DOSE-RESPONSE RELATIONSHIPS IN EXPRESSION OF BIOMARKERS OF CELL PROLIFERATION IN *IN VITRO* ASSAYS AND INHALATION EXPERIMENTS

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□ *Asbestos is a group of naturally occurring mineral fibers which are associated in occupational settings with increased risks of malignant mesothelioma (MM), lung cancers, and pulmonary fibrosis (asbestosis). The six recognized types of asbestos fibers (chrysotile, crocidolite, amosite, tremolite, anthophyllite, and actinolite) are different chemically and physically and may have different dose-response relationships in the development of various asbestos-associated diseases. For example, epidemiologic and lung fiber content studies suggest that the pathogenic potential and durability of crocidolite is much greater than chrysotile asbestos in the causation of human MM. We have used isolated mesothelial cells, the target cells of MM, as well as epithelial cells of the lung, the target cells of lung cancers, in vitro to elucidate the dose-response relationships in expression of early response protooncogenes and other genes critical to cell proliferation and malignant transformation in cells exposed to crocidolite and chrysotile asbestos, as well as a number of nonpathogenic fibers and particles. These studies reveal distinct dose-response patterns with different types of asbestos, suggesting a threshold for effects of chrysotile both in in vitro studies and inhalation experiments. The different patterns of gene expression have been confirmed in lungs of rats exposed by inhalation to these types of asbestos. Experiments also suggest no observed adverse effect levels after evaluation of lung injury, inflammation, and fibrosis at lower concentrations of both types of asbestos.*

Keywords. asbestos, mesothelioma, lung cancer, threshold, proliferation, protooncogenes

INTRODUCTION

The term “asbestos” is used for a group of fibrous, naturally occurring silicate minerals that exhibit properties rendering them useful in commerce. During the past century, asbestos has been mined, processed, and used in thousands of products. Because of its association with lung and pleural diseases in unregulated workplace settings, asbestos is one of the main occupational carcinogens recognized and studied by researchers. Heavy industrial exposure to asbestos causes lung cancer and mesothelioma as well as fibroproliferative lung diseases including pleural and interstitial fibrosis (asbestosis; Mossman and Gee, 1989).

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Asbestos is a group of crystalline 1:1 layer hydrated silicate fibers that are classified into six distinct types based on their different chemical and physical features (Veblen and Wylie, 1993). The six different forms of asbestos include chrysotile, the most common and economically important asbestos in the Northern Hemisphere, and the amphiboles: crocidolite, amosite, anthophyllite, tremolite, and actinolite. Recent studies have demonstrated that toxicity and pathogenicity of different forms of asbestos may be dependent on a combination of physical/mechanical and chemical properties, most importantly their ability to generate oxidative stress (Shukla *et al.*, 2003). The geometry and dimensions of these minerals may govern their deposition and clearance kinetics, biological reactivity, and dissolution in the lung, but chemical and surface properties, including sorption, oxidation/reduction reactions, and charge, also play roles in biopersistence, cellular responses, and pathogenicity (Guthrie and Mossman, 1993). The United States is one of only a few countries in the world, including South Africa, that does not have different standards for regulation and importation of chrysotile versus amphibole asbestos types.

Asbestos Types and Disease Causation in Man

The inhalation of airborne asbestos dust in an occupational setting can produce fibrosis of the lungs and pleura, as well as cancer in the lungs, pleura, peritoneum, and possibly at distant sites (Mossman and Gee, 1989). The extent of lung inflammation and destruction is related to the amount of asbestos retained in the lungs, the fiber type and length, and individual susceptibility (Begin *et al.*, 1986). All forms of asbestos may cause lung fibrosis and there is some evidence to suggest that amphiboles are more potent than chrysotile in causing asbestosis (Mossman and Gee, 1989; Becklake, 1991). It is also likely that an appreciable amount of asbestos must be retained to cause clinically detectable asbestosis. There is, therefore, likely to be a level below which fibrosis will not occur or will be insignificant (Doll and Peto, 1985). Epidemiological studies indicate very clearly that the development of asbestosis requires heavy exposure to asbestos and provides strong evidence that there is a threshold fiber dose below which asbestosis is not seen—this cumulative dose appears to be, at a minimum, in the range of approximately 25 to 100 fiber/ml/years (Dupres *et al.*, 1984; Cookson *et al.*, 1986; Browne, 1994; Weill, 1994; Jakobsson *et al.*, 1995).

Mesothelioma is a rare cancer of the mesothelial cells lining body cavities, including the pleural and peritoneal cavities, and is the classical tumor associated with asbestos exposure (Doll and Peto, 1985; McDonald and McDonald, 1987). The latency for the development of mesothelioma is usually 20 to 40 years or more from time of initial exposure, even though instances of a shorter time lapse have been described. The risk of mesothelioma increases with increasing cumulative exposure. All asbestos fiber types have

been implicated as a cause of mesothelioma, with the risk being greatest for crocidolite (McDonald and McDonald, 1977; Doll and Peto, 1985).

Epidemiological studies on populations exposed to different types of asbestos fibers indicate that both duration and levels of exposure contribute to cancer risk with lower risks of mesothelioma by chrysotile asbestos fibers (Hughes and Weill, 1980). A follow-up study of workers manufacturing chrysotile asbestos cement products by Gardner *et al.* (1986) reported no excess mortality. Comparison of the mortality of workers employed in two asbestos cement manufacturing plants, one having produced more crocidolite than the other (along with chrysotile), added to the evidence that crocidolite caused a greater risk of mesothelioma than chrysotile asbestos (Hughes *et al.*, 1987). A comparison of the exposure-response relationships for lung cancer and mesothelioma in South African amosite and crocidolite mines compared to workers in a chrysotile textile factory showed that the risk of lung cancer and mesothelioma from crocidolite and amosite was higher than in the chrysotile textile factory (Rodelsperger and Woitowitz, 1995). Therefore, epidemiology studies in general have shown that amphibole asbestos fiber types, crocidolite in particular, pose the greatest risk of tumor development.

Chrysotile in mining, processing, and industrial use often contains low concentrations of fibrous tremolite, which may well explain the few cases of mesothelioma associated with this type of asbestos (McDonald and Wagner, 1996; McDonald and McDonald, 1997). Studies on chrysotile miners and millers, in whom the overall frequency of mesothelioma is low, suggest that the risk is mainly determined by the presence of tremolite (Ross and McDonald, 1995). In the 1980s, cohort surveys showed that in mining and in the manufacture of asbestos products, mesothelioma risk was much higher when exposures included other amphiboles, such as crocidolite or amosite, in comparison to chrysotile alone (Hei, 1991).

A multicentered case control study of South African asbestos workers also shows the preponderance of crocidolite-associated cases of mesothelioma, followed by numbers of amosite and a few chrysotile cases, consistent with the view that there is a fiber gradient of mesothelioma potential for South African asbestos (crocidolite > amosite > chrysotile; Rees *et al.*, 1999). One possible explanation for the scarcity or absence of the cancer in South African chrysotile miners and millers may be because of the lack of contaminating fibrous tremolite (Rees *et al.*, 2001).

It is difficult to assess exposure-response relationships in humans because occupational exposures to asbestos typically take place over long periods of time, and concentrations can vary widely. Most exposure-response studies are based on simple cumulative exposure, a time-weighted summation of exposure concentrations, which assumes that the effect of each unit of exposure is proportional to its concentration. This measure does not distinguish between different patterns of exposure, so a person with a long exposure at

low levels can have the same cumulative exposure as a person exposed for a short time at high levels. Despite questions about the validity of these assumptions, cumulative exposure continues to be widely used (Smith, 1992). However, alternative methods for summarizing exposure in Quebec asbestos workers suggest a nonlinear relationship between concentration and lung cancer risk (Vacek, 1998). The exposure measure that provides the best fit to the data is based on a logarithmic function of concentration with a nonzero threshold, raising the possibility that for low exposures to asbestos there may be no detectable increase in lung cancer risk.

The epidemiologic literature cited earlier indicates that, in contrast to amphibole forms of asbestos, chrysotile asbestos is irrelevant or a minor cause of malignant pleural mesothelioma. However, rodent instillation and inhalation studies do not support these views. (Mossman *et al.*, 1990). The fact that chrysotile asbestos is similar in potency to amphibole asbestos after pleural/peritoneal administration or inhalation in rodents may reflect the fact that the latency of tumor development is short (2–3 years) and does not reflect the dissolution of chrysotile fibers over the long latency period (30–40 years) necessary for mesothelioma development in man (Mossman, 1993).

Biopersistence is probably responsible for the much greater tendency of amosite, or crocidolite-induced cancers to progress in man (Guthrie and Mossman, 1993; Browne, 1994). In addition, because of their advantageous aerodynamics, needle-like amphiboles can penetrate much easier into the pulmonary tissue than curly chrysotile fibers (Wrzaszczyk and Owczarek, 1996). With magnesium as its main cation that can be leached from fibers, chrysotile is more unstable and fragments into smaller particles. Therefore, its phagocytosis by macrophages and clearance from the lung tissue are more effective than those processes removing amphiboles (Wrzaszczyk and Owczarek, 1996).

Cell Proliferation and Early Response (*fos/jun*) Protooncogene Expression as Biomarkers of Asbestos Responses in Carcinogenesis

Carcinogenesis is a multistage process that is reflected by initial genetic damage to a cell, called “initiation.” This event is followed by a stage called “promotion” in which genetically damaged cells undergo proliferation, giving them a selective advantage and rendering them more susceptible to additional genetic insults. The accumulation of multiple genetic insults during the long latency period of most cancers is called the “progression” phase of carcinogenesis (Figure 1). Fibrosis caused by asbestos may be an environment favoring the establishment of epithelial/mesothelial cell promotion and progression, as individuals with asbestosis are more likely to develop lung cancers at sites of the development of fibrosis (Mossman and Churg, 1998). Since asbestos is weakly or nonmutagenic except at highly toxic amounts in some *in vitro* models (Hei, 1991), and other factors such as smoking and infection

FIGURE 1 A diagrammatic scheme illustrating the role of sustained protooncogene activation and increased cell proliferation during the long latency period of asbestos-induced diseases. The biopersistence of long amphibole fibers in the lung or at the pleural surface may act as a chronic stimulus for these events. Note that smoking and SV40 Tag, respectively, are important agents initiating DNA damage in lung cancers and mesotheliomas, respectively.

with SV40 Tag (simian virus large T antigen) are known to be transforming and/or carcinogenic influences initiating DNA damage in the development of asbestos-linked lung cancers (Timblin *et al.*, 2001) and mesotheliomas (Bocchetta *et al.*, 2000), respectively, sustained proliferation or tumor promotion by asbestos may be a more accurate signature of response in the establishment of asbestos-induced cancers. For these reasons, we have explored the phenomenon of sustained cell proliferation as a biomarker of asbestos-induced responses in epithelial cells and mesothelial cells *in vitro* and in rodent inhalation models.

In addition to histopathology, there are many quantitative tools to detect increased cell proliferation or unscheduled DNA synthesis, including (1) clonal cell assays or colony-forming assays which document cell proliferation *per se* after plating of individual cells; (2) incorporation of nuclear analogs or labels such as 5' Bromodeoxyuridine (5'BrdU) or ^3H -thymidine; (3) antibodies (Ki67, proliferating cell nuclear antigen) specific to nonresting or dividing cells; and (4) measurement of enzyme activity, that is, ornithine decarboxylase (ODC), which is necessary for cell proliferation. Both crocidolite and chrysotile asbestos cause increased proliferation of tracheal epithelial cells in organ or cell cultures, and longer chrysotile fibers are more potent than shorter chrysotile fibers in inducing both increased ODC activity (Marsh and Mossman, 1988) and incorporation of ^3H -thymidine (Woodworth *et al.*, 1983). At a range of concentrations, nonfibrous particles, including chemically similar analogs of crocidolite and chrysotile, are inactive or much less active than asbestos in all bioassays. Of the few studies using a range of concentrations of asbestos, the lowest concentrations of chrysotile (5 $\mu\text{g}/\text{ml}$ medium) or crocidolite (0.01, 0.05 $\mu\text{g}/\text{cm}^2$) fibers tested caused no significant increases in cell proliferation when compared to untreated control cells (Lemaire *et al.*, 1986; Sesko and Mossman, 1989), indicating a no observed adverse effect level (NOAEL).

A typical dose-response experiment illustrating that crocidolite asbestos fibers are proliferative at lower but toxic at higher concentrations is illustrated in the right-hand panel of Figure 2. In normal rat pleural mesothelial (RPM) cells, increased proliferation (as measured by an increase in colony forming efficiency or CFE in comparison to unexposed cells) is observed at 0.1 $\mu\text{g}/\text{cm}^2$ dish asbestos, but higher concentrations cause dose-related decreases in CFE or growth inhibition. In contrast, SV40-transformed human mesothelial cells (MET5A line) are resistant to the proliferative effects of asbestos (left-hand panel of Figure 2). These different patterns of cell response correlate with the relative resistance of MET5A versus RPM cells to oxidative DNA damage by crocidolite asbestos (Fung *et al.*, 1997).

We have also studied patterns of cell proliferation in lung epithelial and pleural mesothelial cells in relationship to the development of fibrotic lesions in lungs from Fischer 344 rats after inhalation exposures to two

COLONY FORMING ABILITY OF HUMAN MET 5A CELLS AND RAT PLEURAL MESOTHELIAL (RPM) CELLS FOLLOWING EXPOSURE TO ASBESTOS

FIGURE 2 Low concentrations of crocidolite asbestos cause cell proliferation in isolates of normal RPM cells as measured by the CFE assay. In contrast, human MET5A cells, an SV40 Tag-transformed cell line, do not. These trends and responses to the oxidant H_2O_2 suggest that human immortalized cells are more resistant to oxidants as documented previously (Fung *et al.*, 1997).

TABLE 1 Rat Inhalation Studies Using Asbestos

	Crocidolite		Chrysotile	
	High 8.25*[2800]	Low 0.16[60]	High 8.23[2457]	Low 0.18[32]
5'BrdU				
Bronch. Epith.	> (5d)	N.D.	> (5d)	—
Mesothelial	> (20d)	N.D.	> (5d)	—
c-jun	>	—	—	—
odc	>	—	—	—
Hydroxyproline	>	—	—	—

*Time-weighted average concentration (mg/m³ air)

[] = Number of fibers >5 microns/cc air by PCM. From Quinlan *et al.*, 1994, 1995; BeruBe *et al.*, 1996.

concentrations of chrysotile or crocidolite asbestos at identical airborne concentrations of fibers (Quinlan *et al.*, 1994, 1995; BeruBe *et al.*, 1996). Data from lungs evaluated at 5 and 20 days after initial exposures to asbestos and at 20 days postcessation of a 20-day exposure are summarized in Table 1. Increases in cell proliferation of either mesothelial or epithelial cells were observed at only the highest concentrations of asbestos. The pattern of mesothelial cell proliferation in response to crocidolite was protracted in comparison to the early responses of epithelial cells in response to both types of asbestos. Fibrotic lesions and elevations in hydroxyproline, a biochemical marker of collagen synthesis, were only observed after inhalation of crocidolite for 20 days but appeared in both asbestos groups after an additional 20 days in clean air. This pattern is consistent with the observation that fibrosis may progress in the absence of additional exposures to asbestos in man (Mossman and Churg, 1998).

The process of asbestos-induced cell proliferation is causally related to expression of *fos/jun* protooncogenes whose protein products comprise the activator protein-1 (AP-1) transcription factor (Heintz *et al.*, 1993). For example, overexpression of *c-jun* in tracheal epithelial cells results in increases in cell proliferation and transformation (Timblin *et al.*, 1995). Moreover, suppression of the *fos* family member, *fra-1*, using a dominant negative construct reverses the transformed phenotype of rat mesothelioma cells (Ramos-Ninos *et al.*, 2002). Initial *in vitro* studies using epithelial and mesothelial cells showed that both crocidolite and chrysotile asbestos, in comparison to a number of inert particles and nonasbestos fibers, caused increases in mRNA levels of *c-fos* and *c-jun* as well as increased AP-1 to DNA binding (Heintz *et al.*, 1993; Janssen *et al.*, 1994). The patterns of protooncogene expression with different fiber types were dissimilar—crocidolite induced a dramatic response at lower concentrations of asbestos fibers whereas chrysotile has a NOAEL followed by a plateau of response at higher concentrations (Heintz *et al.*, 1993). Erionite, a fiber extremely potent in the causation of mesothelioma in man and rodents, caused more striking elevations in protooncogene expression

FIGURE 3 Dose responses in gene expression (mRNA levels) of various AP-1 family member (*fos/jun*) genes after exposure of a murine lung epithelial cell line (C10) to crocidolite asbestos. As documented previously (Heintz *et al.*, 1993), crocidolite asbestos causes increased mRNA levels of *c-fos* and *c-jun* in RPM cells at lower concentrations than chrysotile asbestos (* $p \leq .05$).

in RPM cells than either type of asbestos (Janssen *et al.*, 1994; Timblin *et al.*, 1998). Follow-up studies with a C10 alveolar type II epithelial cell line are presented in Figure 3, where we used a ribonuclease protection assay to study the dose responses of all members to the *fos/jun* family in response to crocidolite asbestos after 24 hr. These data indicate that the dose-response patterns of individual AP-1 family members are different and nonlinear.

To determine whether *c-jun* and *odc*, a gene encoding the rate-limiting enzyme in polyamine synthesis which is critical in cell proliferation, were increased in lungs of rats after inhalation of either type of asbestos, we performed Northern blot analyses on whole-lung homogenates of rats exposed to asbestos at two concentrations as described earlier (Table 1). These results showed that both markers of proliferation were increased only at high concentrations of crocidolite asbestos. It is unclear whether increased activation of protooncogenes and/or AP-1 occurs directly in cells after stimulation of cell signaling pathways through fiber interactions with growth factor receptors (Zanella *et al.*, 1996, 1999). Alternatively, growth factor gene expression might be elevated through an AP-1-dependent mechanism and stimulate cells through an autocrine-like mechanism.

CONCLUSIONS

Epidemiologic studies have been valuable in suggesting differences in asbestos types in terms of health risks, especially in the causation of malignant mesothelioma. These results, coupled with the results of lung content studies and rodent inhalation experiments, suggest that chrysotile fibers may be less bioreactive because of their increased degradation in lung and dissolution over time. However, the results of short-term inhalation experiments in rodents and *in vitro* mechanistic studies also indicate that on a fiber-to-fiber basis, chrysotile is less apt to cause cell proliferation and increased expression of *fos/jun* protooncogenes in comparison with crocidolite asbestos. These intrinsic differences between fiber types are intriguing and are the subject of future investigation.

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