Induction of c-fos and c-jun Proto-oncogene Expression by Asbestos Is Ameliorated by N-Acetyl-L-Cysteine in Mesothelial Cells

Yvonne M. W. Janssen, Nicholas H. Heintz, and Brooke T. Mossman

Department of Pathology, University of Vermont College of Medicine, Burlington, Vermont 05405

ABSTRACT

Asbestos fibers cause dose-dependent, persistent increases in mRNA levels of c-jun and c-fos proto-oncogenes in rat pleusional mesothelial (RPM) cells, the progenitor cells of asbestos-induced mesothelioma (N. Heintz, Y. M. W. Janssen, and B. T. Mossman. Proc. Natl. Acad. Sci. USA, 90: 3299–3303, 1993). Here we report that addition of N-acetyl-L-cysteine decreases asbestos-mediated induction of c-fos and c-jun mRNA levels in a dose-dependent fashion. Exposure of RPM cells to asbestos causes depletion of total cellular glutathione, a response that can be abolished by pretreatment with N-acetyl-L-cysteine. Pretreatment of cells with buthionine sulfoximine, an agent which diminishes glutathione pools, increases the magnitude of induction of c-fos and c-jun mRNA by asbestos. To determine whether asbestos-induced effects on proto-oncogene expression could be attributed to extracellular generation of active oxygen species (AOS), RPM cells were exposed to H2O2 or xanthine and xanthine oxidase, a generating system of AOS. These oxidative stresses did not decrease cellular glutathione levels nor alter mRNA levels of c-fos or c-jun. However, increased mRNA levels of manganese-containing superoxide dismutase and heme oxygenase were observed, indicating that RPM cells respond to AOS by increased expression of genes encoding antioxidant enzymes. These data indicate that the signaling pathways leading to c-fos/c-jun proto-oncogene induction by asbestos are not triggered directly by formation of extracellular AOS. However, intracellular thiol levels appear to influence the expression of c-fos and c-jun, suggesting a redox-sensitive component in the signaling cascade which modulates gene expression of c-fos and c-jun by asbestos.

INTRODUCTION

Asbestos fibers are a heterogeneous family of naturally occurring mineral silicates which have been used in multiple industrial settings (1). Exposure to asbestos in unregulated workplaces has led to the development of malignant mesotheliomas and lung cancers. The mechanisms by which asbestos induces tumors are poorly understood. Our laboratory has focused on the molecular responses that occur in mesothelial and tracheal epithelial cells in vitro and in vivo after exposure to asbestos, because these cells are the progenitors of asbestos-induced mesotheliomas and bronchogenic carcinomas, respectively.

c-fos and c-jun are early response genes associated with the transition of cells into S phase (2). Induction of c-fos and c-jun is accompanied by increased binding of the transcription factor, AP-1, a heterodimeric protein complex composed of c-fos and c-jun gene products, to DNA. Both crocidolite and chrysotile asbestos cause dose-dependent and persistent increases in the expression of c-fos and c-jun in RPM2 cells (3). In HTE cells, only c-jun expression is induced by asbestos fibers, but c-fos and c-jun can be induced by H2O2 as has been reported in other cell types (4). In contrast, alterations in expression of these proto-oncogenes are not observed after exposure of RPM or HTE cells to nonfibrous and biologically inactive analogues of asbestos or to a variety of inert particles (5).

A number of molecular and cellular events triggered by asbestos fibers appear to be mediated by AOS (6, 7). For example, AOS are involved in asbestos-induced cytotoxicity, inflammation, and the development of asbestosis in rats (6). In addition, AOS are implicated in asbestos-mediated activation of ornithine decarboxylase, a rate-limiting enzyme in the synthesis of polyamines which are necessary for cell proliferation (7). These data suggest that AOS may be related causally to some asbestos-induced cell-signaling events.

Recent work by a number of investigators indicates that the redox status of cells may be important in the regulation of c-fos and c-jun gene expression. For instance, the redox status of c-Jun is important for DNA binding to the AP-1 consensus sequence (8–12). Under oxidative conditions, Jun-Jun homodimers and Fos-Jun heterodimers are unable to bind to DNA. However, upon addition of reducing agents, DNA binding is restored (8, 9, 11, 12). Physical interaction with the Ref-1 protein, which constitutes a major source of redox activity in mammalian cells, is likely to exert this redox regulation in vivo (8, 11, 12). Moreover, escape from redox control by mutating c-fos or c-jun to allow DNA binding under oxidative conditions contributes to cell transformation (9, 10, 12).

In studies here, we determined the intracellular levels of GSH, a small tripeptide with antioxidant properties that is important for maintaining thiol levels (13), in RPM cells after exposure to asbestos or chemical-generating systems of AOS, including xanthine, X/XO, and H2O2. To determine whether thiol levels were important in induction of c-fos and c-jun by asbestos, we pretreated cells with the GSH precursor, NAC, to boost thiol levels, or BSO to deplete GSH. Our results demonstrate that exposure to asbestos depletes GSH levels in RPM cells, an effect abolished by NAC. In addition, NAC pretreatment abolished elevation in c-fos and c-jun mRNA levels after exposure to asbestos. These results suggest that intracellular thiol pools modulate c-fos and c-jun induction by asbestos.

MATERIALS AND METHODS

Chemicals and Asbestos. Reference samples of the National Institute of Environmental Health Science processed crocidolite asbestos [Na2Fe3-](3)(Fe3+).(SiO4)(OH)2] were obtained from the Thermal Insulation Manufacturers Association fiber repository (Littleton, CO). Xanthine and X/XO were purchased from Calbiochem (La Jolla, CA). Fetal bovine serum, HBSS, PBS, and Ham’s F-12 medium were purchased from GIBCO (Grand Isle, NY). [α-32P]dATP (3000 Ci/mmol) was obtained from DuPont New England Nuclear (Boston, MA). Glutathione reductase was purchased from Boehringer Mannheim (Indianapolis, IN). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and Exposure to Test Agents. RPM cells were isolated and propagated as described previously (3, 5). Cells were routinely passaged in complete medium containing 10% fetal bovine serum, and confluent cells were switched to 2% serum-containing medium for 24 h prior to the addition of test agents. Asbestos fibers were suspended in HBSS at 1 mg/ml and triturated 8 times through a 22-gauge needle prior to their addition to medium at nontoxic concentrations (3). H2O2, xanthine (100 μM), and xanthine oxidase (0.1 and

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2 To whom requests for reprints should be addressed, at Department of Pathology, University of Vermont, Soule Medical Alumni Building, Burlington, VT 05405.
3 The abbreviations used are: RPM, rat pleural mesothelial; AOS, active oxygen enzymes; BSO, buthionine sulfoximine; GSH, glutathione; H2O2, hydrogen peroxide; HTF, hamster tracheal epithelium; MnSOD, manganese-containing superoxide dismutase; NAC, N-acetyl-L-cysteine; TPA, 12-O-tetradecanoylphorbol-13-acetate; X/XO, xanthine plus xanthine oxidase.
0.2 units/ml were added directly to medium at concentrations evaluated previously in HTE cells (6). In selected experiments, cells were preexposed to NAC (Sigma), N-acetylsereine (Sigma), or BSO (Sigma) for 20 h before the addition of asbestos. This protocol was necessary both to allow changes in GSH levels to occur prior to exposure to asbestos and to eliminate the possibility that compounds exerted their effects on the asbestos itself. NAC and N-acetylserine were dissolved in HBSS, and the pH was adjusted to 7.4 with NaOH before addition to medium. TPA (Consolidated Midland, Brewster, NY) was added to medium at 100 ng/ml from a stock solution of 1 mg/ml in acetone.

Northern Blot Analysis. At selected time periods following exposure to test agents, RNA was extracted for Northern blot analysis as described previously (3, 5). Fifteen μg of total RNA were electrophoresed in 3-(N-morpholino)-propanesulfonic acid formaldehyde gels overnight, transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH), and hybridized with a labeled cDNA probes (Promega, Madison, WI). Hybridization signals on blots were visualized by exposure to Kodak-X-Omat AR film (Eastman Kodak Co., Rochester, NY) at -80°C with intensifying screens. In addition, selected blots were quantitated on a Betascope Blot Analyzer (Betageen Corp., Waltham, MA). c-fos and c-jun cDNAs were generously provided by R. Gaynor (University of California, Los Angeles, CA). A rat MnSOD cDNA was obtained from Y. S. Ho (Wayne State University, Detroit, MI) (14), and rat HO cDNA was a gift from S. Shibahara (Tohoku University, Sendai, Japan) (15). Glyceraldehyde 3-phosphate dehydrogenase, obtained from P. Jeanteur (Laboratoire de Biochimie, Centre Paul Larraque, France), was used as a housekeeping gene to confirm the homogeneity of RNA loading in individual lanes.

Measurement of GSH. For determination of total GSH levels, cells were washed in PBS, scraped with a rubber policeman, and lysed in 10 mM HCl. GSH was extracted in 5-sulfosalicylic acid. At time periods from 1 to 8 h, total GSH was measured with the use of the 5,5'-dithiobis(2-nitrobenzoic acid) oxidized glutathione reductase recycling assay by quantitating the rate of 5-thio-2-nitrobenzic acid formation spectrophotometrically at 412 nm (16). This assay can also detect proteins including acid soluble protein thiols. Cellular thiol concentrations reflecting GSH levels were obtained from a standard curve and expressed as nmol/mg protein. Protein was measured with the use of the Bradford technique (17).

Statistical Analysis. Data were analyzed by ANOVA with the use of Duncan's procedure for multiple comparisons. All experiments consisted of duplicate determinations per group and were repeated 2-3 times.

RESULTS

Alterations in cellular thiol levels appear to be involved in activation of c-jun by UV or ionizing radiation (18, 19). To determine whether GSH levels modulated c-fos and c-jun gene expression in RPM cells by asbestos, confluent monolayers were pretreated with NAC or BSO. As shown in Fig. 1, addition of NAC (10 mM) or BSO (1 mM) alone did not alter c-fos or c-jun mRNA levels in comparison to untreated controls. However, pretreatment with NAC led to dose-dependent decreases (P < 0.001 by linear trend analysis) in induction of c-fos and c-jun mRNAs by asbestos. mRNA levels of glyceraldehyde 3-phosphate dehydrogenase did not change in these experiments, an indication that NAC does not influence the expression of all genes in a nonspecific fashion (Fig. 1). Pretreatment with N-acetylsereine (1 and 10 mM), a compound structurally similar to NAC but lacking thiol groups, did not alter proto-oncogene induction by asbestos (data not shown).

In parallel studies, cells were pretreated with BSO, an inhibitor of γ-glutamylcysteine synthetase, at a range of concentrations (Table 1). BSO caused dose-dependent increases in both asbestos-induced c-fos and c-jun mRNA levels. However, these values were not elevated significantly from values obtained with use of asbestos alone due to small sample sizes and variability.

To determine whether NAC specifically abrogated c-fos and c-jun expression by asbestos, we tested its effects on c-fos and c-jun induction by TPA, a soluble tumor-promoting compound causing rapid induction of both proto-oncogenes in RPM cells (3). Northern blots of RNA from RPM cells treated with TPA for 1 h, the time point of maximal induction of c-fos and c-jun by TPA (3), showed that pretreatment with NAC did not inhibit induction (Fig. 2).

We next examined whether concentrations of asbestos that induce proto-oncogene expression altered cellular GSH levels. Fig. 3 shows total GSH levels at 8 h (the time point showing the highest levels of proto-oncogene induction by asbestos) in RPM cells exposed to several concentrations of asbestos. NAC (10 mM) and BSO (1 mM) were used in these experiments as positive and negative controls to increase and decrease cellular GSH levels, respectively. As can be seen in Fig. 3, crocidolite caused dose-dependent decreases in total GSH pools. Moreover, pretreatment of RPM cells with NAC allevi-
lated asbestos-induced decreases in GSH in a fashion directly related to the amount of NAC added (P < 0.001 by linear trend analysis).

To determine whether extracellular oxidants mimicked the effects of asbestos on c-fos and c-jun induction in RPM cells, we next performed a series of experiments using H$_2$O$_2$ or X/XO as chemical-generating systems of AOS. Unlike asbestos, these oxidant stresses increased total GSH at 8 h (Fig. 4). However, changes in GSH were unremarkable at a number of earlier time points (data not shown). We next compared the steady-state mRNA levels of c-fos and c-jun in RPM cells after exposure to asbestos, H$_2$O$_2$, or X/XO. Fig. 5 shows a time course study from 2 to 24 h after addition of agents. In contrast to asbestos, which produced protracted increases in the steady-state mRNA levels of both c-fos and c-jun, neither H$_2$O$_2$ nor X/XO altered the mRNA levels of these proto-oncogenes at any time.

To ascertain whether RPM cells were resistant to oxidants, we measured expression of MnSOD and HO, two enzymes involved in the oxidant stress response. Steady-state mRNA levels of these genes are increased in human mesothelial cells after exposure to X/XO or asbestos in vitro (20) and in whole lung homogenates of rats after inhalation of crocidolite asbestos (21). Fig. 6 shows mRNA levels of MnSOD and HO in RPM cells after exposure to asbestos, X/XO, or H$_2$O$_2$. Although asbestos caused increases in mRNA levels of HO, less striking increases in gene expression of MnSOD were observed. In contrast, dramatic increases in MnSOD and HO gene expression were observed after the addition of X/XO, although the time frame of changes differed between the two agents. Consistent with our studies published previously in HTE cells (22), elevations in MnSOD mRNA by X/XO were more protracted, whereas increased HO gene expression was noted at 2 and 4 h (Fig. 6).

![Fig. 2. mRNA levels of c-fos and c-jun levels in RPM cells after exposure to NAC alone and with addition of TPA. RPM cells were exposed to 10 mM NAC for 20 h. Subsequently, cells were treated with 100 ng/ml of TPA for 1 additional h. Some cultures were preincubated with NAC for 20 h, manipulated identically to TPA-treated dishes, and incubated for 1 additional hour in absence of TPA. RNA was extracted for Northern blot analysis and blots were hybridized with c-fos (A) or c-jun (B). Cont., control.](image)

![Fig. 4. Levels of GSH in RPM cells after exposure to crocidolite asbestos, X/XO, or H$_2$O$_2$. RPM cells were treated with test agents for 8 h at the indicated concentrations. Total GSH is expressed in nmol/mg protein. Columns, mean; bars, SEM; n = 2/group. *, P < 0.05 in comparison to untreated controls (0).

![Fig. 3. Levels of GSH in RPM cells after exposure to crocidolite asbestos, NAC, or BSO. RPM cells were exposed to 10 mM NAC for 20 h prior to addition of crocidolite. Cells were subsequently exposed to crocidolite asbestos for 8 h before determination of GSH levels. Untreated cultures or cells exposed to NAC or BSO were evaluated for GSH content after 28 h. Total GSH is expressed in nmol/mg protein. Columns, mean; bars, SEM; n = 2/group. *, P < 0.05 in comparison to untreated controls (0).](image)

**Table 1** c-fos and c-jun mRNA levels in rat pleural mesothelial cells after exposure to crocidolite asbestos (5 μg/cm$^2$ dish) and BSO

<table>
<thead>
<tr>
<th>Agent</th>
<th>c-fos</th>
<th>c-jun</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.7 (0.2)$^a$</td>
<td>1.6 (0.4)</td>
</tr>
<tr>
<td>BSO (1 mM)</td>
<td>1.9 (0.1)</td>
<td>3.6 (1.0)</td>
</tr>
<tr>
<td>Asbestos</td>
<td>4.2 (0.3)$^a$</td>
<td>6.3 (0.9)$^a$</td>
</tr>
<tr>
<td>Asbestos + BSO (0.1 mM)</td>
<td>3.7 (0.5)</td>
<td>4.7 (0.3)</td>
</tr>
<tr>
<td>Asbestos + BSO (0.5 mM)</td>
<td>5.1 (0.6)$^a$</td>
<td>7.0 (0.7)$^a$</td>
</tr>
<tr>
<td>Asbestos + BSO (1 mM)</td>
<td>6.6 (2.2)$^a$</td>
<td>9.2 (2.6)$^a$</td>
</tr>
</tbody>
</table>

$^a$ Results (n = 2/group/duplicate experiments) are expressed in arbitrary units after quantitation of Northern blots as described in the text. SEM are expressed in parentheses.

**DISCUSSION**

Recently we demonstrated that crocidolite asbestos causes prolonged elevation of c-fos and c-jun gene expression in RPM cells at concentrations associated with the stimulation of cell proliferation (3). Proteins encoded by c-fos and c-jun dimerize to form the transcription factor, AP-1, which can bind to the promoter region of intermediate genes required for cell division and other cell functions (2). Here we show that cellular thiol levels modulate induction of c-fos and c-jun by asbestos, events which may be coupled to cell proliferation. These results suggest that thiol levels are important in protecting cells against the proliferative effects of asbestos.
In our studies, low and nontoxic concentrations of asbestos fibers were used as determined previously (5). Thus, our observations on the preventive role of glutathione in c-fos and c-jun induction by asbestos may be consistent with work by Kinnula et al. (23), who showed that the glutathione redox cycle is a major source of protection in RPM cells against low levels of oxidant stress by H$_2$O$_2$. Since catalase is important in protecting these cell types from severe oxidant stress at high concentrations of H$_2$O$_2$ (23), several interrelated pathways of antioxidant metabolism appear to be important in cell defense from AOS (24).

Although little is known about glutathione pools in the cells of the lung, the release of GSH has been documented from rat alveolar macrophages after exposure in vitro to toxic particulates, such as quartz and asbestos (25). Elevations of GSH in cell culture medium correspond with concentration-dependent decreases in cellular GSH levels and are not seen after exposure to latex beads, an inert particle. These observations support our findings with asbestos in RPM cells and suggest that depletion of GSH may occur in target cells of asbestos-induced disease after exposure to a number of pathogenic minerals.

Other investigators have also suggested a role of thiols in the activation of c-fos and c-jun by a variety of different environmental stresses. For example, transcriptional activation of c-jun following UV or $\gamma$-irradiation is blocked by NAC (18, 19). Certain chemical oxidant stresses may activate signaling pathways leading to c-fos or c-jun induction through a common mechanism involving redox control of the AP-1 complex. A recent report suggests that chemicals inducing the AP-1 complex in HpeG2 hepatoma cells act through a common mechanism involving the formation of AOS and the depletion of GSH (26). In these experiments, AP-1-binding activity was induced by increasing intracellular AOS and by lowering GSH levels with BSO or diamide.

In RPM cells, BSO alone failed to induce c-fos or c-jun mRNA at 8 h (Fig. 1; Table 1) or at earlier time periods (data not shown). These experiments suggest that GSH depletion per se is not causally related to proto-oncogene induction at the transcriptional level. However, preexposure to BSO caused dose-dependent increases in c-fos and c-jun mRNA levels by asbestos (Table 1). These results suggest that thiol pools may act as an intracellular buffer against asbestos-mediated induction of early response genes. The propensity of asbestos fibers to generate AOS after phagocytosis by cells (27) and their ability to act as Fenton catalysts after mobilization of iron from fibers (28) may account for the lowering of total cellular GSH.

In previous work by our laboratory and others (6, 7, 29–31), scavengers of AOS, including catalase and the superoxide dismutases, have been used to block the inflammatory and cytotoxic effects of asbestos on a variety of cell types. These results suggest that some of the biological effects of asbestos are mediated by AOS. One goal of work here was to determine whether extracellular AOS caused an increased expression of c-fos and c-jun, as well as a lowering of GSH pools, in a manner similar to crocidolite asbestos. The results of these studies are summarized in Table 2. Overall, the effects of chemically generated oxidants and asbestos are surprisingly disparate. For example, exposure of RPM cells to X/XO or H$_2$O$_2$ fails to alter c-fos and c-jun mRNA or GSH levels, an observation indicating that extracellular AOS per se do not trigger proto-oncogene induction by asbestos. However, increased expression of genes encoding the antioxidant enzymes, MnSOD and HO, by these agents suggests that RPM cells are responsive to chemical-generating systems of AOS.

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RPM cells appear to be a complex process that is triggered by multiple pathways. This induction of gene expression is probably initiated at the cell surface or after phagocytosis and internalization of fibers. Although H2O2 and extracellular AOS generated by X/XO do not directly stimulate c-fos or c-jun at the transcriptional level in RPM cells, our studies indicate that redox control may be important in the cascade of cell-signaling events initiated by asbestos. Recently, we have demonstrated that asbestos activates mitogen-activated protein kinases, one signaling pathway linked to the induction of early response genes (32). The addition of NAC prior to asbestos blocks phosphorylation of mitogen-activated protein kinases, an indication that cellular thiol status affects molecular events occurring upstream of Jun-Jun and Fos-Jun binding to DNA.

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