA damage and loss of mitochondrial function (11). Although these studies and biochemical studies have implicated APE activity in mitochondria, our results here demonstrate APE localization in mitochondria of normal cells of the pleura that are targets of asbestos-induced oxidative stress and carcinogenesis. Future studies are designed to determine the role of increased APE activity in repair from asbestos-associated cell damage and/or proliferation.

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