In Vitro Approaches for Determining Mechanisms of Toxicity and Carcinogenicity by Asbestos in the Gastrointestinal and Respiratory Tracts

by Brooke T. Mossman*

Organ and cell cultures of gastrointestinal and tracheobronchial epithelium have been used to document both the interaction of asbestos with mucosal cells and the sequence of cellular events occurring after exposure of cells to fibers. The biological activity of various types of asbestos *in vitro* is related to surface charge, crystallization, and dimensional characteristics. These factors also influence adsorption of natural secretions and serum components to fibers, a process that ameliorates cytotoxicity. Although mechanistic studies at the cellular level are lacking using epithelial cells of the digestive tract, asbestos appears to elicit a constellation of morphologic and biochemical changes in tracheal epithelium that resemble effects of classical tumor promoters on target cells.

Introduction

Asbestos is implicated in the causation of cancers of the digestive system, airways, and lung. To elucidate possible mechanisms of fiber-induced disease, the interaction of asbestos with epithelial cells of the gastrointestinal and respiratory tracts has been studied in organ and cell cultures. After introduction of asbestos, the uptake of fibers by mucosal cells is observed concomitantly with damage, death, and regeneration of the epithelium (1–3). The extent of these changes, which might be related to the process of carcinogenesis, varies with the type (2–4), charge (5,6), crystallization (7), and size (8,9) of the fibers. Discussed here are experiments *in vitro* that define asbestos-induced cellular responses and their possible relationship to neoplasia in gastrointestinal and respiratory epithelium. Carcinoma, the histologic classification of tumor found most frequently in man, arises from these (i.e., epithelial) cells. Although only one group of researchers has taken an in-depth look at the testing of asbestos in gastrointestinal epithelial cells *in vitro* (4), the

work done with other epithelial cells provides significant data.

Mechanisms of Cell Damage by Asbestos

Because asbestos is a family of fibrous hydrated silicates, each with unique chemicophysical features, the relative toxicity of various types of asbestos has been assessed comparatively in cell and organ cultures derived from intestinal, liver, colonic (4), and tracheobronchial epithelium (2,3,10,11). Cell damage, compared to untreated controls, is assessed by inhibition of growth or colony formation (2,4), release of ^{51}Cr (10) or ^{75}Se (11) from prelabeled cultures, and quantitation of lysosomal or cytoplasmic enzymes in medium (12). Although the relative sensitivity of different cell lines varies, chrysotile is more toxic when compared at equal concentrations to the amphiboles, amosite and crocidolite.

Surface Charge and Cytotoxicity

To elucidate mechanisms of asbestos-induced cytotoxicity, the chemical and structural composition of asbestos has been altered experimentally by leaching and adsorption of macromolecules.

*Department of Pathology, University of Vermont, College of Medicine, Burlington, VT 05405.

These changes are thought to occur after inhalation or ingestion of fibers *in vivo*. For example, unlike a soluble material of defined chemical composition, asbestos is insoluble and comprised of a number of extractable elements that are removed after prolonged periods of time *in vivo* (*13–15*).

After incubation of chrysotile in hydrochloric acid, substantial amounts of cations, including Mg^{2+} and Ca^{2+}, are released from fibers (*4*). Accordingly, the surface charge of fibers, as measured by the zeta potential, is altered. Experiments by Light and Wei (*5,6*) have indicated the important role of surface charge in asbestos-induced toxicity by quantitating hemolysis in red blood cells (RBC) after adding natural and leached asbestos.

Under physiological conditions, the surface charges of chrysotile and crocidolite asbestos are similar in magnitude, but opposite in polarity (+44.5 mV for chrysotile and −43.5 mV for crocidolite). Chrysotile causes distortion and hemolysis of RBC, whereas crocidolite is only weakly hemolytic. After leaching, however, the surface charge of chrysotile decreases as does its hemolytic activity. In contrast, the hemolytic potential for crocidolite increases proportionately as it becomes more negative in charge. Leaching chrysotile in hydrochloric acid also inhibits its cytotoxicity in epithelial cells from liver (*4*) and trachea (*10*) while cytotoxicity of leached crocidolite is enhanced.

In additional experiments by Light and Wei (*6*), dipalmitoyl phosphatidylcholine (DPPC), the main component of pulmonary surfactant, a substance secreted by the lung, was incubated with unleached fibers before their addition to RBC. Under these circumstances, DPPC decreases the surface charge of both chrysotile and crocidolite with a proportional inhibition of hemolysis. Thus, asbestos fibers appear to adsorb macromolecules that can ameliorate their cytotoxicity. These results are supported by those of Desai and Richards (*16*) showing a selective adsorption and retention of serum proteins by chrysotile and crocidolite after incubation in culture medium. The addition of fibers to cell cultures in serum, versus medium containing no serum, also decreases the growth-inhibitory effects of asbestos (*2*)(Fig. 1).

Fiber Size and Cytotoxicity

Addition of asbestos to a number of cell types in culture is associated with release of lysosomal and cytoplasmic enzymes into the medium (*12,17*). In macrophagelike cells, the amount of cell contact and phagocytosis correlate directly with a decrease in number of viable cells, whereas cultures of fibroblasts, a relatively nonphagocytic cell type, are more resistant to asbestos (*18*). The dimensions of the fiber also determine how the cell will react. For example, toxicity is reduced substantially when larger fibers and particles are reduced to a submicron size range (*8,9*). In general, shorter, smaller fibers can be phagocytized, whereas macrofibers cannot be enveloped completely. Under the latter circumstances, one might postulate that damage to membranes results in cell death, and remnants of cellular debris can be seen in association with fibers that are phagocytized incompletely (Fig. 2). Recent data from our laboratory suggest that oxygen-free radicals are generated when longer fibers are encompassed by tracheal epithelial cells (*11*). Moreover, levels of superoxide dismutase (SOD), an enzyme that converts the superoxide radical (O_2^- to H_2O_2 and O_2, appear to be altered in cells after exposure to asbestos. More importantly, cell damage by longer fibers can be prevented by addition of scavengers of oxygen-free radicals to cultures.

Surface Area and Crystallization

The mechanisms of cell lysis by asbestos are also related to specific surface area (*19*) and crystallization of the fiber (*7*). Dimensional characteristics appear important in determining the amount of cell contact, whereas the role of structure is enigmatic. Palekar and colleagues compared the hemolytic and cytotoxic potential of structurally modified forms of amosite, an amphi-
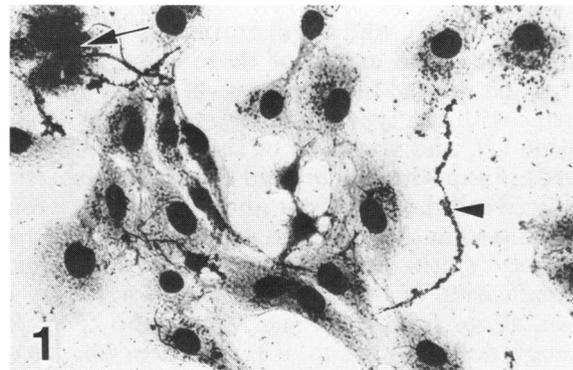

FIGURE 1. When chrysotile asbestos (5 µg/mL F12 medium, 10% calf serum) is added to monolayer cultures of tracheal epithelial cells, cytotoxic alterations such as sloughing of cells, multinucleated cells (arrow), and prominent cytoplasmic granules are observed. The adsorption of basic dye appears as beads on the fiber (arrowhead). Giemsa, × 1035.

bole asbestos (7). These minerals occurred in various developmental stages of crystallization and included both species with structural and surface
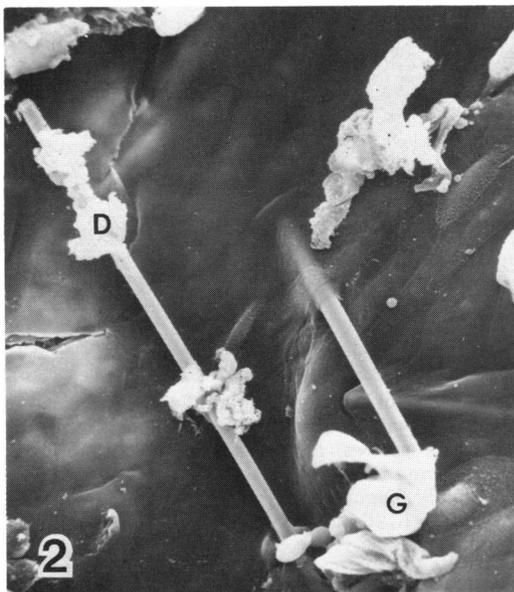

FIGURE 2. Larger fibers (i.e., >10 μm length) are phagocytized incompletely by human bronchial epithelial cells, and are more cytotoxic than smaller fibers (i.e., <2 μm) *in vitro*. Note the cellular debris (D) and cell ghost (G) in association with the fiber. This scanning electron micrograph was furnished by Craig Woodworth, Department of Pathology, UVM College of Medicine, ×560.
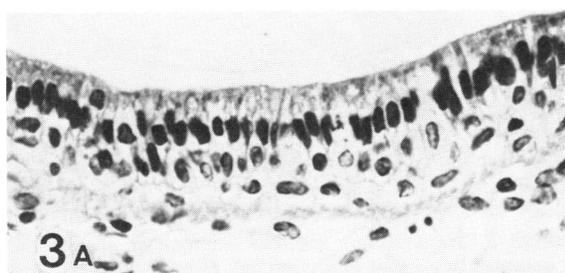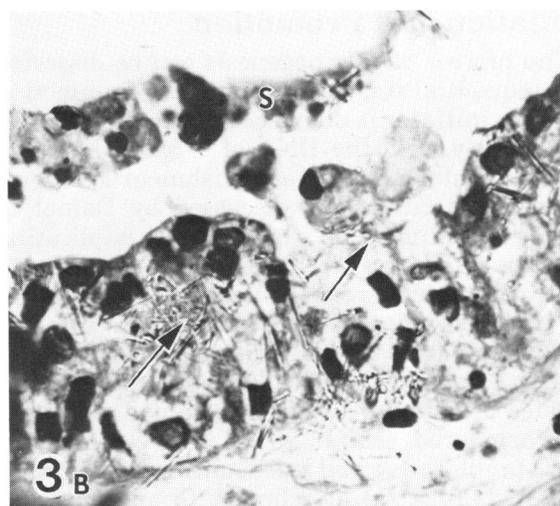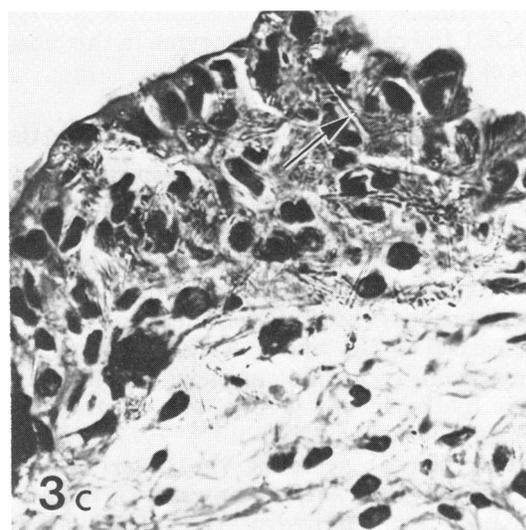

FIGURE 3. Sequential morphologic changes in organ cultures of hamster tracheal epithelium after exposure to crocidolite asbestos. Tissues are exposed to asbestos for 1 hr before transfer to asbestos-free medium for extended periods of time: (A) culture without addition of asbestos at 1 week after preparation, ×1400; (B) at 72 hr after addition of crocidolite (arrows, 4 mg/mL medium), sloughing of superficial cells (S) occurs, ×1600; (C) a hyperplastic lesion is observed at 1 week after addition of asbestos. Note the accumulation of refractile crocidolite (arrow) within the mucosa, ×1400. Hematoxylin and eosin.

defects and more "asbestiform" varieties, i.e., long, perfect fibers with high tensile strength. All samples were characterized for surface area, charge and chemical content. When assayed at comparable surface areas, hemolytic activity and cytotoxicity in cell cultures were related directly to the degree of crystallization. Amosite exhibited the greatest biological activity, whereas the most nonasbestiform mineral was inert.

Injury, Promotion and Carcinogenesis

After injury and death of epithelial cells of the gastrointestinal and respiratory tracts, luminal cells are sloughed and replaced by basal cells, the presumed progenitors of carcinoma (Fig. 3). Be-

cause malignant transformation of cells occurs specifically during the DNA synthetic phase of the cell cycle (20), asbestos-induced proliferation of cells might be an intrinsic mechanism of carcinogenesis. In the next several paragraphs, the concepts of initiation and promotion in carcinogenesis will be discussed. Moreover, we will describe the properties of asbestos that suggest its role as a tumor promoter in the development of gastrointestinal and bronchogenic carcinomas. The experiments in our laboratory have been performed using epithelial cells from the respiratory tract; however, since these cells are similar structurally and functionally to mucin cells of the gut, our results might apply to events occurring in the gastrointestinal mucosa after ingestion of asbestos.

Initiation and Promotion

The process of carcinogenesis can be dissected into sequential stages of initiation and promotion (21). An initiator is defined as an agent interacting directly with the DNA of a cell, whereas a promoter influences the establishment and development of a tumor. As discussed by Daniel in these proceedings (22), the evidence implicating asbestos as a mutagenic agent in intestinal and other cell types is weak; thus its role as an initiator of carcinogenesis is questionable. On the other hand, work from our laboratory shows that the properties of asbestos in cell and organ cultures of trachea are similar to classical tumor promoters such as phorbol esters (23, 24). These substances cause proliferative and biochemical alterations in cells including: (a) attachment to and entrance into target cells; (b) stimulation of cellular division; (c) inhibition of normal cell differentiation; and (d) increased activity of ornithine decarboxylase (ODC), the rate-limiting enzyme in the biosynthesis of polyamines.

Uptake of Asbestos by Mucosal Cells

Fibers penetrate intestinal and respiratory mucosa both in vivo and in vitro (1, 25, 26) and remain in epithelial cells for extended periods of time (Fig. 4 and 5). These processes not only allow continuous cellular exposure to the dust, but also permit the access into cells of chemical carcinogens adsorbed to the fibers. As an example, organic material, including carcinogenic polycyclic aromatic hydrocarbons (PAH), are natural contaminants of asbestos (27).

In experimental systems, the association of PAH with the fiber surface appears to enhance cellular uptake of the hydrocarbon. Thus, asbes-
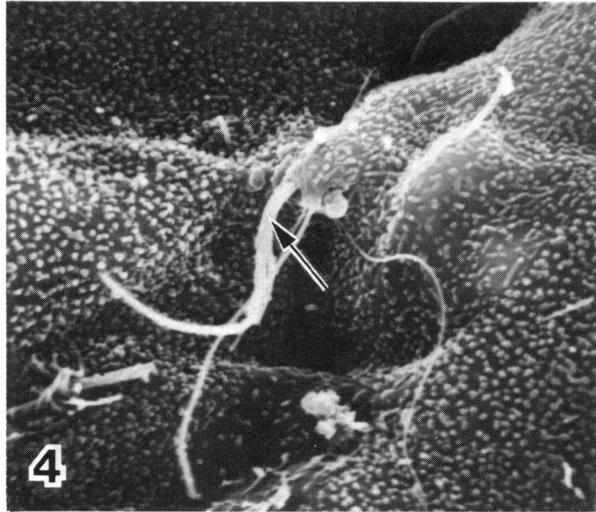

FIGURE 4. Human bronchial explant 24 hr after exposure for 1 hr to chrysotile asbestos (1 mg/mL medium). Note the long fibers (arrows) protruding from the epithelial cells. This scanning electron micrograph was furnished by Craig Woodworth, Department of Pathology, UVM College of Medicine, × 6120.
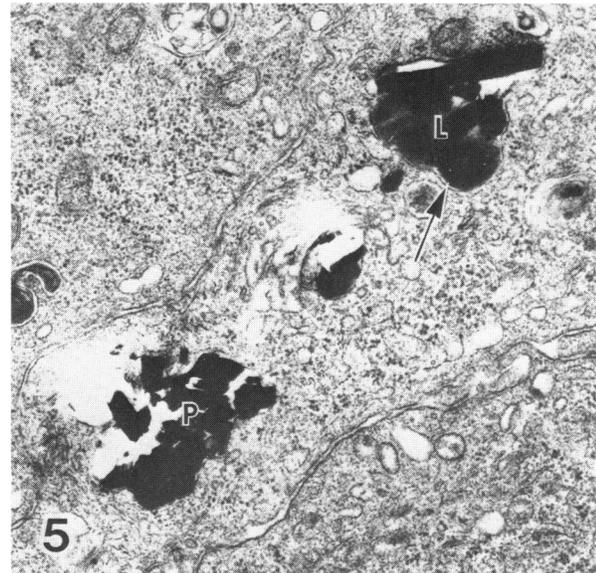

FIGURE 5. Transmission electron micrograph of crocidolite asbestos in a basal epithelial cell of hamster trachea. The asbestos occurs in membrane-bound (arrow) phagosomes (P) and phagolysosomes (L). Uranyl acetate and lead citrate, × 7500.

tos might be cocarcinogenic by facilitating the entrance of documented chemical carcinogens into cells. For example, Lakowicz and colleagues (20, 29) document by fluorimetry the increased transfer of PAH to artificial membranes when chemicals are adsorbed to asbestos. On the other

hand, dispersions and particles of PAH alone do not transfer rapidly to membranes.

When ³H-BaP is adsorbed either to crocidolite or chrysotile asbestos before its addition to cultures of tracheal epithelial cells, approximately 70% of the total BaP introduced enters the cell within 1 hr., whereas 50% remains intracellular and unmetabolized at 8 hr. (*30*). In contrast, if identical amounts of BaP are added directly to medium, an influx of only 20% is observed and cells retain 5% of the initial amount at 8 hr. Under the former conditions, alkylation of BaP to DNA is increased for as long as 5 days after introduction of asbestos adsorbed to BaP. Increased uptake, retention, and alkylation of BaP to DNA are not observed when BaP is added 1 hr. after the asbestos (*31*).

Stimulation of Cell Replication by Asbestos

After exposure to amosite or crocidolite asbestos, tracheal epithelial cells *in vitro* show increased incorporation of ³H-thymidine, an indication of DNA synthesis, and basal cell hyperplasia (*32*). Although enhanced uptake of ³H-thymidine and morphologic changes are also observed in monolayers of cells exposed to either crocidolite of chrysotile (*33*), proliferative alterations appear more transiently in organ cultures exposed to the latter type of asbestos.

An increase in cell replication could contribute in several ways to carcinogenesis. First, it would give an "initiated" cell (i.e., one that is committed to becoming malignant) a selective advantage over normal cells. Second, since cells are transformed by chemical carcinogens only during DNA synthesis, a cell preparing for division is more susceptible to transformation.

Induction of Squamous Metaplasia by Asbestos

Squamous metaplasia is defined as the replacement of a normal mucin-secreting epithelium by keratinizing squamous cells. This lesion, although reversible, can be considered preneoplastic. The appearance of squamous metaplasia in the gastrointestinal or respiratory tract could be a promoting influence in carcinogenesis by the following mechanisms. First, mucin secretion by differentiated epithelial cells is interrupted, thus removing the protective barrier against asbestos and other carcinogens.

Second, the interaction and uptake of fibers by squamous epithelium are observed frequently *in*

vitro (Fig. 6) (*34*); therefore, increased entrance into, and retention of asbestos by, squamous epithelium is a possibility. Last, the basal cells of squamous lesions are actively dividing (*32*), a process encouraging their transformation by carcinogens in general.

Stimulation of Ornithine Decarboxylase (ODC) by Asbestos

The magnitude of induction of ODC, the first and rate-limiting enzyme in the biosynthesis of polyamines, relates directly to the tumor-promoting capabilities of a number of phorbol compounds in mouse skin (*35*). When chrysotile or crocidolite is added to tracheal epithelial cells, ODC is increased in a dosage-dependent fashion; however, the magnitude and latency of response differ from that observed with the phorbol, 12-tetradecanoylphorbol-13-acetate (TPA) (*33*). The increase in enzyme induction occurs concomitantly with a mitogenic response as measured by uptake of ³H-thymidine.

An increased synthesis of polyamines might be important in carcinogenesis because it is linked intrinsically to cell division. For example, critical cellular levels of polyamines must exist for cell division to occur.
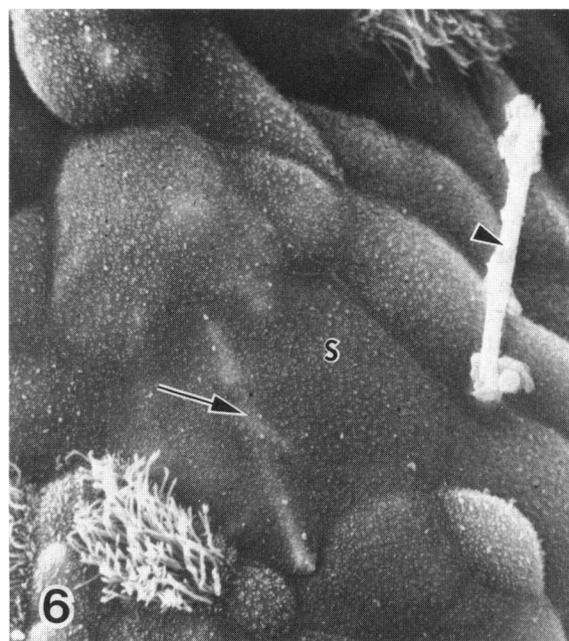

FIGURE 6. Surface of a hamster tracheal explant at 4 weeks after addition of amosite asbestos (4 mg/mL medium). The superficial cells are becoming squamous (S). Note the fiber within the cell (arrow) and the fiber (arrowhead) on the epithelial surface. Scanning electron micrograph, ×2000.

Summary and Conclusions

Asbestos interacts with mucosal cells of the gastrointestinal and respiratory tracts, although its composition and cytotoxicity are modified by acidity and coating with natural secretions such as mucin and surfactant. The biological activity of the fibers is determined by both surface charge, crystallization, and dimensional characteristics. After damage and death of superficial cells of the respiratory tract, replacement occurs via actively dividing basal cells. These latter cell types become squamous and exhibit increased synthesis of polyamines.

The role of asbestos in carcinogenesis appears epigenetic and can be compared to that of a classical tumor promoter. A number of experimental and epidemiologic studies support this observation. For example, various types of asbestos do not cause single-strand breakage of DNA (30) and are not mutagenic in a number of cell types (22). Moreover, asbestos, unless combined with PAH, is not carcinogenic in hamster tracheal implants (36,37) and is only weakly carcinogenic in rats after repeated intratracheal instillations (23).

Compelling epidemiologic evidence also indicates that asbestos acts synergistically with cigarette smoke to induce tumors of the respiratory tract. Persons occupationally exposed to asbestos (smokers and nonsmokers as a group) have an eightfold higher incidence of bronchogenic carcinoma than the general population. While the increase (1.5- to 4-fold) in disease among nonsmoking asbestos workers is small, smokers have an 80- to 90-fold greater predisposition (38,39).

Although asbestos acts like a promoting agent in the airways and lungs, there is little experimental evidence documenting this capability in the gastrointestinal tract. Mechanistic studies to evaluate the promoterlike activity of asbestos in organ and cell culture of esophagus, stomach, and/or intestine should be encouraged.

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