Asbestos Causes Stimulation of the Extracellular Signal-regulated Kinase 1 Mitogen-activated Protein Kinase Cascade after Phosphorylation of the Epidermal Growth Factor Receptor

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Abstract

Asbestos fibers are human carcinogens with undefined mechanisms of action. In studies here, we examined signal transduction events induced by asbestos in target cells of mesothelioma and potential cell surface origins for these cascades. Asbestos fibers, but not their nonfibrous analogues, induced protracted phosphorylation of the mitogen-activated protein (MAP) kinases and extracellular signal-regulated kinases (ERK) 1 and 2, and increased kinase activity of ERK2. ERK1 and ERK2 phosphorylation and activity were initiated by addition of exogenous epidermal growth factor (EGF) and transforming growth factor-α, but not by isoforms of platelet-derived growth factor or insulin-like growth factor-1 in mesothelial cells. MAP kinase activation by asbestos was attenuated by suramin, which inhibits growth factor receptor interactions, or tyrophostin AG 1478, a specific inhibitor of EGF receptor tyrosine kinase activity (IC50 = 3 nm). Moreover, asbestos caused autophosphorylation of the EGF receptor, an event triggering the ERK cascade. These studies are the first to establish a MAP kinase signal transduction pathway is initiated after phosphorylation of a peptide growth factor receptor following exposure to asbestos fibers.

Introduction

Exposure to asbestos has been linked to the development of both malignant (lung cancer, mesothelioma) and nonmalignant (asbestosis) diseases, all of which involve a dysregulation of cell proliferation (1). Asbestos fibers cause persistent increases in mRNA levels of the proto-oncogenes c-jun and c-fos in target cells of disease in vitro and in vivo, as well as increases in DNA binding activity of the activator protein-1 transcription factor (2). Because expression of both c-fos and c-jun is required for transition through the G1 phase and entry into the S phase of the cell cycle, and functional overexpression of c-jun can lead to increased cell replication and morphological transformation of tracheal epithelial cells (3), transcriptional activation of immediate early response proto-oncogenes by asbestos may be linked to chronic cell proliferation and carcinogenesis.

Materials and Methods

Cell Culture and Treatments. RPM cells were isolated by gentle scraping of the parietal pleura of Fischer 344 rats and propagated as described previously (2). Cells were maintained in DMEM/F-12 containing 10% fetal bovine serum, hydrocortisone (100 ng/ml), insulin (2.5 µg/ml), transferrin (2.5 µg/ml), and selenium (2.5 ng/ml). Twenty-four h before exposure to minerals, growth factors, or TPA, cells were switched to medium containing reduced serum (0.5% fetal bovine serum). Particles were suspended in HBSS at 1 mg/ml, triturated 8 times with a 22-gauge needle, and then added to medium for final concentrations between 1.25 and 25 µg/cm2 of culture dish. Reference samples of National Institute of Environmental Health Sciences-processed crocidolite and chrysotile (Jeffery Mines, Montreal, Quebec, Canada) asbestos fibers were obtained from the Thermal Insulation Manufacturers Assurers Fiber Repository (Littleton, CO). Riebeckite and antigorite, the nonfibrous (≤3:1 length to diameter ratio) chemically similar analogues of crocidolite and chrysotile asbestos, do not induce alterations in cell proliferation and were used to determine the specificity of asbestos-induced ERK activation by fibers (8). TPA (Consolidated Midland, Brewster, NY) was added to culture medium at 100 ng/ml from a 1 µg/ml stock solution in acetone (0.1% final concentration). EGF, PDGF-AA, PDGF-BB, TGF-α, and IGF-1 were purchased from United Biomedical Inc. (Lake Placid, NY).

MAP Kinase Western Blots. Assays were performed as described previously using polyclonal rabbit antibodies 1913.2 or 1913.3 raised to the carboxy terminus of the Xenopus laevis Mr 42,000 MAP kinase (peptide sequence KElIFEETARFQPGY; Refs. 9 and 10). Antibodies 1913.2 and 1913.3 recognize both the Mr 42,000 (ERK2) and the Mr 44,000 (ERK1) mammalian MAP kinases, as well as their phosphorylated forms, which have reduced mobility on SDS-polyacrylamide gels.

ERK2 in Vitro Kinase Assays. RPM cells were washed twice in ice-cold PBS and then lysed in cold NP40 immunoprecipitation buffer [1% NP-40, 10 mM HEPES (pH 7.4), 2 mM EDTA, 50 mM NaCl, 50 mM NaF, 0.1% 2-mercaptoethanol, 1% aprotinin, 0.2 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride]. Lysates were centrifuged at 14,000 rpm for 10 min, and the supernatant was removed to a clean tube. ERK2 was immunoblotted using a monoclonal antibody (Clone 8D11, Cell Signaling). MAP kinase activity was determined using a 32P-glycerokinase phosphorylation assay (Promega). The assay is based on the formation of 32P-glycerokinase substrate under conditions that allow autophosphorylation of ERK2, and was performed as described previously (8).

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3 The abbreviations used are: MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; RPM, rat pleural mesothelial; TPA, 12-0-tetradecanoylphorbol 13-acetate; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; IGF, insulin-like growth factor; PKC, protein kinase C; EGF-R, EGF receptor.
noprecipitated using an anti-ERK2 (C-14) antibody (Santa Cruz) at a 1:100 dilution. The washed immunoprecipitates were incubated for 20 min at 30°C in kinase buffer [20 mM HEPES (pH 7.5), 2 mM 2-mercaptoethanol, 5 mM MgCl₂] containing 5 μCi [γ-32P]ATP and 5 μg of GST-Myc (1–300) bacterial fusion protein or 10 μg myelin basic protein per reaction as a substrate for ERK activity (11). Incorporation of 32P to the GST-Myc or myelin basic protein substrate was visualized by autoradiography and quantitated on a Bio-Rad phosphoimage analyzer.

EGF-R in Vitro Kinase Assays. Procedures were modified from Sorokin et al. (12). RPM cells were washed twice in ice-cold PBS and then lysed in cold Triton-X extraction buffer [1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 10 mM Tris (pH 7.6), 1% aprotinin, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 0.5 μM microcystin]. Lysates were centrifuged at 14,000 rpm at 4°C for 10 min, and the supernatant was removed to a clean tube. EGF-R was immunoprecipitated using an anti-EGF-R antibody from the IgG 151 AE4 hybridoma, a subclone of IgG 151 BH6. The washed immunoprecipitates were incubated for 10 min at 30°C in 25 μl of kinase buffer plus 5 μCi [γ-32P]ATP. Incorporation of 32P to the EGF-R was visualized by autoradiography and quantitated on a phosphoimage analyzer. Western blots were performed using anti-EGF-R antibody F1 (a gift from the laboratory of J. J. M. Bergeron, McGill University, Montreal, Quebec, Canada) to confirm the presence of immunoprecipitated EGF-R in each sample.

Results

Asbestos Fibers Cause a Persistent Phosphorylation of ERK1 and ERK2 and Increased Activity of ERK2. Fig. 1A shows the time course of ERK phosphorylation induced by crocidolite asbestos (5 μg/cm²), the most pathogenic type of asbestos in the causation of mesothelioma (1), or by the phorbol ester tumor promoter TPA (100ng/ml).

![Fig. 1](image-url)

Fig. 1. Asbestos induces phosphorylation of ERK1 and ERK2 proteins as shown by Western blot analysis (A) and increases in ERK2 activity (B–D). A, time course of ERK response comparing controls (0) with crocidolite asbestos (Croc.) at 5 μg/cm² and with TPA at 100 ng/ml. S, lane showing protein standards. (B–D) ERK2 activity is increased with TPA or EGF, but not PDGF, stimulation at 45 min. C, ERK2 kinase activity is increased in a dose-dependent manner by both crocidolite and chrysotile asbestos (8 h incubation). D, ERK2 kinase activity is not increased above control values in response to nonfibrous particulates (riebeckite and antigorite at 1.25–10 μg/cm²).
STIMULATION OF ERK MAP KINASE CASCADE BY ASBESTOS

EGF and TGF-α Stimulate Prolonged Phosphorylation of ERK1 and ERK2 but PDGF Isosforms and IGF-I Do Not. Differential expression of growth factor receptors, including EGF-R, and the production of many growth factors have been reported in both normal and transformed mesothelial cells and in mesothelial cells after exposure to asbestos fibers in vitro (14–17). However, no experimental evidence to date has demonstrated a causal involvement of growth factors or their receptors in cellular responses to asbestos. As EGF stimulates transcription of c-jun and c-fos in other cells (18) through a mechanism of action involving EGF-R dimerization and autophosphorylation (19), we hypothesized that the EGF-R was a potential target site for asbestos-induced activation of the MAP kinase pathway.

Because RPM cells possess features of both epithelial and mesenchymal cells and have EGF, PDGF, and IGF receptors (14–17), we screened EGF and other candidate growth factors for their ability to activate the ERK proteins by initiating a signaling cascade at the RPM cell surface. RPM cells were exposed to receptor grade EGF at a range of concentrations and time points up to 8 h. TPA (100 ng/ml), as well as all concentrations of EGF examined, caused band shifting of ERK1 and ERK2 at all time points. Fig. 2A shows the time course from 0 min to 2 h. At 15 min, untreated cells that were handled in an identical manner demonstrate phosphorylation of the ERK proteins in all experiments. This response is observed consistently and is similar to the early induction of c-fos and c-jun observed in RPM cells at early time points (2). The response appears to be a nonspecific response to mechanical manipulations as has been observed by others (3).

We next examined RPM cells after exposure to TGF-α, a peptide that is regulated differently than EGF but which interacts with the same cell surface receptor. RPM cells treated with TGF-α at a range of time points and concentrations exhibited ERK1 and ERK2 band shifting at all doses and times examined (Fig. 2B). These results indicate that stimulation of the EGF-R with EGF or TGF-α phosphorylates and activates ERKs over an extended time course in a manner similar to asbestos.

PDGF is a dimeric molecule consisting of A and/or B polypeptide chains. All PDGF isosforms (PDGF AA, AB, and BB) and IGF-I were examined here over a range of concentrations for time periods up to 4 h. At times ranging from 30 min to 4 h, neither control, PDGF-treated, nor IGF-1-treated cells showed ERK phosphorylation (data not shown). The lack of increase in ERK2 kinase activity experiments using PDGF-BB (Fig. 1B) also indicates that PDGF does not induce an ERK response in RPM cells.

Blocking Growth Factor Receptors Inhibits the ERK Response to Asbestos. To assess the causal role of cell surface receptors in ERK activation by asbestos or EGF, we examined effects of suramin, a polysulfonated naphthylea hexaion that has been shown to inhibit growth factor receptor interactions at doses ranging from 10−6 to 10−3 M in numerous other cell types (20). The coaddition of suramin prevented the induction of ERK phosphorylation by crocidolite or EGF (Fig. 3A). Untreated cells (Fig. 3A, Lane 1) and cells treated with suramin (Fig. 3A, Lanes 2 and 3) did not show activation of ERKs. In contrast, cells exposed for 8 or 24 h to EGF (Fig. 3A, Lane 4) or crocidolite (Fig. 3A, Lanes 6 and 10) exhibit ERK activation. These phosphorylation events were not observed if suramin was added with the stimulating agents to the culture media (Fig. 3A, Lanes 5, 7–9, and 11–13). Suramin inhibits PKC activity in cell lysate systems (20). Thus, to rule out possible artifacts of PKC inhibition by suramin in the RPM cell system, the ERK response to TPA in the absence and presence of suramin was examined. At 1 h, activation of ERKs by TPA was not abolished even at highest concentrations of suramin (5 mM; Fig. 3B).

To examine whether the EGF-R is directly involved in ERK activation by asbestos, we used tyrphostin AG 1478, a specific inhibitor of the EGF-R tyrosine kinase (21). Fig. 4A shows that AG 1478 alone at 0.1, 1, or 10 μM has no effect on the ERKs and does not inhibit the

ng/ml). TPA is a PKC agonist known to activate MAP kinases (13) and is used here as a positive control (2). In contrast to untreated cells at each time point, exposure of RPM cells to 100 ng/ml TPA for 1, 2, or 4 h results in activation of ERK1 and ERK2, as evidenced by decreased mobility of the molecules indicative of phosphorylation (9, 10). These responses to TPA are diminished at 8 h and absent after 24 h. The early and transient ERK response to TPA contrasts with the more protracted response to crocidolite asbestos, in which phosphorylation of the ERKs is first apparent at 4 h after exposure to asbestos and increases at 8 and 24 h (Fig. 1A, Lanes 8, 11, and 14). The pattern and time frame of ERK responses to TPA or crocidolite correspond to the kinetics of induction of c-fos and c-jun transcription by these agents (2).

To determine whether the ERKs are also catalytically activated by asbestos fibers, immune complex kinase assays were performed on ERK2 immunoprecipitated from asbestos-treated RPM cells. ERK2 kinase assays confirm that MAP kinase is enzymatically activated by crocidolite and chrysotile asbestos (Fig. 1B–D). Activity was also increased with TPA or EGF (but not PDGF-BB; Fig. 1B). Furthermore, at 24 h, ERK2 activity was increased by crocidolite asbestos but not by its nonfibrous analogue riebeckite.

To examine the dose dependence of the ERK response in cells exposed to asbestos fibers, immune complex kinase assays were performed with RPM cells that had been treated for 1 h with TPA or EGF, or for 8 with crocidolite or chrysotile asbestos (Fig. 1C). A dose-related increase in ERK2 kinase activity was observed in cells treated with increasing concentrations of crocidolite or chrysotile asbestos.

RPM cells also were exposed to crocidolite or chrysotile asbestos (each at 10 μg/cm2) and their nonfibrous analogues riebeckite and antigorite over a range of concentrations (1.25–10 μg/cm2; Fig. 1D). EGF (5 ng/ml) and chrysotile and crocidolite asbestos elevated the kinase activity of ERK2 as quantitated by phosphoimage analysis. However, the nonfibrous particles riebeckite and antigorite failed to increase the ERK2 activity above control levels in untreated cells. These data support the importance of fibrous geometry in reactivity.

Fig. 2. EGF (A) and TGF-α (B) cause protracted band shifts of the ERK1 and ERK2 proteins.

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Authentication of the EGF-R intracellular domain (22). The finding that the increase the tyrosine kinase activity of the EGF-R and cause aggregation of the receptor was diminished below untreated control levels at 2 h (Fig. 5, Lane 10). At both 4°C and 37°C, asbestos at concentrations (5 @g/cm²) were completely abolished in the presence of AG 1478 (Fig. 4B, Lanes 7 and 8).

Asbestos Activates EGF-R Kinase Activity. On the basis of findings with suramin and AG 1478, we next examined the ability of crocidolite asbestos or EGF to modulate the in vitro kinase activity of the EGF-R as a possible site of initiation of signals leading to activation of ERKs (Fig. 5). In these experiments, the EGF-R was immunoprecipitated after addition of agents, and its kinase activity was evaluated by performing in vitro autophosphorylation assays with the receptor as substrate. Experiments were performed at 4°C (to slow the reaction rate at which the EGF-R autophosphorylates), as well as at 37°C. Asbestos was added for 2 h, because this is the minimal time to allow for significant numbers of fibers to settle onto and make contact with the cells. At 4°C, stimulation of RPM cells with EGF for 1 or 5 min produced a large increase in EGF-R phosphorylation (Fig. 5, Lanes 4 and 5). At 37°C, this increase was evident within 5 or 15 s (Fig. 5, Lanes 8 and 9), and phosphorylation of the receptor was diminished below untreated control levels at 2 h (Fig. 5, Lane 10). At both 4°C and 37°C, asbestos at concentrations (5 μg/cm²) inducing ERK phosphorylation and activity increased the phosphorylation of the EGF-R.

Discussion

Our studies indicate that the EGF-R may be a site at which asbestos fibers initiate the MAP kinase signaling cascade. Results here are consistent with reports showing that in addition to specific ligand, various agents, including divalent cations and cationic polypeptides, increase the tyrosine kinase activity of the EGF-R and cause aggregation of the EGF-R intracellular domain (22). The finding that the EGF-R is phosphorylated in RPM cells in response to asbestos is the first reported observation linking interaction of asbestos fibers with a growth factor receptor on the surface of a target cell of disease. Moreover, we demonstrate a functional response (i.e., activation of ERKs triggered after fiber-induced phosphorylation of EGF-R) that may be critical to the advent of abnormal cell proliferation and differentiation, features of asbestos-induced diseases (1). Our studies with asbestos fibers support other findings that ERKs can be phosphorylated and activated in response to a number of external stimuli that promote growth and differentiation. These factors include ionizing radiation and H₂O₂, which activate MAP kinases by the production of active oxygen species (23), as well as agents that induce oligomerization of receptor tyrosine kinases (19). Asbestos fibers, which generate active oxygen species from cells or by redox reactions on the fiber surface (1), also may phosphorylate the EGF-R through an oxidant-dependent mechanism, as has been suggested in recent studies using H₂O₂ (24). We are presently exploring these possibilities, as well as more protracted time courses of EGF-R phosphorylation by asbestos, using an EGF-R kinase activity assay.

The protracted activation of ERKs induced by asbestos may also be explained by the direct interactions of asbestos fibers with cells in culture. After initial addition to medium, fibers continue to settle onto cells over a number of hours and may trigger sequential and additional signaling cascades. Because fibers are insoluble and not metabolized, they remain in contact with cells and thus serve as persistent sources of signals. In addition to signal transduction events that are mediated through the EGF-R and phosphorylation of ERKs, it is possible that asbestos may activate other ERK-independent pathways to provide additional signaling stimuli to the nucleus (25).

Fig. 3. A, suramin blocks ERK activation by EGF and crocidolite asbestos. B, suramin does not block ERK phosphorylation by TPA.

Fig. 4. The EGF-R inhibitor AG 1478 inhibits activation of ERKs by EGF and crocidolite asbestos in a dose-related manner but does not effect ERK activation by TPA. A, Western blot analysis. B, ERK2 immune complex kinase assay.
Because of their large surface area and rigid structure, it is unlikely that asbestos fibers interact with the EGF-R with the specificity attributed to ligand. However, in receptor binding studies, we have found that crocidolite asbestos interferes with the binding of EGF to its receptor in membrane preparations. This interference appears to be due to interactions of the fibers with the membranes and/or with the EGF-R itself. In binding studies between fibers and EGF in the absence of membranes, there was no significant binding of EGF to the asbestos. Therefore, the interference with binding is not due to sorption of the EGF to the fibers. The ability of crocidolite to block the binding of growth factor to receptor makes it unlikely that the temporal delay between fiber exposure and ERK1/2 response is due to crocidolite inducing autocrine feedback on cells, because these growth factors would presumably be blocked as well.

One hypothesis of carcinogenesis, based on microscopic studies in vitro, is that asbestos is genotoxic due to direct interaction of fibers with chromosomes due to their penetration of the discontinuous nuclear membrane during mitosis (4, 5). A number of reports indicating that asbestos does not cause cell transformation or genotoxicity in a variety of standard bioassays and the inability to demonstrate asbestos fibers within mesothelial cells in rodent inhalation experiments or in tissues from patients with mesothelioma have brought this theory into question (reviewed in Ref. 1). Recently, chronic inflammation and mitogenesis have been proposed as more plausible mechanisms to explain the carcinogenicity of inhaled fibers (26). Our studies here provide a mechanistic framework for a signal transduction pathway that triggers chronic cell proliferation by asbestos after interaction of fibers with the cell surface. Moreover, because inhibition of ERK responses to asbestos could be achieved with use of AG1478 (a tyrophostin inhibiting EGF-R activation), additional strategies based on perturbation of asbestos-induced signaling for development of therapeutic agents in the treatment of asbestos-associated tumors may be plausible (27).

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