Asbestos Induces Activator Protein-1 Transactivation in Transgenic Mice

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ABSTRACT

Activation of activator protein (AP-1) by crocidolite asbestos was examined in vitro in a JB6 P+ cell line stably transduced with AP-1-luciferase reporter plasmid and in vivo using AP-1-luciferase reporter transgenic mice. In in vitro studies, crocidolite asbestos caused a dose- and time-dependent induction of AP-1 activation in cultured JB6 cells. The elevated AP-1 activity persisted for at least 48 h. Crocidolite asbestos also induced AP-1 transactivation in the pulmonary and bronchial tissues of transgenic mice. AP-1 activation was observed at 2 days after intratracheal instillation of the mice with asbestos. At 3 days postexposure, AP-1 activation was elevated 10-fold in the lung tissue and 22-fold in bronchial tissue as compared with their controls. The induction of AP-1 activity by asbestos appeared to be mediated through the activation of mitogen-activated protein kinase family members, including extracellular signal-regulated protein kinase, Erk1 and Erk2. Aspirin inhibited asbestos-induced AP-1 activity in JB6 cells. Pretreatment of the mice with aspirin also inhibited asbestos-induced AP-1 activation in bronchial tissue. The data suggest that further investigation of the role of AP-1 activation in asbestos-induced cell proliferation and carcinogenesis is warranted. In addition, investigation of the potential therapeutic benefits of aspirin in the prevention/amelioration of asbestos-induced cancer is justified.

INTRODUCTION

Exposure to asbestos is associated with the development of pulmonary fibrosis, lung cancer, and other malignant cancers (1). In addition, exposure to the members of the amphibole subgroup (crocidolite and amosite) is associated with increased incidence of malignant mesothelioma and bronchogenic carcinoma (1). The mechanisms of asbestos-induced fibrosis and carcinogenesis are unclear. It is generally believed that asbestos-mediated free radical reactions may play an important role (2–6). Asbestos can stimulate cells to generate ROS via reactions mediated by iron present on the surface of asbestos. This transition metal catalyzes the reduction of hydrogen peroxide (H2O2) to generate hydroxyl radical (•OH). •OH radical can damage various cellular components, causing DNA strand breaks, protein modification, and lipid peroxidation (6). Asbestos can trigger a number of molecular and cellular events via reactions mediated by ROS (6–9).

Recent studies have demonstrated that ROS are responsible for asbestos-stimulated pro-inflammatory cytokine expression, enhanced toxicity, development of asbestosis, and carcinogenesis (3, 6, 10, 11). ROS are implicated in asbestos-induced activation of nuclear transcription factor kB (12, 13) and of ornithine decarboxylase, a rate-limiting enzyme in the synthesis of polyamines that are required for cell proliferation (10). Exposure to asbestos can lead to increased accumulation of diacylglycerol and stimulation of protein kinase C activity, suggesting that asbestos may trigger signaling events leading to cell proliferation (14).

Previous studies have shown that asbestos fibers cause dose-dependent and persistent increases in the expression of c-fos and c-jun in rat pleural mesothelial cells (7, 8). These proto-oncogenes are the early response genes involved in the transition from G1 phase to S phase in the cell cycle (15, 16). Members of the fos and jun gene family can dimerize to form AP-1. AP-1 is a transcription factor composed of homodimers and/or heterodimers of Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, Fra-2, and FosB2) gene families (16–19). This transcription factor regulates the transcription of various genes with the consensus DNA sequence TGA/C(T)CA, designated as a TRE in their promoter region (20). Many stimuli, including the tumor promoter TPA and ROS, regulate AP-1 binding to the DNA of the promoter region of a number of intermediate genes that govern inflammation, proliferation, and apoptosis (15, 16, 21). AP-1 and its regulated gene expression have been reported to play a key role in preneoplastic-to-neoplastic transformation in cell culture and animal models (22, 23). AP-1 is also known to be involved in tumor progression and metastasis (24, 25).

Earlier studies have shown that asbestos is able to cause elevated expression of c-fos and c-jun in rat pleural mesothelial cells via a protein kinase C pathway (7, 14). However, it has been reported that the binding of AP-1 protein to DNA does not always result in induction of transcription (26). AP-1/DNA binding activity measured by gel-shift assay does not necessarily correlate with AP-1 transcription activity (26). Therefore, in the present study we used transgenic mice as an animal model to study the possible activation of AP-1 by asbestos (27, 28).

The JB6 mouse epidermal cell lines, a well-developed cell culture system for studying tumor promotion and antitumor promotion, were chosen as an in vitro model in these studies. This cell system of clonal genetic variants that are P′ or P″ allows the study of genetic susceptibility to transformation, promotion, and progression at the molecular level. These P′, P″, and transformed variants are a series of cell lines representing earlier-to-later stage of preneoplastic-to-neoplastic progression. P″ variants gain P′ phenotype upon transfection with mutated p53 (29). The P″ cells gain transformed phenotype irreversibly upon TPA, epidermal growth factor, or other tumor promoter treatment or with c-jun overexpression (30, 31). Transformed variants grow under anchorage-independent conditions and are tumorigenic in nude or BALB/c mice in the absence of tumor-promoting conditions. One of the few molecular events known to distinguish P″ and P′′ cellular responses to tumor promoters is the activation of AP-1-driven transcription activity in P′′ cells but not in P′ cells (22). Furthermore, the induced AP-1 activity appears to be required in the tumor promoter-induced transformation in P′′ cells (31).

Recent studies have shown that aspirin and its related compound salicylic acid inhibit the activation of AP-1 and neoplastic transformation induced by tumor promoter TPA and UVB in JB6 cells and in AP-1-luciferase transgenic mice (32, 33). We therefore evaluated the potential inhibitory role of aspirin in asbestos-induced transactivation of AP-1 in transgenic mice. This in vivo evaluation of aspirin was
considered important because of the potential inhibitory actions of aspirin in the development and progression of cancer in asbestos-exposed populations.

In the present studies, we attempted to answer the following questions: (a) Does asbestos induce AP-1 activation both in JB6 cells transfected with AP-1-luciferase reporter plasmid and AP-1-luciferase reporter transgenic mice? (b) If it does, which signal transduction pathways are involved in asbestos-induced AP-1 activity? (c) Does aspirin inhibit asbestos-induced AP-1 activation in vitro in cell culture and in vivo in transgenic mice?

**MATERIALS AND METHODS**

**Chemicals.** Eagle’s MEM was from Whittaker Biosciences (Waldersville, MD); FBS, gentamicin, and l-glutamine was from Life Technologies, Inc. (Gaithersburg, MD); aspirin was from Sigma Chemical Co. (St. Louis, MO). The luciferase assay system was from Promega (Madison, WI). PhosphoPlus MAPK Antibody Kit and MEK 1 inhibitor (PD98059) was from New England BioLabs, Inc. (Beverly, MA).

**Cell Culture.** The JB6 P1-AP-1-luciferase stable transfecnt cells (JB6/ APx/B; Refs. 34 and 35) were cultured in Eagle’s MEM containing 5% FCS, 2 mm l-glutamine, and 50 μg of gentamicin/ml. The cells were grown at 37°C in a 5% CO2 atmosphere.

**Assay of AP-1 Activity in Vitro.** A confluent monolayer of JB6/APxB cells was trypsinized, and 5 × 10^4 viable cells (suspended in 1 ml of Eagle’s MEM supplemented with 5% fetal bovine serum) were added to each well of a 24-well plate. Plates were incubated at 37°C in a humidified atmosphere of 5% CO2. Twelve hours later, cells were cultured in Eagle's MEM supplemented with 0.5% FBS for 12–24 h to minimize basal AP-1 activity and then exposed to asbestos in the same medium to monitor the effects on AP-1 induction. The cells were lysed and extracted with 200 μl of 1× lysis buffer supplied in luciferase assay system by the manufacturer. Luciferase activity was measured using a Monolight luminometer, model 3010 (Analytical Luminescence Laboratory, Sparks, MD). The results are expressed as relative AP-1 activity compared with controls.

**Animals and Administration of Asbestos.** Two times TRE-luciferase reporter transgenic mice were originally established by Rincon and Flavell. C57BL/6J male mouse carrying the 2 TRE-luciferase transgene was crossed with DBA2 (SASCO, Omaha, NE 68101) females by Huang (St. Louis, MO). The luciferase assay system was from Promega (Madison, WI). The luciferase activity started at 6 h after incubation, and the elevated AP-1 activity persisted for at least 72 h. 

**Transactivation of AP-1 by Crocidolite in AP-1-Luciferase Reporter Transgenic Mice.** Because the binding of AP-1 protein to DNA does not always result in an induction of transcription, the AP-1 DNA binding activity measured by gel-shift assay may not correlate with AP-1 transcription activity in some cases (26). We used AP-1-luciferase reporter transgenic mice to determine whether crocidolite leads to the elevation of AP-1 transcription activity in vivo. The transgenic mice were exposed intratracheally to crocidolite asbestos (100 μg/mouse) (1 mg/ml in 0.9% NaCl). At intervals of 1, 2, and 3 days after exposure, the lung and bronchiolar tissues were dissected, and their luciferase activities were measured according to the method described (30).

**RESULTS**

**Time Course and Dose-Response of Crocidolite-induced AP-1 Activity.** To examine whether asbestos could induce AP-1 activation in a stable AP-1-luciferase reporter plasmid-transfected JB6 P1 cell line, we exposed 5 × 10^4 cells to 7.5 μg of crocidolite asbestos dissolved in 1 ml of MEM. This asbestos concentration was found optimal for AP-1 activation in vitro by a series of experiments with varying concentrations (0 to 30 μg/ml) of asbestos at different designated time periods (6–72 h). Crocidolite asbestos caused a significant dose-dependent AP-1 activation (Fig. 1). The activation reached a maximum at a crocidolite concentration of 7.5 μg/ml (≈4 μg/cm2). An additional increase above 7.5 μg/ml resulted in a slight decrease of AP-1 activation. Therefore, this concentration was selected as the optimal concentration required for studies on time course and selective inhibition of AP-1. At intervals from 6 to 72 h, luciferase activity was tested. Induction of AP-1 activity started at 6 h of incubation, and thereafter, AP-1 activity increased steadily to the maximum of 2-fold at 36 h (Fig. 2). The elevated AP-1 activity persisted for at least 72 h.

**Immunoblot for Phosphorylated Erks, JNK, and p38 Kinase.** Phospho-specific antibodies against Erks, JNK, and p38 kinase proteins provided in each assay kit were used to normalize the phosphorylation assay by using the same-transferred membrane blot.

**Statistical Analysis.** Data presented are the means ± SE of values compared and analyzed using a one-way ANOVA. Statistical significance was determined by two-tailed Students t test for paired data and considered significant if P ≤ 0.05.

![Fig. 1. Dose-response of crocidolite-induced AP-1 activity in JB6 P1 cells.](https://example.com/fig1.png)
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Fig. 2. Time course of crocidolite asbestos-induced AP-1 activity in JB6 P+ cells. JB6/AP+ cells (5 \times 10^4 in 1 ml of MEM plus 5% FBS) were seeded into each well of a 24-well plate. After overnight culture at 37°C, cells were cultured in MEM plus 0.5% FBS for 12 h. The cells were then exposed to 7.5 \mu g/ml/well (4 \mu g/cm²) crocidolite asbestos prepared in the same medium. Other experimental conditions were the same as those described in the legend to Fig. 1. Results, presented as relative AP-1 induction compared with the untreated control cells, are means of eight assay wells from two independent experiments; bars, SE. * significant increase from control (P ≤ 0.05).

at 1 day after exposure (data not shown), but increased significantly at 2 or 3 days after exposure (Fig. 3). At 3 days postexposure, the induction of AP-1 activation by crocidolite was 10 times higher in lung tissue and 22 times higher in bronchiolar tissue than that of the control groups.

Inhibition of Asbestos-induced AP-1 Activity by Aspirin in JB6 Cells. It was reported that aspirin inhibits both UVB and tumor promoter TPA-induced activation of AP-1, and it also blocks TPA-induced neoplastic transformation in JB6 cells and transgenic mice (32, 33). To determine whether aspirin has any inhibitory effects on crocidolite asbestos-induced AP-1 activation, JB6 cells were pretreated for 30 min with aspirin before exposure to asbestos. As shown in Fig. 4, aspirin exhibited a significant dose-dependent inhibitory effect on crocidolite-induced AP-1 activation. At a concentration of 1 mM aspirin, the AP-1 activation was completely blocked to the basal control values, whereas additional aspirin reduced AP-1 activation below the basal level.

Inhibition of Transactivation of AP-1 by Aspirin in Vivo. To determine the inhibitory effect of aspirin on crocidolite asbestos-induced AP-1 activation in vivo, AP-1-luciferase transgenic mice were administered aspirin (120 mg/kg) i.p. 30 min before exposure to crocidolite, followed by subsequent administration i.p. every 12 h until the termination of the experiment. Aspirin significantly inhibited crocidolite asbestos-induced transactivation of AP-1 activity in bronchiolar tissues (Fig. 5). These results demonstrate that application of aspirin not only blocks crocidolite asbestos-induced AP-1 activity in a cell culture model but also inhibits AP-1 transactivation in vivo.

Fig. 3. Crocidolite asbestos induces the transactivation of AP-1 in AP-1-luciferase transgenic mice. The AP-1-luciferase transgenic mice were intratracheally instilled with 100 \mu g of crocidolite asbestos suspended in 0.1 ml of 0.9% sterile saline. At 2 or 3 days after exposure, the mice were sacrificed, and the lung and bronchiolar tissues were removed. The luciferase activity of the tissue was measured as described in "Materials and Methods." The results, presented relative to the level of luciferase activity of control groups, are means of eight mice; bars, SE. * significant increase from control; **, significant increase from 2 days after exposure (P ≤ 0.05).

Fig. 4. Inhibition of crocidolite asbestos-induced AP-1 activity by aspirin in JB6 cells. JB6/AP+ cells (5 \times 10^4) suspended in MEM plus 5% FBS were added to each well of 24-well plates. After overnight culture at 37°C, the cells were cultured in MEM with 0.5% FBS for 12 h. The cells were pretreated with different concentrations of aspirin for 30 min and then exposed to 7.5 \mu g/ml (4 \mu g/cm²) crocidolite asbestos. After a 24-h exposure at 37°C and 5% CO₂, the AP-1 activity was measured using luciferase activity assay. The results, presented relative to the level of luciferase activity of control groups, are means of eight assay wells from two independent experiments; bars, SE. * significant increase from control; **, significant decrease from asbestos alone (P ≤ 0.05).

Fig. 5. Blocking of crocidolite asbestos-induced AP-1 activity by aspirin in vivo. The AP-1-transgenic mice were i.p. injected with 120 mg/kg aspirin 30 min before intratracheal instillation of 100 \mu g of crocidolite asbestos suspended in 0.1 ml of 0.9% sterile saline and followed by sequential applications of the same dose of aspirin every 12 h. Control group received 0.1 ml of 0.9% NaCl alone. Forty-eight h later, the mice were killed, and the luciferase activity of lung and bronchiolar tissues was measured as described in "Materials and Methods." The results, presented relative to the level of luciferase activity of control groups, are means of eight mice; bars, SE. * significant increase above control; **, significant decrease from asbestos alone (P ≤ 0.05).
Activation of Erks but not JNK and p38 Kinase by Crocidolite Asbestos. Because the MAPK family, including Erks and JNKs as well as p38 kinase, are the upstream activator kinases responsible for the c-Jun phosphorylation and AP-1 activation (36–41), we tested which class of MAPK is involved in the AP-1 activation by crocidolite. We examined the influences of crocidolite asbestos on the phosphorylation of Erk1, Erk2, JNK, and MAPK p38. Using antibodies specific for the above MAPK family and phospho-specific for the phosphorylated MAPK family, we studied Erk1, Erk2, JNK, and MAPK p38. Crocidolite asbestos did not affect the phosphorylation levels of JNK and MAPK p38 proteins (data not shown). But crocidolite significantly stimulated the phosphorylation of Erk1 and Erk2 (Fig. 6A) proteins. As shown in Fig. 6A, crocidolite-induced phosphorylation of Erk1 and Erk2 is in a time-dependent manner, peaking in 15 min. These results suggested that induction of AP-1 activity by asbestos might be through an activation of an Erk-dependent, JNK- and p38-independent pathway.

Additional studies using MEK 1 inhibitor support this hypothesis. MEK 1 is an upstream activator of Erks. MEK 1 inhibitor has been shown to act as a highly selective inhibitor of MEK 1 activation (42, 43). As shown in Fig. 7, at a concentration of 20 μM MEK 1 inhibitor, the AP-1 activation induced by asbestos was completely inhibited. These results provide further evidence in support of the induction of AP-1 activity by asbestos, which might be through an Erk-dependent pathway.

Inhibition of AP-1 by Aspirin Is Not Through the Inhibition of Erk1 and Erk2. To test whether aspirin inhibits AP-1 through the inhibition of Erk1 and Erk2, JB6 cells were pretreated with aspirin for 30 min. We found that pretreatment of cells with aspirin did not effect asbestos-induced phosphorylation of Erk1 and Erk2 proteins (Fig. 8). These results indicated that inhibition of AP-1 by aspirin is through an Erk1- or Erk2-independent pathway. This result also suggests that kinases upstream of Erk1 or Erk2 are not likely to be involved in aspirin-mediated inhibition of AP-1.

DISCUSSION

The results obtained in the present study show that asbestos is able to induce AP-1 activation in cell cultures and in AP-1-luciferase reporter transgenic mice. The induction of AP-1 activity by asbestos may be through an Erk1- or Erk2-dependent and JNK- or MAPK p38-independent pathway. Aspirin exhibits an inhibitory effect on asbestos-induced AP-1 activation in both in vitro and in vivo models. This inhibitory effect is not mediated through an Erk1- or Erk2-dependent pathway.

Previous studies using different model systems have suggested the important role of AP-1 activation in preneoplastic-to-neoplastic transformation in cell culture and animal models (16, 31–33). AP-1 is a critical mediator of tumor promotion involved in a diversity of processes. This transcription factor is able to alter gene expression in response to a number of stimuli including the tumor promoter TPA, epidermal growth factor, tumor necrosis factor-α, interleukin 1, and UV irradiation (16). Some of the genes known to be regulated by AP-1 are involved in the immune and inflammatory responses, tumor promotion, and tumor progression. These include cytokines such as interleukin 1, tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor, collagenase IV, and stromelysin (44–47).

Over expression of c-Jun in JB6 P+ cells causes neoplastic transformation. Inhibition of AP-1 activity by either pharmaceutical agents, such as fluorocinolone acetonide or retinoic acid, or molecular biological inhibitors, such as dominant-negative c-Jun and dominant-negative phosphatidylinositol-3 kinase, was found to block tumor promoter-induced neoplastic transformation (23, 31, 32, 34, 35, 48).

Exposure to asbestos causes the development of pulmonary fibrosis, bronchogenic carcinoma, and malignant mesothelioma (1). Mesothelioma and other asbestos-associated diseases, such as lung cancer and asbestosis, are characterized by chronic inflammation and cell proliferation. Using mesothelial cells, earlier studies have reported that asbestos is able to cause increased expression of c-fos and c-Jun (7). These studies provided important information regarding the mechanisms in which asbestos triggers cell-signaling cascades leading to transactivation of proto-oncogene. However, the induction of c-Jun by aspirin was not through the inhibition of Erk1 or Erk2.
expression is not necessarily predictive of AP-1 activation (49). AP-1/DNA binding activity measured by gel-shift assay does not always correlate with AP-1 transcription activity (26), and there is a lack of in vivo studies to support this finding. Recently, a transgenic mouse, which expressed a 2× TRE luciferase in all cell types, was developed (27, 28). The development of this transgenic mouse makes it possible to study the role of AP-1 activation in tumor promotion and investigate the mechanism of action for possible therapeutic agents. Results obtained from the present study show that asbestos is able to cause AP-1 activation in JB6 cells, as well as in pulmonary tissues of transgenic mice. Maximal AP-1 activation was 10-fold in lung tissue and 22-fold in bronchiolar tissue 3 days after intratracheal instillation of crocidolite. These findings are consistent with the previous report that asbestos causes bronchogenic carcinoma and provide further evidence that asbestos may act as a mitogen in carcinogenesis by persistently activating the early response gene pathway. However, which cell type(s) that are involved in responding AP-1 activation in these complex tissues are not clear. Additional studies are required to answer this question.

The observation of asbestos-induced AP-1 in transgenic mice also makes it feasible to study the possible pharmaceutical inhibition of the activation of this transcription factor. In the present study, aspirin was tested. Our results indicated that pretreatment of AP-1 reporter transgenic mice with aspirin markedly inhibited asbestos-induced AP-1 transactivation in bronchiolar tissue. As mentioned in the introduction, aspirin and its related compounds may have considerable potential as therapeutic or preventative agents against inflammation and cancers. Long-term use of aspirin in humans has been reported to protect against colon cancer (40% decrease in risk) and other digestive system cancers (50). In animal studies, aspirin and other nonsteroidal anti-inflammatory drugs were found to inhibit chemically induced tumors of the colon, pancreas, bladder, breast, liver, skin, and various sarcomas (51–56). Because AP-1 is a critical regulator in cell growth and proliferation and is a major mediator of development of human cancer (16), the inhibitory action of aspirin on asbestos-induced AP-1 activation suggests that aspirin may have the potential to serve as a prevention or attenuation drug for asbestos-induced fibrosis or carcinogenesis. Further study in this direction may lead to the development of efficient therapeutic and preventative strategies against asbestos-induced diseases.

The signal transduction pathways leading to transcription factor activation have been studied extensively in the last several years. It is believed that extracellular signals, such as TPA and UV radiation, induce the activation of MAPK pathways (Erks, JNKs, and p38). AP-1 is a downstream target of these three MAPK members (38–41, 57, 58). Activation of MAPKs occurs through the phosphorylation of threonine and tyrosine by upstream MAPK kinases (36, 59). If asbestos-induced AP-1 activation occurs through one or more of the above signal transduction pathways, then the phosphorylation of the MAPK(s) should increase. Our data indicate that Erk1 and Erk2 appear to be involved in AP-1 induction activated by asbestos, whereas JNK and p38 kinase are not. Using specific MEK1 inhibitor to block the Erks activity, the AP-1 activation was also blocked. The time period required for maximal Erks induction (15–30 min) was much shorter than that of AP-1 induction (24–36 h) in the cell culture system. It is possible that: (a) Erks are regulators several steps upstream of AP-1; (b) AP-1 transcriptional activity requires not only the active form of Fos/Jun proteins but also quantitative accumulation of fos/jun gene products; and (c) AP-1 activity measured by luciferase assay also requires quantitative accumulation of luciferase proteins in the cells. Aspirin showed no effect on phosphorylated protein levels of Erk1 or Erk2. This indicated that inhibition of AP-1 activation by aspirin is through an Erk1- or Erk2-independent pathway. These studies also suggested that kinases upstream of Erk1 or Erk2 are probably not involved in the aspirin-mediated inhibition of AP-1 activation.

In conclusion, the results obtained in the present study show that asbestos was able to cause AP-1 activation in JB6 cells and AP-1-luciferase reporter transgenic mice. The induction of AP-1 activation above control was 10-fold in lung tissue and 22-fold in bronchiolar tissue. Aspirin significantly inhibited asbestos-induced AP-1 activation in both in vivo and in vitro systems. It has been proposed that activation of AP-1 may be associated with the carcinogenic effect of asbestos. If this hypothesis is correct, our data suggest that activation of AP-1 may play a pivotal role in the development of human lung cancer caused by asbestos. The inhibitory effect of aspirin on asbestos-induced AP-1 activation suggests that this widely prescribed drug may have the potential to be a therapeutic or preventative drug against asbestos-induced fibrosis and carcinogenesis. Further investigation of this possibility is warranted.

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