p53, Cip1, and Gadd153 Expression following Treatment of A549 Cells with Natural and Man-made Vitreous Fibers

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DNA damage induced by chemicals and ionizing radiation is associated with the expression of negative regulators of the cell cycle. The arrest of cells in G1 and G2 phases of the cell cycle provides time for DNA repair. Asbestos fibers are carcinogenic when inhaled by both humans and animals; however, the mechanism by which the fibers exert their effect is unknown. This work was undertaken to determine whether the expression of DNA damage-inducible genes differs between crocidolite, a fiber positive for lung tumors, and JM 100 glass microfiber, which is negative for lung tumors when inhaled by rats. Temporal and dose-related expressions of p53, Cip1, and Gadd153 proteins were determined in cultured A549 cells treated with either Union Internationale Contre le Cancer crocidolite or JM 100 for 20 hr and cultured in fresh media. Immunolabeled DNA was analyzed by flow cytometry, and the increased number of protein-expressing cells was determined by subtracting the expression in unexposed cells from exposed cells. Crocidolite induced the expression of all three proteins with a maximum expression after approximately 18 hr in fresh media. At a similar time point, JM 100 did not markedly induce the three proteins. Crocidolite also induced a dose-dependent increase in the number of cells in the G2 phase of the cell cycle. These results show that asbestos behaves like ionizing radiation and genotoxic chemicals by inducing proteins associated with DNA damage and cell-cycle arrest. The clear difference in response between crocidolite and JM 100 may help elucidate the mechanism of action of toxic and nontoxic fibers. — Environ Health Perspect 105(Suppl 5):1143-1145 (1997)

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Introduction

Asbestos induces the early response genes c-fos, c-jun, nuclear factor kappa B (NF-κB), and c-myc in a variety of pulmonary cells (1,2). The protein products of c-fos and c-jun form the transcription factor activator protein-1; NF-κB is also a transcription factor. Both of these factors are regulated by the intracellular redox state and are associated with the inducible expression of a wide variety of genes involved in oxidative stress and cellular response mechanisms (3). These early-response genes are part of a more general cascade of genes induced by exposure to toxicants. Ionizing radiation also induces the early-response genes c-fos, c-jun, c-myc, and NF-κB (4-6); the DNA damage-inducible genes p53, Gadd45, and Cip1 are also expressed (7,8). This work was conducted to determine whether crocidolite, a known human carcinogen (9), could also induce these DNA damage-inducible genes. A glass microfiber (JM 100), which has been shown to be free of carcinogenic potential in a number of inhalation studies in rats (10), was also used for comparison.

Material and Methods

A549 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were incubated at 36.5°C in 5% CO₂ in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 μg/ml penicillin/streptomycin. Cells within passages 7 and 8 and in log-phase growth were used in all experiments.

The colony-forming efficiency of A549 cells treated with Union Internationale Contre le Cancer (UICC) crocidolite (MRC Toxicology Unit, Leicester, U.K.) or JM 100 (Manville, Denver, CO) was determined; 5 × 10⁵ cells were plated per 25-cm² culture flask. After 24 hr at 37°C in a humid atmosphere of 5% carbon dioxide and filtered room air, the medium was replaced with RPMI containing five different concentrations of test material (0, 5, 10, 15, 25, and 50 μg/ml). Cultures were incubated as above for 20 hr, after which the cells were washed in buffered saline. The cultured cells were replated at a density of 1.25 × 10⁶ cells per well of a six-well plate. After 8 days, the number of colonies that formed in each well was determined. Relative colony-forming efficiency was calculated as the ratio of the number of colonies formed in treated versus control cultures (11).

The temporal pattern of the expression of p53, Cip1, and Gadd153 was determined in A549 cells in log-phase growth. Cells were plated and cultured for 20 hr before their exposure to 2.0 μg/cm² UICC crocidolite or JM 100 for an additional 20 hr. Media were then changed and cells harvested by trypsinization at various time periods. The temporal pattern of protein expression was determined in a single experiment. The results were used to determine the appropriate period postexposure for dose-response relationship delineation. For the dose–response studies, triplicate cultures of cells were treated in a similar fashion, exposed to graded doses of crocidolite or JM 100 (0.0, 0.1, 0.5, 1.0, 2.0, and 3.0 μg/cm²), and harvested 18 hr after the fiber-exposure period.

The harvested cells were rinsed in Dulbecco’s phosphate-buffered saline (DPBS), fixed, and stored in 70% methanol at −20°C. Cells were immunostained (12) for p53 using Clone DO-7 (Ab-6, Oncogene Sciences, Uniondale, NY), Cip1 using Clone EA10 (Oncogene Sciences), or Gadd153 (R20, Santa Cruz Biotechnology, Santa Cruz, CA). These antibodies were labeled with the appropriate biotinylated secondary antibodies and streptavidin fluorescein conjugates (Vector Laboratories, Burlingame, CA). Cell-cycle status was determined by staining with 25 μg/ml propidium iodide in DPBS containing 100 μg/ml RNase A, and was analyzed by the

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Abbreviations used: DPBS, Dulbecco’s phosphate-buffered saline; NF-κB, nuclear factor kappa B; UICC, Union Internationale Contre le Cancer.
MacCycle program (Phoenix Flow Systems, San Diego, CA). The percentages of positively stained cells were determined by flow cytometry on a FACStar Plus (Becton Dickinson, San Jose, CA) using the channel subtraction method of the CellQuest program. These analyses were conducted on files containing 10,000 events.

Results
Crocidolite and JM 100 samples displayed a dose-dependent toxicity toward A549 cells (Figure 1). The cytotoxicity was more marked in the JM 100 sample than in the crocidolite sample. Temporal changes in the cell cycle were evident in the crocidolite-exposed cells. There was a persistent elevation of the number of cells in the G2 phase and a persistent decrease in the number of cells in G1 phase of the cell cycle (data not shown). The increase in the number of cells in the G2 phase of the cell cycle 18 hr after crocidolite exposure was dose dependent (Figure 2). The expression of p53, Cip1, and Gadd153 proteins increased with time after exposure in cells treated with crocidolite (data not shown). The protein response of cells exposed to JM 100 was much less marked (data not shown). There was a dose-dependent increase in the expression of p53 (Figure 3), Cip1 (Figure 4), and Gadd153 (Figure 5) in crocidolite-exposed cells, but not in those cells treated with JM 100.

Discussion
Crocidolite asbestos can induce genes associated with DNA damage and cell-cycle arrest. In this respect, crocidolite behaves in a similar fashion as other carcinogens such as ultraviolet light (13), ionizing radiation (14), 4-nitroquinoline-1-oxide (15), and methyl methanesulfonate (15). p53 protein can be induced by the presence of DNA strand breaks (16); however, p53 protein can also be induced by other factors such as low oxygen conditions and elevated temperatures (17). In a similar fashion, p21 can be induced by DNA damage via a p53-mediated pathway and by p53 independent pathway unrelated to DNA damage. These p53 independent inducers of p21 include cellular senescence, tumor growth factor beta, and oxidative stress (18,19). The mechanism by which crocidolite induces the expression of p53, Cip1, and Gadd153 proteins is unknown, but may be a result of DNA damage. Crocidolite induced a noticeable cell-cycle arrest in the G2 phase of the cell cycle with no apparent disturbance of the G1 phase of the cell cycle. This pattern of cell-cycle arrest would not have been predicted from the protein expression where p53 and Cip1
proteins are closely associated with arrest of cells in G1. However, recent studies have indicated a role for p53 in G2 arrest (20).

The markedly reduced ability of JM 100 glass microfber to induce p53, Cip1, and Gadd153 proteins is of interest because JM 100 was more toxic to cultured A549 cells than crocidolite on an equal mass/unit area basis (11). The expression of these proteins appears to be independent of cytotoxicity.

Crocidolite is a known human carcinogen (9), but JM 100 inhalation exposures in rats have not produced an elevated risk of lung or pleural tumors (10). This protein response may also be independent of fiber size. The crocidolite and JM 100 samples had approximately equal numbers of fibers per microgram, whereas the JM 100 sample had approximately 33% more fibers that were longer than 15 mp (12). These fibers are believed to be the most pathogenic for inducing pulmonary and pleural neoplasia (21). The durability of crocidolite in the lung is much higher than that of JM 100; however, biopersistence is an unlikely factor to explain the differences in protein response because the duration of the culture is only a small fraction of the half-life of these materials.

Crocidolite has a high iron content compared to JM 100. This may be a factor in the expression of p53, Cip1, and Gadd153 proteins but not a factor in the cytotoxic potential of the fibers, as JM 100 is more cytotoxic than crocidolite. Lund and Aust (22) showed that crocidolite fibers can induce hydroxyl radicals via the Fenton reaction, which has been postulated as the mechanism of induction of DNA damage detected in vitro. Changes in the redox potential of cells alter the expression of p53 protein (23). The possibility that the iron content of the fiber is a major factor in the induction of these proteins is being tested in vitro using amosite, which contains iron, and erionite, which contains little iron. Amosite and erionite induce pulmonary neoplasia in rats exposed to these materials by inhalation (10); studies of the inducible proteins using iron-rich and iron-poor fibers will delineate the potential role of iron in these responses.

However, the role of hydroxyl radicals in elevated protein expression and cytotoxicity may be different, as both crocidolite and JM 100 readily produce hydroxyl radicals in vitro (24).

These results show that asbestos behaves like genotoxic chemicals and ionizing radiation by the induction of proteins associated with DNA damage and cell-cycle arrest. The clear difference in response between crocidolite and JM 100 may help elucidate the mechanisms of action of toxic and nontoxic fibers.

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