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In recent years, it has become apparent that minerals can trigger alterations in gene expression by initiating signaling events upstream of gene transactivation. These cascades may be initiated at the cell surface after interaction of minerals with the plasma membrane either through receptorlike mechanisms or integrins. Alternatively, signaling pathways may be stimulated by active oxygen species generated both during phagocytosis of minerals and by redox reactions on the mineral surface. At least two signaling cascades linked to activation of transcription factors, i.e., DNA-binding proteins involved in modulating gene expression and DNA replication, are stimulated after exposure of lung cells to asbestos fibers *in vitro*. These include nuclear factor κB (NFκB) and the mitogen-activated protein kinase (MAPK) cascade important in regulation of the transcription factor, activator protein-1 (AP-1). Both NFκB and AP-1 bind to specific DNA sequences within the regulatory or promoter regions of genes that are critical to cell proliferation and inflammation. Unraveling the cell signaling cascades initiated by mineral dusts and pharmacologic inhibition of these events may be important for the control and treatment of mineral-associated occupational diseases. — *Environ Health Perspect* 105(Suppl 5):1121–1125 (1997)

Key words: asbestos, gene expression, protein kinases, NFκB, transcription factors

Introduction

Asbestos fibers are a group of fibrogenic and carcinogenic minerals that have been extensively studied by toxicologists and biologists in an effort to understand the mechanisms of fiber-induced diseases [reviewed in (*1–3*)]. In recent years, the development of sophisticated techniques in molecular and cellular biology has allowed us to study gene expression in cells of the respiratory tract that are affected in asbestos-associated diseases *in vivo* and the pathways controlling regulation of gene expression in these cells *in vitro*.

Various types of asbestos have been assessed for their genotoxicity, using karyotypic and morphologic approaches in a number of rodent and human cell lines [reviewed in (*1,4*)]. These studies reveal differences in responses to asbestos in various cell types, and disparate results in that asbestos appeared to cause chromosomal and mutational changes in some, but not other cell lines examined. Alternate theories were advanced which suggest that asbestos fibers, particularly high iron-containing amphiboles such as crocidolite and amosite, damage DNA indirectly through release of active oxygen species (AOS) and formation of oxidative and mutagenic DNA lesions (*5–8*), or directly after penetration of the nuclear membrane by fibers during mitosis (*9*).

The discovery that asbestos and erionite fibers, but not a variety of other nonpathogenic particulates, cause increased and protracted mRNA levels of the early response protooncogenes, c-*fos* and c-*jun* and increased activator protein-1 (AP-1) binding to DNA in both tracheal epithelial and pleural mesothelial cells (*10–12*) and the knowledge that transactivation of these genes is regulated by upstream cell signaling events in other cell types (*13,14*) prompted us to examine the mechanisms of cell signaling in asbestos-exposed mesothelial, tracheal epithelial, and alveolar type II epithelial cells. Thus far, we have focused on two major pathways, the nuclear factor κB (NFκB) and mitogen-activated protein kinase (MAPK) cascades.

NFκB is a transcription factor important in regulation of a number of genes intrinsic to inflammation, proliferation, and lung defense (*15*). Genes that contain the NFκB-binding cis-regulatory elements in their promoter or intronic regions include various interleukins, nitric oxide synthase, certain adhesion molecules, and the protooncogene c-*myc*. Protein subunits that bind to nuclear NFκB elements are members of the Rel family of genes, which may be differentially and specifically activated in various cell types. Members of the Rel proteins may occur in complexes found in cytosols with inhibitory proteins (IKB-α, IKB-β, and bcl-3). After proteolytic degradation of these inhibitory subunits, homodimeric or heterodimeric complexes then may bind to specific consensus DNA sequences for NFκB DNA. These protein complexes can be detected using electrophoretic mobility shift assays (EMSA).

AP-1 is a family of accessory transcription factors that interact with other regulatory DNA sequences called TPA (12-*O*-tetradecanylphorbol-13-acetate)-response elements (referring to the active phorbol ester that classically induces the expression of AP-1 in a variety of cell types) (*14*) or AP-1 sites. The family of transcription factors that interact with AP-1 sites includes both homo- (Jun/Jun) and heterodimeric (Fos/Jun) complexes encoded by various members of the c-*fos* and c-*jun* families of protooncogenes. Although the functional

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Abbreviations used: AOS, active oxygen species; AP-1, activator protein-1; BrdU, 5'-bromodeoxyuridine; EGFR, epidermal growth factor receptor; EMSA, electrophoretic mobility shift assays; ERK1, ERK2, extracellular signaling kinases; HTE, hamster tracheal epithelial; JNK1, JNK2, stress-related protein kinases; MAPK, mitogen-activated protein kinase(s); MAPK, MEKK kinase kinase; NAC, *N*-acetylcysteine; NFκB, nuclear factor κB; RLE, rat alveolar type II epithelial cells, RPM, rat pleural mesothelial; TNF-α, tumor necrosis factor alpha; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; UV, ultraviolet.

ramifications of c-*fos* and/or c-*jun* transactivation may be cell-type specific, Fos and Jun proteins are considered early-response gene products that may regulate the expression of other genes required for progression through the cell cycle (*14*), programmed cell death, i.e., apoptosis (*16*), or transformation of cells (*17*).

Activation of the MAPK cascade involving phosphorylation and dephosphorylation of a number of proteins leads to transactivation of c-*jun* and a number of interrelated transcription factors (*18–20*). The MAPK cascade includes the extracellular signaling-related kinases (ERK1, ERK2), the stress-activated protein kinases (JNK1, JNK2) and p38 (Figure 1). Selected arms of this cascade can either be induced by TPA, causing phosphorylation of the Raf protein through a Ras-dependent mechanism or a Ras-independent mechanism in the case of tumor necrosis factor alpha (TNF-α), or other cellular stresses including endotoxin and heat shock (*21*). A series of MAPK kinase kinase (MEKK) and MAPK kinases (MEK) may then become phosphorylated differentially dependent upon the stimulus. Most intriguing, the balance of activation between ERKs, JNKs and p38 may govern

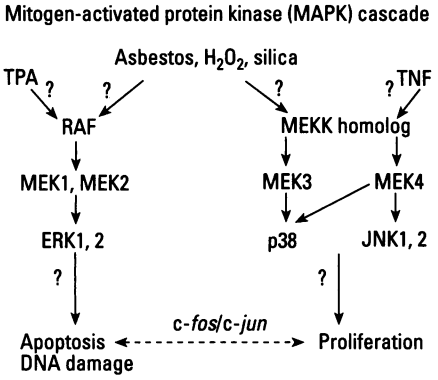

Figure 1. The mitogen-activated protein kinase (MAPK) cascade consists of distinct phosphorylation cascades, which in the case of ERK activation, may be triggered by interaction of growth factors with their receptors, activation of membrane-associated receptor tyrosine kinases, and the sequential activation of Ras and Raf proteins. Raf then phosphorylates MEK1 and MEK2, which in turn phosphorylate ERKs. In contrast, Ras activation may contribute to, but is not essential for, JNK or p38 activation, but upstream kinases including both an MEKK and MEK3 and MEK4 have been identified in this pathway. Concentrations of asbestos and H_2O_2 that cause apoptosis are potent activators of the MAPK cascade in RPM and RLE cells, and H_2O_2 causes early and transient increases in JNK activation. TPA and TNFα are also agents inducing ERK and JNK activation, respectively, in a variety of cell types.

differentiation and apoptosis in malignant cells (PC12 neuroblastoma line) (*22*).

In our study we demonstrate that asbestos fibers cause induction of multiple signaling pathways. We first present data showing that crocidolite asbestos causes NFκB activation in hamster tracheal epithelial (HTE) cells (*23*). We then describe work showing activation of the ERK MAPK cascade by crocidolite asbestos after phosphorylation of the epidermal growth factor receptor (EGFR) (*24*). Last, we summarize how cell signaling events, particularly the balance between ERK and JNK activities in the MAPK pathway, may be important in induction of cell proliferation (*25,26*) or apoptosis (*27*), with phenotypic end points occurring in pulmonary epithelial and pleural mesothelial cells after exposure to asbestos.

Methods

Cell Cultures and Exposure to Asbestos

A diploid line of HTE cells previously isolated in our laboratory was maintained in Ham's F-12 medium (GIBCO, Grand Island, NY) containing 10% newborn bovine serum, 50 units of penicillin, and 50 μg of streptomycin per ml (*23*). All cells were used at confluency at passages between 38 and 48, and growth medium was replaced with medium containing 2% serum to arrest cell growth at 24 hr before addition of test agents.

The National Institute of Environmental Health Sciences' preparation of processed crocidolite asbestos was obtained from the Thermal Insulation Manufacturers Association Fiber Repository (Mountain View, CO) and weighed out in 5-mg aliquots before use. After heat sterilization in a dry oven overnight, samples were diluted in Hank's balanced salt solution and added directly to medium at 1.25 or 5.0 μg/cm^2 dish. The latter concentration of crocidolite induces both c-*jun* expression and AP-1 DNA binding activity in HTE and rat pleural mesothelial (RPM) cells (*10–13*) and increased ERK MAPK phosphorylation and activity in the latter cell type (*24*). A variety of nonfibrous analogs of asbestos have been used routinely as negative controls in these assays and do not induce these events.

Electrophoretic Mobility Shift Assays

At periods from 2 to 24 hr after addition of asbestos, HTE cells were isolated for preparations of nuclear extracts as described by Staal et al. (*28*). Electrophoretic mobility shift assays (EMSA) were performed using 2 to 4 μg of nuclear protein (*23*). The DNA binding buffer consisted of 40 mM HEPES buffer, 4% Ficoll 400, 200 ng poly(dI)•(dC) per μl, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.175 pmol of a ^{32}P-end-labeled double-stranded oligonucleotide containing a DNA consensus NFκB site (*23*). Protein extracts were incubated in DNA-binding buffer for 20 min at room temperature before electrophoresis on a 5% polyacrylamide gel that was then dried and visualized by exposure to Kodak X-Omat film. Radioactivity in retarded binding complexes was quantitated using phosphorimaging (*23*).

To determine the identity and specificity of EMSA complexes, a 40-fold molar excess of unlabeled NFκB binding oligonucleotide or an unlabeled oligonucleotide containing a consensus AP-1 binding sequence (fat soluble element) was induced in the binding reactions. We also used antibodies specific to the p50 or p65 members of the NFκB family (SC-109 and SC-114, respectively, at 1 mg/ml from Santa Cruz Biotechnology, Santa Cruz, CA) to identify the proteins present in the retarded complexes. In these experiments, we added 2 μl of each antibody for an additional 30 min after incubating nuclear proteins in DNA-binding buffer.

Statistics

Data were analyzed by analysis of variance using Duncan's procedure to correct for multiple comparisons.

Results and Discussion

Figure 2 shows a time-course study demonstrating increases in protein complexes that bind to the NFκB consensus sequence in HTE cells exposed to crocidolite asbestos, compared to sham control cells. Note that several gel-shift complexes are observed that increase in intensity in response to asbestos. In the experiments shown in Figure 3 the specificity of gel-shift complexes in HTE cells exposed to asbestos was determined. Lane 1 in Figure 3A represents nuclear proteins from HTE cells exposed to 5 μg/cm^2 asbestos after incubation in DNA-binding buffer. Lanes 2 and 3 show incubation of nuclear proteins in DNA-binding buffer containing a 40-fold molar excess of unlabeled NFκB-binding oligonucleotide or an oligonucleotide containing an AP-1 consensus binding site, respectively. The appearance of all complexes are abolished with use of excess cold NFκB. The two predominant

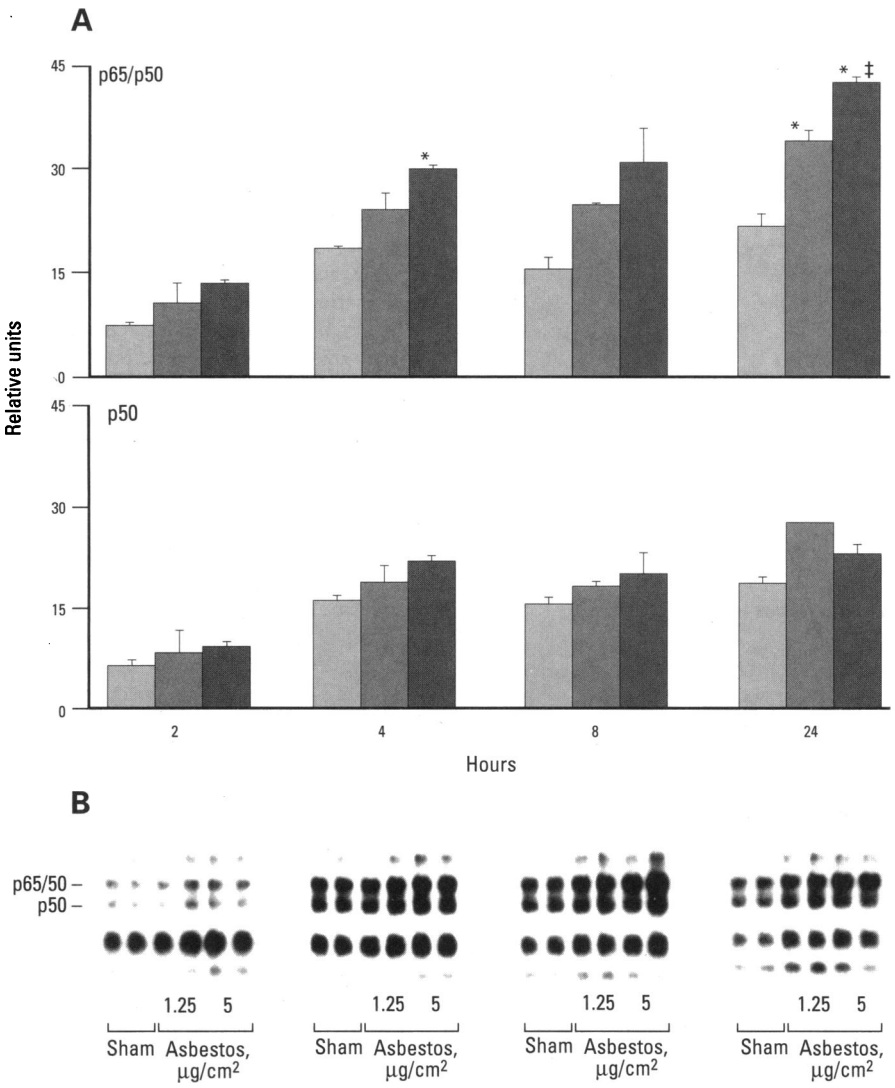

Figure 2. Crocidolite asbestos causes increases in p65/50 and p50 protein complexes binding to the NFκB-binding consensus DNA sequence in HTE cells. Reproduced with permission from Janssen et al. (*23*). $n=2$ duplicate lanes per group. All experiments were performed in duplicate. *Significantly different from sham group ($p<0.05$). ‡Significantly different from the 1.25 µg/cm^2 asbestos group at the same time point ($p<0.05$).

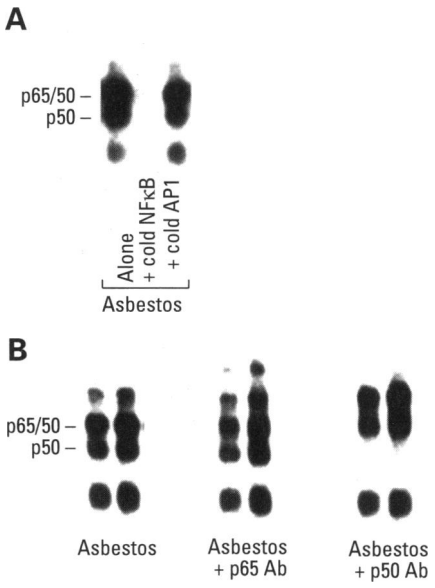

Figure 3. Specificity of binding to the NFκB consensus sequence in asbestos-exposed (5 µg/cm^2 dish) HTE cells. (*A*) Competitive inhibition of complex formation in presence of cold NFκB, but not AP-1 binding oligonucleotide. (*B*) Supershift assays using antibodies (Ab) recognizing p65 and p50 to identify proteins in complexes. Reproduced with permission from Janssen et al. (*23*). $n=2$ duplicate lanes per group. All experiments were performed in duplicate.

complexes in HTE cells, identified in supershift assays shown in Figure 3B, comprise p65/50 and p50 protein subunits. Note that in the duplicate lanes presented in the right panel of Figure 3B, both upper complexes are supershifted upward with use of the p50 antibody. Although similar trends in elevations of both gel-shift complexes were noted over time in HTE cells exposed to asbestos (Figure 2), statistical increases ($p< 0.05$) were only observed in p65/p50. These changes were ameliorated with preaddition of *N*-acetylcysteine (NAC), an agent boosting cellular glutathione levels, to HTE cells (*12*), which suggests that oxidative stress caused by asbestos may be important in increased binding of p65/p50 and p50 complexes to the NFκB consensus sequence. In all studies, NAC alone was added to cells for 18 hr and diminished both crocidolite-induced c-*fos* and c-*jun* mRNA levels and AP-1 to DNA-binding activity.

Figure 4 presents a hypothetical construct of how asbestos fibers or AOS liberated from fibers or phagocytosis may initiate the NFκB signaling pathway as well as the MAPK cascade. As emphasized in the "Introduction," stimulation of both of these cell signaling pathways was induced in mesothelial cells and other cell types by oxidant stresses including hydrogen peroxide (*29–31*), arsenite (*31,32*), and chromium (*33*). Based on our observations that elevations of cellular glutathione levels by NAC can prevent both crocidolite asbestos-induced NFκB (*23*) and ERK MAPK activation (*29*), one might suggest that oxidants contribute to initiation of both cell signaling pathways induced by asbestos.

How AOS elaborated by asbestos may initiate these signaling pathways is suggested in recent studies using RPM cells. In these cells, both elevations of ERK1 and ERK2 phosphorylation and increases in ERK2 *in vitro* kinase activity induced by crocidolite asbestos can be blocked by pretreatment of cells with suramin, an agent causing internalization of growth factor receptors, or by using the tyrphostin AG1478, a compound inhibiting phosphorylation of the EGFR (*24*). These data suggest that interaction of fibers directly with the EGFR or phosphorylation of the EGFR by AOS, as has been observed in cells after addition of hydrogen peroxide or ultraviolet (UV) irradiation (*34*), is critical to initiation of the ERK MAPK cascade by asbestos fibers. The possibility that the EGFR also is integral to activation of the NFκB cascade by asbestos is currently being explored using similar approaches in

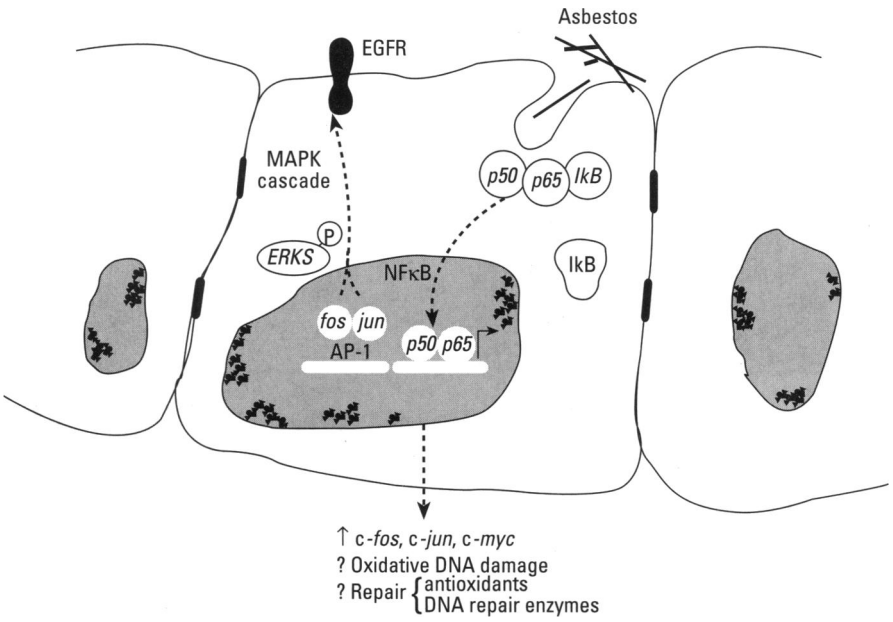

Figure 4. Illustration of how asbestos fibers interact with cells to elicit ERK MAPK and NFκB cascades and transcriptional activation of early response genes. One mechanism of activation in mesothelial cells may be through phosphorylation of the EGFR.

both RPM and rat alveolar type II epithelial (RLE) cells (*35*).

Silica, especially when freshly ground, also elaborates AOS (*36*), and activates the NFκB pathway in a manner similar to that in asbestos (Driscoll et al., unpublished data). Although this mineral has not been examined for its ability to stimulate the various arms of the MAPK cascade as diagrammed in Figure 1, it is likely that MAPK activation may occur in certain cell types of the respiratory tract (epithelial, fibroblasts) that are affected in silicosis (*37*). In view of observations in malignant cells indicating that ERK activation occurs selectively after mitogenic stimuli (*38*) whereas JNK/p38 activation governs apoptosis occurring in response to stress (*21*), we have developed dual labeling *in situ* cell-imaging techniques to quantitate both proliferation and apoptosis in RLE cells (*39*) after exposure to asbestos TPA or TNF (*40*). Preliminary data suggest that these morphologic end points may reflect patterns of ERK and JNK activation in epithelial cells of the respiratory tract. In brief, crocidolite asbestos at concentrations (5 μg/cm^2 dish) causing apoptosis but not cell proliferation as determined by labeling with an antibody to 5′-bromodeoxyuridine, selectively stimulates the ERK pathway in this cell type as does TPA, an agent inducing apoptosis that is classically used as a positive control for ERK activation in other cell types. In contrast, TNFα, an agent inducing JNK activation, also causes dramatic cell proliferation in RLE cells whereas asbestos (5 μg/cm^2) fails to cause increases in JNK activity over a range of time points examined. These data are exciting in that they suggest that ERK activation is linked to apoptosis rather than to cell proliferation in this cell type. Moreover, this observation is supported by the fact that pretreatment of RPM cells with an MEK1,2 inhibitor compound, which selectively blocks ERK activation, inhibits the development of apoptosis by asbestos (*29*). Thus, manipulating different arms of the MAPK cascade using pharmacologic inhibitors may be feasible in controlling phenotypic outcomes of asbestos exposure in cells of the respiratory tract.

In summary, multiple cell-signaling pathways may be stimulated by mineral dusts. Probable mediators in these pathways are oxidants that phosphorylate either receptors on the cell surface or other signaling proteins occurring upstream of transcription factor interaction with DNA. AOS may also be important in the degradation of proteins in these cascades or in the initiation of lipid peroxidation cell signaling pathways yet to be characterized after exposure of cells to minerals (*41*). These experiments are fruitful areas for further investigation into how asbestos and other mineral dusts alter gene expression.

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