

Abstracts - EPA

The following abstracts from the Asbestos Mechanisms of Toxicity Workshop are available below:

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- [Influence of Fiber Type, Size, and Number in Human Disease: Conclusions from Fiber Burden Analysis](#) (Andrew Churg, M.D., Ph.D.)
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Arnold Brody, Ph.D.

Mechanisms of Pulmonary Fibrosis

We have been studying asbestos fiber deposition patterns, translocation pathways, and mechanisms of asbestos fiber toxicity. Chrysotile, crocidolite, and amosite fibers of varying lengths, from fragments to tens of microns long deposit at all levels of the respiratory tract. Fibers inhaled past the mucociliary escalator accumulate primarily at alveolar duct bifurcations (ADB), thus explaining the pattern of developing asbestosis as the lesions begin near the bronchioles and spread peripherally along the alveolar ducts over time, demonstrating a localized dose response. Inhaled fibers that deposit on the Type I alveolar epithelial surfaces are rapidly translocated by these cells to the interstitial vascular and lymphatic spaces from where they can reach the pleura and other anatomic compartments. All of the fiber types activate the fifth component of complement in the alveolar lining layer, thus producing C5a, which is a potent chemotactic factor for inflammatory cells. The inhaled fibers rapidly (within hours) activate the expression of a number of genes that code for peptide growth factors which mediate cell growth and matrix production. Our data, using tumor necrosis factor alpha receptor knockout (TNF- α RKO) mice and a fibrosis-resistant mouse strain, support the hypothesis that asbestosis and probably other fibroproliferative processes at the alveolar level and in the airway walls are mediated by specific growth factors according to the following scenario: 1) Deposition of toxic fibers rapidly upregulates expression of TNF- α . 2) TNF- α controls the expression of the platelet-derived growth factor (PDGF) isoforms by macrophages, epithelial and interstitial cells, and PDGF- α receptors are increased in the alveolar interstitium. Transforming growth factor alpha (TGF- α) expression in macrophages and epithelial cells appears quickly after exposure as well. 3) PDGF is the most potent mitogenic agent for mesenchymal cells yet described, and TGF- α mediates epithelial cell proliferation through the EGF receptor. 4) In very new experiments, we show that TNF- α upregulates the expression of transforming growth factor beta (TGF- β), the most powerful inducer of extracellular matrix proteins by mesenchymal cells. 5) Our as yet unpublished data show that reactive oxygen species (ROS) generated by chrysotile asbestos fibers activate latent TGF- β to its biologically active form.

In summary, these data support the postulate that inhaled asbestos fibers upregulate the expression of TNF- α , PDGF, and TGF- α , which control epithelial and mesenchymal cell proliferation as well as TGF- β which mediates extracellular matrix production. ROS generated by iron in the asbestos fibers activates TGF- β to its active form. It appears that all of the asbestos varieties induce these cellular and molecular responses that mediate the pathogenesis of asbestosis.

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Vincent Castranova, Ph.D.
Mechanisms of Fiber-Induced Lung Disease

A critical question in fiber research is the relative contribution of chemical properties vs physical dimensions to the potential pathogenicity of an inhaled fibrous particle. To address this question, it is essential to obtain fiber samples of discrete lengths for investigation. Recently our laboratory has developed a method, which utilized a dielectrophoretic classifier, to separate fiber fractions of narrowly defined lengths. The objective of the present study was to analyze the effects of fiber length on the ability of rat macrophages to phagocytize these fibers and to determine the potency of fibers of various lengths to activate nuclear transcription and cytokine production and to elicit cytotoxicity. Glass fibers (JM-100) were separated into five discrete size fractions (lengths of 3, 4, 7, 17, and 33 μm). Fibers ≤ 7 μm long were phagocytized by rat macrophages *in vitro*, while fibers ≥ 17 μm in length were too long to be completely engulfed, resulting in frustrated phagocytosis. There was a clear distinction in the bioactivity and cytotoxicity of fibers too long to be completely engulfed compared to shorter fibers. Glass fiber fractions having 17 μm or 33 μm lengths exhibited similar cytotoxicity on macrophages *in vitro*, measured as lactate dehydrogenase release or inhibition of zymosan-stimulated chemiluminescence. However, these long fibers had a toxic potency nearly two orders of magnitude greater than fiber fractions of 3, 4, and 7 μm lengths. Bioactivity was measured as the ability of glass fiber fractions to activate the DNA binding of transcription factors, nuclear factor kappa B (NF κ B) and activator protein-1 (AP-1), to phosphorylate MAP kinases, to activate the gene promoter for tumor necrosis factor alpha (TNF α), and to increase TNF α production by rat or mouse macrophages *in vitro*. Long-fibers (17 μm) were significantly more potent bioactivators than short fibers (7 μm). This bioactivation was inhibited by N-acetyl-L-cysteine, an antioxidant, indicating that the generation of oxidants contributed to this induction. These results suggest that length plays an important role in the potential pathogenicity of fibrous particles with effects being magnified when fibers are too long to be phagocytized completely.

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Andrew Churg, M.D., Ph.D.
Influence of Fiber Type, Size, and Number in Human Disease: Conclusions from Fiber Burden Analysis

Fiber burden studies are a potentially valuable tool in understanding the effects of asbestos on the lung, since such studies allow direct measurement of the types, numbers, and sizes of fibers seen in the lung and correlation with disease patterns, but the limitations of fiber burden studies must be remembered. Differences in preparative methods, instrumentation and counting techniques prevent direct comparisons of either fiber concentrations or fiber sizes between labs. However, if comparisons are made within the data provided by a given lab, then reproducible patterns of fiber burden and disease are observed.

Fiber burden studies have shown quite clearly that amphiboles are retained in and accumulate in lung tissue to a much greater extent than chrysotile. Estimated half lives for amosite and crocidolite are on the order of decades, whereas the estimated half live for chrysotile is a few months. Chrysotile usually contains the amphibole tremolite, which also shows preferential retention. Thus analysis of worker cohorts always shows significant amounts of amphibole, no matter what the nominal exposure. Even in chrysotile miners and millers the preponderant fiber is tremolite. A corollary of this observation is the finding that the lungs of workers from some nominally "chrysotile-only" industries such as textiles often contain a considerable burden of amosite or crocidolite, even though only small amounts of amosite or crocidolite were used in the plants. Given the much greater mesothelial carcinogenicity of amphiboles, this observation confounds attempts to attribute mesotheliomas to chrysotile exposure. The lack of biopersistence of chrysotile is probably the reason for its relatively weak mesothelial carcinogenicity.

Fiber burden studies have shown that everyone in the population has asbestos fibers in their lung, fibers that are inhaled from ambient air. This burden is largely short fibers of chrysotile and tremolite, with a much smaller number of fibers of amosite and crocidolite. Despite the fact that the numbers of fibers in everyone's lung is fairly high in numeric terms, there is no evidence that this "background" load causes disease.

Fiber burden studies have also shown that there is a striking difference between chrysotile (with its accompanying tremolite) and amosite or crocidolite in the relationship of fiber concentration and disease pattern. Disease always appears at burdens considerably higher than those found in the

general population. The development of asbestosis requires very high burdens of fibers of any type. For amosite and crocidolite, mesothelioma appears at a considerably lower burden than does asbestosis. **However, for chrysotile (and its accompanying tremolite), mesothelioma and asbestosis require the same, very high, fiber load.** Further, induction of asbestosis with chrysotile appears to require a much higher burden than does induction of asbestosis with amosite or crocidolite. These observations suggest that “chrysotile-induced mesothelioma” is purely a historic problem, because the required exposures are massive.

Some data suggest that tremolite is really the agent of “chrysotile-induced” mesothelioma. The tremolite in the lungs of those exposed to chrysotile ore or processed chrysotile products is a fairly short fiber of relatively low aspect ratio, and few long fibers are present. This is probably the reason that “chrysotile” exposure produces so few mesothelioma. By contrast, the tremolite seen in various environmental settings such as part of Turkey, Corsica, and in the region of Libby, Montana (regardless of what exact name is applied to the Libby amphibole), is a long, often thin, fiber that is closer to amosite in size characteristics. Such long fiber tremolite behaves more like amosite and is a relatively potent mesothelial carcinogen; it also is fibrogenic at sufficient doses.

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Bice Fubini, Ph.D.

Influence of Chemical/Physical Properties of Asbestos Fibers on Biological Activity

The mechanism(s) of asbestos toxicity and the differences in pathogenicity among the various asbestiform fibres are still partially obscure. One single molecular message, starting from the fiber surface and progressing to tumour development, has not been found, probably because several fiber features and multiple “mineral surface/living matter” interactions take place, during the development of asbestos related health effects. However, a large body of research in the past decade has evidenced a close relationship between some physical-chemical features (e.g. free radical generating surface sites) and adverse cellular responses.

The various paradigms on the physical-chemical side, on the basis of which research has been developed – fibrous habit, aspect ratio, iron ions release and deposition, free radical generation, biopersistence - will be revisited on the assumption that each of these feature does play a specific role in toxicity, while the overall pathogenic potential of a given fiber sample is build up by the concomitant effects of all of them. The effect of surface modifications and the variability among different asbestos types will be discussed in view of understanding their different dose-response relationships and envisaging possible routes for decontamination.

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Tom Hei, Ph.D.

Genetic Effects of Asbestos Fibers

Although the association between exposure to asbestos fibers and the development of lung cancer and mesothelioma has been well-established in man, the precise mechanisms by which asbestos produces malignancy are still not clear. Various *in vitro* and *in vivo* studies, however, have suggested that fiber dimensions, surface properties, and physical durability are important criteria for the carcinogenicity of the fibers. Studies using oncogenic transformation as an endpoint have shown that asbestos fibers can induce malignantly transformed foci in certain rodent cells and that oxygen radicals are important in the toxicity, oncogenic transforming and mutagenic effects of asbestos fibers. The mutagenic effects of asbestos in mammalian cells have been demonstrated using several model systems that can detect large multilocus deletions. These findings provide a direct link between chromosomal abnormalities that have frequently been demonstrated in fiber exposed human and rodent cell lines and carcinogenicity *in vivo*. There is evidence to suggest that K-ras mutations may be linked to lung cancer among asbestos workers who also smoke. However, an independent role for ras mutations in the etiology of asbestos-mediated lung cancer and mesothelioma is not conclusive. Microarrays have been used to identify differentially expressed genes associated with fiber carcinogenesis. There is recent evidence that down-regulation of p21^{Cip1} and the integrin receptor-associated gene *BigH3* may be causally linked to the neoplastic process induced by asbestos fibers. This lecture will provide a comprehensive overview of the recent advances in the mechanisms of fiber

carcinogenesis.

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Charles H. Hobbs, D.V.M.

Fiber Durability and Biopersistence – Assessment and Role in Asbestos Toxicology

Biopersistence is the term used to describe the ability of materials, including fibers, to persist in the lung. In the case of fibers, biopersistence of fibers longer than 20µm is of particular interest due to their association with asbestos induced pulmonary disease, mesotheliomas, and lung tumors. Clearance of materials from the lung is by dissolution or via the mucociliary escalator and trachobronchial tree or via lymphatics. In addition, long fibers may break apart. Recently, a protocol using a five-day inhalation of fibers by rats, followed by serial sacrifice of animals over 12 months, has been used to determine the biopersistence of fibers of several types, including asbestos. The biopersistence or half-life of fibers in the lung with lengths >20 as determined by this protocol has been shown to correlate well with the pulmonary pathology of these fibers following chronic inhalation in rats. This short-term inhalation protocol has recently been used to determine the biopersistence of chrysotile, crocidolite, amosite, and tremolite asbestos fibers. Chrysotile fibers L>20µm are much less biopersistent as determined by this protocol than fibers L>20µm of crocidolite, amosite, and tremolite. These findings support the lower observed toxicity of chrysotile fibers in animal inhalation studies and epidemiological studies of human populations. This short-term inhalation study protocol appears to be very useful for comparing the biopersistence of various fiber types including asbestos, especially if it is coupled with determining histopathological changes in lung, or with a longer term inhalation study (i.e., 90 days) that defines pulmonary pathology.

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Agnes B. Kane, M.D., Ph.D.

Molecular Pathogenesis of Malignant Mesothelioma

Asbestos fibers may act as direct or indirect carcinogens. Direct genotoxic and clastogenic effects of asbestos have been detected in *in vitro* assays. Asbestos fibers also stimulate persistent inflammation and release of oxidants, cytokines, and growth factors from inflammatory cells. The potential action of SV40 virus as a co-factor with asbestos fibers in the induction of malignant mesothelioma is controversial.

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Eugene McConnell, D.V.M.

Influence of Fiber Type, Size and Exposure in the Cancer and Non-Cancer Response to Asbestos Fibers (Animal Studies)

From a toxicologist's standpoint asbestos is an unusual material and does not conform to standard toxicological principles. It does not cause disease due to its chemical composition or form metabolites, as do most chemicals. Rather it is the physical nature of asbestos that imparts its' pathogenicity. The only characteristic that is in common with chemicals is "dose", which is also a seminal component of the toxic response of asbestos. Characteristics such as its' fibrous nature, size of the fibers and duration in the lung are the primary drivers of its' pathogenicity. In animals it is clear that asbestos has to reach the "deep lung", i.e. to the level of the terminal bronchioles and alveoli, to cause disease. Lesions in the upper respiratory tract and airways have not been observed even under the most severe exposure scenarios. Fibrous and nonfibrous particulates that are deposited in the airways are rapidly removed by the mucociliary escalator, are swallowed, and excreted in the feces. Whether asbestos reaches the deep lung or not by inhalation is entirely dependent on its size. If asbestos fibers are of a size to reach the deep lung, they must reside there in sufficient numbers and for a long period to cause irreparable disease, e.g. fibrosis and cancer. The fibers of most import are those that are long and biopersistent. Short fibers are cleared from the deep lung in a relatively short period and therefore, do not cause disease unless the "dose" exceeds the lungs physiological clearance mechanisms, primarily phagocytosis by resident macrophages. The importance of fiber size in relation to lung cancer has been demonstrated by inhalation and other routes of exposure for all three major forms of asbestos, i.e. chrysotile, amosite and crocidolite, long fibers being much more potent. Providing that a sufficient quantity of long asbestos fibers reach the deep lung, they can produce fibrosis and cancer. However, they must reside there for most of the animal's life. The residence time is termed

“biopersistence” and is a direct reflection of the fibers’ solubility in the lung milieu. The solubility of a given fiber is dependent on its chemical and physical properties. *in vitro* and *in vivo* studies have shown that all three types of asbestos are biopersistent in the lung, albeit chrysotile is less so. The above features of the toxicity of asbestos have to be considered in concert, and no single parameter can be used in isolation of the others.

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Brooke T. Mossman, Ph.D.

Asbestos-Induced Cell Activation and Proliferation

In contrast to a number of nonpathogenic particles and synthetic fibers, asbestos and erionite fibers target cell signaling proteins leading to increased expression of early response protooncogenes (AP-1 family members of the *fos/jun* family), proliferation, and transformation. Using normal rodent mesothelial and pulmonary epithelial cells, we have shown that the Extracellular Signal-Regulated Kinases (ERKs1/2 and ERK5) are preferentially stimulated by asbestos through an oxidant-dependent pathway and linked causally to expression of *c-fos*, *c-jun* and *fra-1* as well as cell proliferation *in vitro*. These responses are dose-related and observed at lower concentrations of crocidolite in comparison to chrysotile asbestos. In rodent inhalation experiments, increased expression of ERKs1/2, *c-jun* and *c-fos* and epithelial cell proliferation after exposure to asbestos has also been confirmed by immunohistochemistry and laser capture microdissection/TaqMan (LCM). Modification of the ERK cascade using a dominant negative MEK1 targeted to lung epithelium (CC10-dnMEK1) significantly inhibits both bronchiolar epithelial cell proliferation and fibrosis without modifying inflammation, suggesting a critical importance of epithelial cell signaling (Manning et al., in preparation for publication). Recent work documents that transformation of rat pleural mesothelial (RPM) cells is characterized by ERK-dependent Fra-1 expression and increases in Activator Protein-1 (AP-1) DNA binding complexes exhibiting Fra-1 heterodimers. Moreover, inhibition of ERK phosphorylation or transfection with a dominant negative *fra-1* reverses the transformed phenotype of mesothelioma cells and their growth in soft agar. Microarray (Affymetrix) data comparing RPM cells with and without exposure to crocidolite asbestos and rat mesotheliomas indicates that *fra-1* and several genes (CD44, High Mobility Group Protein, PAI-1) associated with proliferation and migration are increased in mesotheliomas and in mesothelial cells after acute exposure to asbestos. Data from experiments using siRNA techniques also show that expression of several of these genes is *fra-1*-dependent, supporting a critical role of Fra-1 and AP-1 in asbestos-increased signaling pathways leading to cell proliferation and transformation. Supported by NIH grants R01 ES/HL09213, T32 071222 and P01 HL67004.

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