Cigarette smoke increases amosite asbestos fiber binding to the surface of tracheal epithelial cells

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Churg, A., J.-P. Sun, and K. Zay. Cigarette smoke increases amosite asbestos fiber binding to the surface of tracheal epithelial cells. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L502–L508, 1998.—Binding of asbestos fibers to the cell surface appears to be important in the initiation of intracellular signaling events as well as in initiation of particle uptake by the cell. We have previously shown that cigarette smoke increases the uptake of asbestos fibers by tracheal epithelial cells in explant culture. Whether smoke acts by increasing surface binding of fibers is not known. In this study, we exposed rat tracheal explants to air or cigarette smoke and then to a suspension of amosite asbestos. Explants were harvested after 1 or 24 h of dust exposure and washed by repeated dips in culture medium to remove loosely bound fibers, and the number of fibers adhering to the apical cell surfaces was determined by scanning electron microscopy. Smoke-exposed explants retained significantly more surface fibers than air-exposed explants. After four washes, binding levels were similar at 1 and 24 h. The smoke effect was still present when incubations were carried out at 4°C, but binding was decreased ~25%. Preincubation of the asbestos fibers with iron chloride to increase surface iron increased fiber binding in both air- and smoke-exposed explants, whereas preincubation of the fibers with the iron chelator deferoxamine decreased binding after air exposure and completely eliminated the smoke effect. Inclusion of mannitol or catalase in the medium or preincubation of the explants with GSH produced decreases in binding of 10–25% in air-exposed explants and generally greater decreases in smoke-exposed explants. We conclude that 1) amosite binding is a very rapid process that does not require active cellular metabolism, 2) cigarette smoke increases adhesion of fibers to the epithelial surfaces, and 3) iron on the asbestos fiber appears to play an important role in binding, probably through an active oxygen species-mediated process.

Article:
THERE IS RECENT EVIDENCE to suggest that binding of asbestos fibers (and presumably other types of particles) to the cell surface may lead to direct activation of the extracellular signal-regulated kinases (ERKs) and to transcription of a variety of genes associated with cell proliferation and inflammatory responses as well as to apoptosis (15, 19). This process appears to proceed via activation of the epidermal growth factor receptor and is driven, at least in part, by active oxygen species (AOS) (15; see DISCUSSION). As well, particle binding to the surface of pulmonary epithelial cells is believed to be the first step in particle uptake (internalization) (1, 8, 10, 17), and uptake is associated with a variety of deleterious effects including inflammatory cytokine secretion (9), genotoxicity (reviewed in Ref. 14), and, after particle transport to the interstitium, interstitial fibrosis (8).

Although it is clear that particle binding is important in the development of pathological abnormalities, the factors that control particle adhesion to the cell surface are poorly understood. To further complicate matters, asbestos occurs in two physically and chemically different forms that appear to bind quite differently. Chrysotile is a magnesium silicate that carries a positive surface charge. Amphibole types of asbestos (amosite and crocidolite) are magnesium iron and sodium magnesium silicate that carry a negative surface charge. Brody et al. (4) and Gallagher et al. (11), using scanning electron microscopy (SEM), observed that adhesion of chrysotile fibers, carbonyl iron spheres, or aluminum spheres, all of which carry a positive surface charge, to red blood cells or alveolar macrophages could be decreased by neuraminidase, suggesting that these particles bound to negatively charged sialic acid residues. They found that neuraminidase treatment had no effect on the adhesion of crocidolite asbestos or glass spheres, implying that negatively charged particles did not bind to sialic acid residues but that these substances did nonetheless bind to cell membranes.

Brown and colleagues (5, 6) investigated the adhesion of chrysotile, crocidolite, and particularly amosite (also a negatively charged fiber) asbestos to various cultured cell lines using gradient centrifugation of fractionated radiolabeled cells in an attempt to sort out free fibers from fibers bound to membranes. Adhesion varied considerably from cell line to cell line. With V79 cells, amosite adhesion increased with the addition of increasing amounts of serum to the medium; addition of fibronectin mimicked the serum effect, and Arg-Gly-Asp (RGD)-containing pentapeptides blocked adhesion, implying that fibronectin was the adhesion mediator. Adhesion was decreased in calcium-magnesium-free medium. Changing the fiber charge from negative to positive by coating the fiber with poly-L-lysine increased adhesion. Brown et al. concluded that the coating of negatively charged fibers with serum-derived fibronectin and subsequent adhesion to the cellular RGD receptor was the primary mode of fiber adhesion.

Boylan et al. (3) used rabbit mesothelial cells in culture to examine the adhesion of crocidolite asbestos and wollastonite, another negatively charged fibrous calcium silicate. They incorporated a fluorescent dye in the cell membrane and, using confocal scanning microscopy, counted fibers with a fluorescent rim as internalized and nonfluorescent fibers as adherent. Boylan et al. observed that adhesion of crocidolite fibers to the surface could be decreased by treatment with trypsin-EDTA, whereas actual uptake of crocidolite fibers into the cells was mediated via the vitronectin-binding integrin-αvβ3. Serum and exogenous vitronectin in-
creased uptake, but fibronectin had no effect on either adhesion or uptake.

Stringer et al. (22) employed A549 cells, a pulmonary epithelial cell line, in culture to look at the adhesion of TiO2, Fe2O3, air particulates, and quartz. They used a flow cytometric technique and measured right-angle scatter to define the amount of particle adhesion. Particle adhesion was found to be calcium dependent for TiO2 and Fe2O3 but calcium independent for quartz. Heparin and polynsinosic acid markedly decreased particle adhesion, but polyanion chondroitin sulfate did not, suggesting that adhesion was mediated by scavenger-type receptors and that the receptors were distinct from those found on alveolar macrophages.

This whole set of observations suggests that different particles bind to very different receptors. For positively charged fibers and particles, binding may be primarily mediated by simple electrostatic interactions with negatively charged moieties on the cell surface, but charge binding has not been shown for negatively charged fibers and particles. Specific integrins may be important in fiber and particle binding; however, the available data are inconsistent and also problematic because binding occurs in serum-free systems where there are no adhesive proteins to coat the fibers. Thus the exact receptors and mechanisms of particle binding remain obscure.

If rat tracheal explants are briefly submerged in a mineral dust suspension and then maintained in a culture medium one, two, or four times. The explant was then exposed to either room air or cigarette smoke (five 20-ml puffs of whole smoke) for 10 min in a humidified 2-liter chamber. All explants were then submerged, epithelial side up, in a 5 mg/ml suspension of Union Internationale Centre le Cancer amosite asbestos in DMEM without serum for 1 h. In some experiments, the fibers were treated to modify available surface iron, AOS scavengers were included with the fiber suspension, or the explant GSH levels were changed as detailed in Manipulation of GSH levels. At the end of this time, the explants were very carefully lifted from the medium so as not to disturb the asbestos fibers adherent to the surface. Some explants were maintained in an air plus 5% CO2 organ culture with basal feeding in an incubator at 37°C for 24 h, whereas other explants were removed from the amosite suspension and immediately washed to remove dust as described in Determination of fiber binding.

Surface iron manipulations. Surface iron levels were increased by incubating amosite asbestos overnight at room temperature with a freshly prepared mixture of 1:1 10 mM iron(II) and 10 mM iron(III) chloride. After the amosite was washed in saline, surface iron levels were evaluated with the dithionite-citrate-bicarbonate method (18). The iron-loaded or native amosite was mixed with sodium dithionite in a citrate-bicarbonate buffer at 80°C for 30 min, the mineral particles were centrifuged out, and the supernatant was treated with o-phenanthroline. Absorbance was measured at 510 nm. A blank containing the color reagent but no iron was used as the reference standard. This method reduces all surface iron to the ferrous form and hence provides a measure of both ferrous and ferric surface iron.

Two verses surface iron was rendered non-redox active by incubation overnight with the iron chelator deferoxamine (DFX; Desferal, Ciby-Geigy) at a concentration of 10 mM. Fibers were washed two times in saline before use.

Manipulation of GSH levels. To examine the effects of boosting cellular antioxidant protection, explants were first incubated with 10 mM GSH (Sigma) for a 1-h period, then washed and exposed to smoke or air and asbestos as described in Dust exposure. Intracellular GSH levels were determined by HPLC as described below. Conversely, to deplete intracellular GSH, rats were given an intraperitoneal injection of diethylmaleate (Sigma) at a dose of 1 mg/kg body weight 2 h before death.

GSH levels in the lungs and explants were assayed by a modification of the method of Anderson (2) and Newton et al. (21). The tissues were stored, frozen in 5% t-sulfosalicylic acid, then thawed and homogenized in 5% t-sulfosalicylic acid. The homogenate was centrifuged to remove solid debris. The supernatant was derivatized with N-ethylmorpholine and monobromobimane in the dark. HPLC analysis was performed with a Whatman ODS-3 4-µm × 25-cm column and methanol-acetic acid buffers. A fluorescence detector system was used, with an excitation wavelength of 394 nm and detection wavelength of 480 nm. The detection limit was <100 pg, with a signal-to-noise ratio of 5:1.

Scavenging of AOS. To examine the role of AOS, experiments were performed in which mannitol (1 mM; Sigma), a hydroxyl radical scavenger, or catalase (1600 U/ml; Boehringer Mannheim), a hydrogen peroxide scavenger, was added to the dust suspension.

Effects of temperature. To examine the effects of temperature on surface binding, air or smoke and subsequent asbestoses exposures were carried out at 4°C. Initial experiments showed poor cellular preservation at 24 h at 4°C so that adhesion levels were only measured after 1 h in the cold.

Determination of fiber binding. Exposure of the explants to asbestos for 1 h, as described in Dust exposure, results in initial coating of the epithelial surface with fibers. To remove fibers not bound to the surface and count actual adherent fibers, each explant was carefully and slowly dipped in fresh culture medium one, two, or four times. The explant was then dried under vacuum and prepared for SEM. SEM photographs of randomly selected fields were taken at a magnification of ×1,000. The proportion of the surface occupied by
fibers (areal fraction of fibers) was determined with a 42-point transparent overlay, counting points that fell on fibers versus points that fell on tissue (13). This approach avoids problems such as dealing with touching fibers and irregular surfaces that occur if one attempts to count numbers of fibers per unit area and is simple and relatively rapid to apply.

Each group contained four or five explants, and differences among groups were determined with analysis of variance with the SYSTAT statistical package (23).

RESULTS

Figure 1 shows a representative scanning micrograph illustrating fibers adherent to the tracheal surface. The mucous cells are artificially prominent, and the cilia have collapsed in this air-dried preparation.

Figure 2 shows the effects of smoke on fiber surface adhesion immediately after the 1-h dust incubation (i.e., 1 h after smoke exposure), and Fig. 3 shows the effects after 24 h of organ culture (25 h after smoke exposure). At 1 h, significantly increased adhesion is seen in smoke-exposed explants after two or four washes, and at 24 h, significantly increased adhesion is seen in smoke-exposed explants after one, two, and four washes. The differences between smoke and air exposure are more apparent at 24 h and vary from two- to threefold.

On the basis of the data from Figs. 1 and 2, new experiments were run as described in MATERIALS AND METHODS for AOS effects, and segments were subjected to four washes. Figure 4 shows data for asbestos fibers loaded with iron. Incubation with 10 mM iron chloride...
overnight boosted surface iron levels from 110 ± 6.0 µg/g dust to 250 ± 6.0 µg/g dust (mean ± SD) and increased the number of adherent fibers by 30% in air-exposed explants and 42–47% in smoke-exposed explants. Conversely, as shown in Fig. 5, loading the fibers with 10 mM DFX decreased the number of bound fibers by 25% in air-exposed explants and 47% in smoke-exposed explants, completely abolishing the smoke effect.

Figure 6 shows the effects of including mannitol in the incubation medium. Mannitol reduced adhesion by 20% in air-exposed explants and 20–25% in smoke-exposed explants. Catalase (Fig. 7) had minimal effects on the adhesion of fibers to air-exposed explants (a 15% decrease at 24 h and a 4% increase at 1 h) and a 20–25% decrease in adhesion to smoke-exposed explants.

Loading the explants with GSH as described in MATERIALS AND METHODS boosted cellular GSH by close to 10-fold (0.10 ± 0.01 and 0.92 ± 0.07 µg/mg tissue for untreated and GSH-treated explants, respectively).

Boosting GSH levels produced less than a 10% decrease in fiber adhesion in air-exposed explants and about a 25% decrease in adhesion to smoke-exposed explants (Fig. 8). Treatment with diethylmaleate as described in MATERIALS AND METHODS decreased GSH to undetectable levels but had no effect on fiber adhesion (Fig. 9).

Exposure of the explants at 4°C produced about a 25% decrease in binding with both air and smoke; smoke enhancement of binding was still present (Fig. 10).

DISCUSSION

In this study, we have attempted to set up a simple system of measuring surface binding of asbestos fibers. Our approach of defining a “bound” particle as one resistant to removal by washing may be criticized as arbitrary, but, as indicated in the introduction, there is no agreement in the literature on the question of how binding measurements should be made from a technical point of view, nor is there even agreement about what constitutes a bound fiber. Indeed, review of the various papers discussed in the introduction will reveal that all approaches to particle adhesion, which is in
itself a labile and manipulable phenomenon, are fairly arbitrary because, with harsh enough treatment, one can remove all fibers or particles from the cell surface. As is evident from Figs. 2–10, the approach used here does appear to produce quite reproducible results, and we found that increasing the number of washes to 8 or 10 did not produce appreciable changes in binding (data not shown). It should be appreciated, as well, that uptake levels in the first 24 h are very low with this system (8, 16), so that we do not have to make a correction for fibers that have entered the epithelial cells.

A further advantage of using tracheal explants is that the cultured cells maintain polarization and apical differentiation, features that are frequently lost in monolayer cultures and that appear to be important in determining binding and uptake (8). Thus, although tracheal explants do not provide a direct model of uptake in the alveoli, they do provide a realistic system for examining features related to uptake and to extracellular signaling mechanisms, and the system is directly relevant to the development of both fibrogenic and neoplastic pulmonary disease.

One of the facts established by our experiments is that binding, at least binding as defined here, is established quite rapidly because, after four washes, there is very little difference in the number of bound fibers in explants exposed to air plus dust for only 1 h and those exposed for 24 h, and this is also true (with slightly greater variability) of the smoke-exposed explants. Exposure to asbestos or to asbestos and smoke at 4°C had remarkably little effect on binding; smoke enhancement of binding was still present and binding in the presence of smoke or air was decreased by ~25% compared with a 37°C exposure. These observations imply that binding does not require active cellular metabolism or cytoskeletal rearrangement but might proceed via changes in lipid-based cell membrane receptors and/or chemistry because these processes are not dramatically slowed at 4°C.

Our data also confirm our hypothesis that smoke affects particle binding; there is a consistent difference in fiber adhesion between smoke-exposed and air-exposed tracheal segments, with greater adhesion after smoke exposure. Thus one way in which smoke increases particle uptake appears to be by increasing...
surface adhesion, making more particles available for eventual internalization. The mechanism behind the smoke effect is not entirely clear, but surface iron clearly plays an important role because boosting surface iron increased adhesion and chelating surface iron decreased adhesion with both air and smoke exposures. The results with smoke exposure were more marked, and, in fact, DFX treatment completely abolished the smoke effect. These observations may imply that surface iron is catalyzing the formation of AOS [or possibly reactive nitrogen species (7, 12)]. This idea is supported by the findings that mannitol, a hydroxyl radical scavenger, and catalase, a hydrogen peroxide scavenger, decreased adhesion. Boosting GSH levels also decreased adhesion, possibly by scavenging AOS, although it is unclear why decreasing cellular GSH did not have the opposite effect.

For the most part, the effects of modifying surface iron or scavenging AOS were greater with smoke than with air exposure, perhaps implying that surface iron plays its greatest role in the presence of exogenous AOS or reactive nitrogen species, which are also formed in smoke (7). However, these conclusions must be viewed with caution. A recent review by Gold et al. (12) on the chemical reactivity of chelator-treated amosite and...
crocidolite asbestos concluded that DFX diminishes the redox reactivity of surface iron on the fibers, but they could find no clear correlation among formation of hydroxyl radicals, reduction of exogenous hydrogen peroxide, and the nominal amount or oxidation state of surface iron. As well, it is obvious that AOS can only be a partial determinant of fiber binding because AOS scavengers and iron chelators never reduced binding below ~75% of the control values after air exposure. It should be appreciated, also, that our results apply only to amosite and probably crocidolite asbestos; whether smoke increases chrysotile asbestos binding is not known, but, if it does, both the positive surface charge and the small amount of iron in chrysotile suggest that a different mechanism would be involved.

Last, it is intriguing to consider the results recently reported by Jimenez et al. (15). These authors observed that DFX and catalase decreased ERK activation by crocidolite asbestos fibers and concluded that asbestos-mediated via oxidant species. Combining their results, appears to be a cell-surface effect, is probably mediated via oxidant species. Combining their results with ours raises the possibility that the level of asbestos-induced extracellular signaling may be more specifically a function of the number of fibers bound to the surface and that this function is determined by the generation of AOS or other oxidant species.

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