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Assessment of the Pathogenic Potential of Asbestiform vs. Nonasbestiform Particulates (Cleavage Fragments) in *In Vitro* (Cell or Organ Culture) Models and Bioassays

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Abstract

Asbestos fibers are highly fibrous silicate fibers that are distinguished by having a large aspect (length to diameter) ratio and are crystallized in an asbestiform habit that causes them to separate into very thin fibers or fibrils. These fibers are distinct from nonasbestiform cleavage fragments and may appear as thick, short fibers which break along cleavage planes without the high strength and flexibility of asbestiform fibers. Since cleavage fragments of respirable dimensions have generally proven nonpathogenic in animal studies, little data exists on assessing well-characterized preparations of cleavage fragments in *in vitro* models. The available studies show that cleavage fragments are less bioreactive and cytotoxic than asbestiform fibers.

Keywords

Asbestiform; Cleavage; Fibers; Fibrous

1. Introduction

'Asbestos' is a commercial and regulatory designation for a family of naturally occurring asbestiform fibers. Asbestos fibers are recognized as human carcinogens and also cause pleural and pulmonary fibrosis, i.e., asbestosis in occupationally exposed individuals (Mossman et al., 1990; Mossman and Churg, 1998; Mossman and Gee, 1989). Mineralogical and biological differences exist between various types of asbestos fibers, and much research has focused on the characteristics of fibers that are associated with the causation of lung disease. The different types of asbestos include chrysotile [$Mg_6\ Si_4\ O_{10}\ (OH)_8$], the only asbestos in the serpentine family of minerals, and other types of asbestos classified as amphiboles. These include crocidolite [$(Na_2\ (Fe^{3+})_2(Fe^{2+})_3\ Si_8\ O_{22}\ (OH)_2$], asbestiform grunerite or amosite [$(Fe,Mg)_7\ Si_8\ O_{22}\ (OH)_2$], anthophyllite [$(Mg,Fe)_7\ Si_8\ O_{22}\ (OH)_2$], tremolite [$Ca_2\ Mg_5\ Si_8\ O_{22}\ (OH)_2$], and actinolite [$(Ca_2\ (Mg,Fe)_5\ Si_8\ O_{22}\ (OH)_2$]. These formulae are indeed ideal, and natural amphiboles differ to varying degrees from these as the chemical environment, pressure and temperature at the time of formation control the mineral chemistry. Other factors such as shear stresses and directed pressures determine whether or not an amphibole that crystallizes is asbestiform. Although various types of asbestos are different chemically, structurally and

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biologically, they are common in that they are highly fibrous silicate minerals that are crystallized in an asbestiform habit, causing them to separate into thin fibers or fibrils (Klein, 1993; Veblen and Wylie, 1993). In addition, asbestos fibers are distinguished by having large aspect (length to diameter) ratios, generally from 20:1 or higher for fibers > 5 microns in length. Smaller fibers (<0.5 microns in width) appear by microscopy as very thin fibrils, as defined by the American Society of Testing Materials in 1990. In contrast, nonasbestiform cleavage fragments, although sometimes elongated with aspect ratios of >3:1 which can be defined as fibers, have widths much larger than asbestos fibers of the same length. Though the more common nonasbestiform analogs of asbestos share the same, or essentially the same chemical composition, they do not share the same crystal structure (the crystals form or grow differently).

Cleavage fragments of amphiboles lack the tensile strength of asbestos amphiboles and are traditionally regarded by mineral scientists as distinctly different from asbestos fibers, primarily based on their morphology, and lack of strength or flexibility. For example, in the report of the Committee on Nonoccupational Health Risks of Asbestiform Fibers commissioned by the National Research Council (National Research Council, 1984), cleavage fragments were categorized as distinctive from asbestiform fibers, i.e.: "*Cleavage* refers to the preferential breakage of crystals along certain planes of structural weakness. Such planes of weakness are called cleavage planes. A mineral with two distinct cleavage planes will preferentially fracture along these planes and will produce *acicular* fragments. Minerals with one cleavage plane produce *platy* fragments and those with three or more cleavage planes yield *polyhedral* fragments…. Cleavage cannot produce the high strength and flexibility of asbestiform fibers" (National Research Council, 1984).

These definitions were also recognized by the members of the panel of the Health Effects Research-Asbestos Research in their report on Asbestos in Public and Commercial Buildings (Health Effects Institute-Asbestos Research, 1991). Because epidemiologic and animal studies have not suggested that nonasbestiform amphiboles or cleavage fragments are pathogenic or biologically active, they have not been used in many *in vitro* models, except as negative or nonpathogenic controls for testing of asbestos fibers. Moreover, the results of numerous epidemiologic, animal, and *in vitro* studies, have led scientists to conclude that short asbestos fibers (< 5 microns in length) are inactive or much less active biologically than long, thin asbestos fibers (ATSDR, 2003; Health Effects Institute-Asbestos Research, 1991). Thus, it is unlikely that cleavage fragments of respirable dimensions (i.e., less than 3 microns in diameter) will be pathogenic or targeted extensively for *in vitro* fiber testing in the future. The results of limited work with these minerals from our laboratory and others are summarized below.

2. Advantages and caveats of *in vitro* mineral studies

In vitro studies have been used historically to compare the effects of different types of minerals on cells or organ (explant) cultures (Mossman and Begin, 1989). Regardless of cell type, asbestos fibers, in comparison to a variety of other nonpathogenic, synthetic or naturally occurring fibers (glass, cellulose, etc.) or particles, have been most biologically active in these models. In addition to elucidating the properties of minerals (size, fibrous morphology, surface charge, chemical composition, etc.) that are associated with toxicity (cell injury or death), DNA damage, proliferation and/or alterations in cell function that may be predictive of their pathogenic potential, *in vitro* studies have shed light on the complex features of bioreactive minerals that may be important in reactions with cells and their ability to cause disease. Cell and organ culture models are also much more inexpensive than animal testing. Thus, they have been suggested as screening tools for new synthetic fibers developed for industry.

However, there are also caveats that must be recognized in *in vitro* work with minerals. First, dependent upon the cells used in these models, cell type and species-specific responses may

exist. Thus results from lab to lab working with the same mineral might be inconsistent. Although the most appropriate *in vitro* cell types to use in these models are normal cells of respiratory tract origin, i.e., epithelial or mesothelial, these are notoriously difficult to isolate and maintain in a differentiated state for prolonged periods of time. It also should be acknowledged that concentrations of minerals used in short term *in vitro* assays, where weighed amounts of fibers or particles are precipitated on cells, do not mimic normal clearance patterns and long-term dissolution patterns after inhalation into the human lung, factors that are important in dosimetry and disease causation (Mossman et al., 1990). Lastly, different minerals are generally evaluated in *in vitro* studies on an equal weight basis, which might be misleading based on the facts that different weights of dissimilar fiber types or particles may reflect vastly different total numbers of fibers and surface areas. Regardless of these caveats, however, *in vitro* studies have helped to establish mechanisms of fiber carcinogenesis and differentiated between responses to asbestos fibers and nonasbestiform particles.

3. Studies using tracheal explants

In comparison to cell cultures, tracheal explant cultures can be maintained for weeks in a differentiated state in which the respiratory epithelium is maintained in a normal, mucociliary phenotype. We have used this model to show that crocidolite and chrysotile fibers (asbestos) and long glass fibers cause squamous metaplasia, a reversible but often premalignant lesion, and increased DNA synthesis, a signature of injury and proliferation of fibers that might be important in tumor promotion and progression and/or repair (Woodworth et al. 1983). In contrast, the non-fibrous mineral analogs of these asbestos types, riebeckite (similar in chemistry to crocidolite) and antigorite (similar in chemistry to chrysotile) failed to induce these changes at a range of concentrations and exposure times. Though a number of these riebeckite and antigorite particles were elongated, they were thick, short single crystal cleavage fragments. These studies highlight the importance of fibrous geometry, crystal growth and aspect ratio in bioreactivity.

4. Studies using cell types of lung or pleural origin

The antigorite and riebeckite preparations used in the Woodworth et al. (1983) study (above) were also evaluated in cell cultures of hamster tracheal epithelial cells (HTE) for their ability to induce ornithine decarboxylase (ODC), an enzyme associated with cell proliferation and tumor promotion in mouse skin models of cancer, with asbestos fibers (Marsh and Mossman, 1988). These studies showed that crocidolite and chrysotile (fibers> 10 microns in length) fibers stimulated ODC, but neither of the two nonasbestiform (cleavage fragment) preparations were bioreactive. Subsequent studies revealed that both antigorite and riebeckite were less potent than crocidolite (asbestos) in stimulating survival or proliferation of HTE cells in a colony-forming assay (CFE) in which proliferation was measured directly over a 7 day period in low-serum containing medium (Sesko and Mossman, 1989). Experiments in HTE cells also revealed that antigorite and riebeckite were less cytotoxic than crocidolite or chrysotile to these cells when release of radioactive chromium, a marker of cell damage, was measured (Mossman and Sesko, 1990).

Another exciting development in our laboratory was the observation that crocidolite (asbestos) generated Reactive Oxygen Species (ROS) which have been linked to cell injury, inflammation, mutagenesis, and the development of many cancers, (Shukla et al., 2003). In a study in which we isolated alveolar macrophages (AMs) from rodents and measured release of the ROS, superoxide, after addition of crocidolite and riebeckite (nonasbestiform analog of crocidolite) to these cells, as well as nonasbestiform mordenite (note that all particle diameters and/or fiber lengths were measured by scanning electron microscopy), the nonasbestiform particles were taken up, i.e., phagocytized, by cells, but were much less bioreactive than

crocidolite at comparable concentrations, only causing release of superoxide at concentrations 5-to 10-fold higher than asbestos in the rat cells and never causing significantly increased release in the hamster macrophages (Hansen and Mossman 1987). It should be emphasized that lung epithelial cells, mesothelial cells and fibroblasts are target or progenitor cells of lung cancers, mesotheliomas and pulmonary fibrosis, respectively, and that alveolar macrophages are inflammatory cells that first encounter asbestos and may contribute to and/or alternatively, be important in lung defense from pathogenic minerals. This is an important question that has yet to be resolved by scientists. However, alveolar macrophages are studied because these cells accumulate in the lung at sites of deposition of inhaled particles or fibers and responses of alveolar macrophages to dusts are known to produce ROS after phagocytosis of minerals.

In recent years, we have used riebeckite and antigorite preparations as nonasbestiform control minerals to determine whether early response proto-oncogene (*fos/jun* cancer- causing genes) (Janssen et al., 1994) or signaling pathways leading to activation of these genes (Janssen et al., 1997; Zanella et al., 1996; Zanella et al., 1999) are selectively induced by asbestiform, cancer-causing fibers (crocidolite and chrysotile asbestos, erionite) in HTE cells, rat lung epithelial cells (RLE) and isolates of normal rat pleural mesothelial cells (RPM). These studies have consistently revealed that these nonasbestiform minerals are inactive, regardless of endpoint. Moreover, they are incapable, in contrast to asbestos fibers, of causing alterations in cell proliferation or death in RPM cells (Goldberg et al., 1997).

Comparative studies in HTE and RPM cells with well-characterized mineral samples of crocidolite and chrysotile (asbestos) and 3 mineral samples containing various proportions of fibrous talc have also been useful in illustrating fundamental differences in response to asbestos fibers and fibrous talc preparations based on various dose parameters including equal weight concentrations, equivalent surface areas and numbers of fibers > 5 microns in length (Wylie et al., 1997). Using the CFE assay described above to document proliferative potential (increased numbers of colonies as compared to untreated control cells) or cytotoxicity (decreased numbers of colonies as compared to untreated control cells), exposure of RPM cells to both asbestos types, but not fibrous talcs, elicited cytotoxicity in RPM cells that was more striking at higher weight concentrations of asbestos. In contrast, HTE cells proliferated in response to asbestos at nontoxic lower concentrations, but not to fibrous talcs. Since cell responses could not be correlated directly with the presence of mineral fibers > 5 microns in length or aspect ratios, mineral type rather than fiber length *per se* appeared to be a more important determinant of bioreactivity. This study suggests that while fiber morphology is important, it is not the only factor important in biologic responses. This has also been noted by critics of Stanton's famous pleural implantation studies in rats (Oehlert, 1991; Wylie et al., 1987).

5. Studies using *in vitro* models of non-respiratory cells

As detailed above, cytotoxicity testing in cells of non-respiratory origin was used decades ago to determine differences in fiber-cell interactions and the ability of asbestos fibers to induce cell death or lysis. Since dead cells can not give rise to cancers, the extrapolation of these results, especially to mechanisms of cancer causation, is questionable. However, studies by Palekar and colleagues (Palekar et al., 1979) used sheep red blood cells (RBC) and Chinese Hamster Ovary (CHO) cells to test the hemolytic potential and cytotoxicity of 4 samples of cummingtonite-grunerite including amosite asbestos fibers, and 3 other samples of various crystallization habits, predominantly asbestiform cummingtonite, acicular cummingtonite, and acicular grunerite. At the same surface areas of dose, these minerals were found to be hemolytic and cytotoxic in this same order, again showing the increased potency of amphibole asbestiform fibers.

6. Summary and Conclusions

The results summarized above represent a large body of work showing that nonasbestiform minerals are less potent than asbestos fibers in a number of *in vitro* bioassays. In most assays, these cleavage fragments or non-fibrous minerals are virtually inactive. These observations have been incorporated into the conclusions of several panel reports that should be recognized by regulatory agencies. For example, the HEI-Asbestos Research Panel (page 6–75, 1991) concluded: “Good evidence exists that thick fibers (>2 to 3 microns in diameter) are less harmful than thin fibers” and “Support for the importance of fiber length in the production of biological effects has been obtained from the use of non-fibrous analogues of asbestos and other fibers. In general, these materials produce no detectable biological effects, or do so only at high dose levels”.

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