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Effects of Crocidolite and Chrysotile Asbestos on Cellular Uptake and Metabolism of Benzo(a)pyrene in Hamster Tracheal Epithelial Cells

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The incidence of bronchogenic carcinoma is increased substantially in asbestos workers who smoke. We used several approaches to determine possible mechanisms of synergism at the cellular level between asbestos and the polycyclic aromatic hydrocarbon (PAH), benzo(a)pyrene (BaP), a chemical carcinogen in cigarette smoke. Specifically, we hypothesized that cellular uptake and metabolism of BaP might be facilitated when the hydrocarbon was coated on asbestos. In addition, we were interested in whether asbestos, alone or in combination with BaP, caused single strand breakage of DNA in epithelial cells of the airway.

UICC reference samples of crocidolite and chrysotile were coated with ^{3}H-BaP before their addition to monolayers of hamster tracheal epithelial cells. In comparative studies, ^{3}H-BaP at identical amounts was added to cells in culture medium. At intervals thereafter, uptake of BaP by cells was documented by scintillation spectrometry and by autoradiography. In addition, cells and media were assayed by use of high pressure liquid chromatography (HPLC) to demonstrate the water-soluble metabolites of BaP. The integrity of DNA was monitored by alkaline elution at intervals after exposure of tracheal cells to various concentrations of asbestos, BaP and BaP-coated asbestos.

A rapid transfer of BaP to cells occurred after addition of BaP-coated asbestos to cultures. When BaP was adsorbed to both types of fibers before their addition to cultures, 70% of the total BaP introduced entered the cell within 1 hr; 50% remained intracellular after 8 hr. In contrast, if identical amounts of BaP were added directly to medium, an initial influx of 20% was observed and cells retained only 5% of the initial amount at 8 hr. Little or no alteration in metabolism of BaP was observed under these circumstances. Minimal single-strand breakage of DNA was observed after administration of BaP, but not after chrysotile or crocidolite. Moreover, BaP-coated asbestos did not cause breakage of DNA in excess of the amounts induced by BaP alone. Our results suggest that asbestos not only facilitates the transfer of PAH into cells but also increases the retention of hydrocarbons by the "target" cell of the respiratory tract. These processes might be intrinsic mechanisms of asbestos-induced (co)carcinogenesis.

Introduction

Asbestos workers who smoke have an 80- to 92-fold higher incidence of bronchogenic carcinoma than the nonsmoking general population (*1, 2*). In contrast, the nonsmoking asbestos worker has a substantially smaller risk (2- to 4-fold) of developing tumors than tobacco smokers with no occupational exposure (10-fold higher risk). A number of experimental studies support these epidemiologic observations. For example, after intratracheal instillation of asbestos into rodents, bronchogenic neoplasms are not observed unless PAH are adsorbed to the surfaces of fibers (*3-5*). Although PAH alone are carcinogenic, more tumors are induced when they are administered on a variety of fibers and particles (*6*).

Particulates in the atmosphere can serve as "condensation nuclei" for effluent PAH that are generated as products of combustion (*7*). After inhalation, particles could either increase the retention of PAH within the respiratory tract or elute the chemical carcinogens to "target" cells of the airways. Alternatively, since minerals such as asbestos are

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cytotoxic, they could alter the normal cellular metabolism of PAH. The studies described here are *in vitro* approaches to address these hypotheses.

Methods

Hamster Tracheal Epithelial Cells (HTE-B)

HTE-B is a nontumorigenic cell line isolated and cloned from neonatal hamster tracheal epithelium (*8*). The line retains the ability to synthesize and secrete glycoproteins characteristic of mucin (*9*). Cultures of HTE-B are maintained routinely in Ham's F12 medium supplemented with 10% calf serum, mycostatin (25 U/mL) and Garamycin (100 μg/mL, M.A. Bioproducts, Walkersville, MD). Passages 40-50 were used for these studies.

Adsorption of BaP to Asbestos

Labeled ^{3}H-BaP (specific activity 17 Ci/mmole, Amersham, Arlington Heights, IL) or unlabeled BaP (Aldrich Chemical Co., Milwaukee, WI) were suspended in acetone in a round-bottom flask containing various amounts of either crocidolite or chrysotile asbestos (both UICC reference samples). After evaporation of the solvent, BaP-coated asbestos was added to cells for various time periods in serumless Eagle's Minimal Essential Medium (GIBCO, Grand Island, NY). Before adsorption of BaP, organic contaminants were removed from asbestos by extraction with ethyl acetate in a Soxhlet apparatus.

Autoradiography

Crocidolite (10 μg/mL medium) coated with ^{3}H-BaP (100 μCi) was added to monolayers of HTE-B cells at 10 μg/mL, and cultures prepared for autoradiography after exposure for 15 min, 1 hr, 6 hr and 24 hr. Cells were fixed *in situ* in 2.5% glutaraldehyde, post-fixed in 0.1*M* osmium tetroxide and embedded in Epon by standard techniques (*10*). Sections (1 μm) were prepared by using a microtome and adhered to gelatin-coated slides. Slides were dipped in Kodak NTE-B emulsion (Rochester, NY) and developed after 1 week by methods reported previously (*11*). Sections were stained with toluidine blue.

Uptake and Metabolism of ^{3}H-BaP

At 1, 2, 4, and 8 hr after exposure of cells to ^{3}H-BaP, either solubilized in culture medium or coated on asbestos (ca. $2 \times 10^{-7}M$ BaP on 3 μg chrysotile or 10 μg crocidolite/mL medium), the cellular content of ^{3}H-BaP was measured by scintillation spectrometry. After repeated rinses in 0.9% NaCl to remove extracellular BaP, cells were removed from culture flasks using a rubber policeman, pelleted by centrifugation and solubilized in NCS (New England Nuclear, Cambridge, MA). Aliquots of NCS were counted in Aquasol (New England Nuclear, Boston, MA) in a Beckman scintillation counter. In addition, medium was analyzed to identify water-soluble metabolites of BaP. Aliquots (250 μL) were diluted with 750 μL of 0.2*M* sodium acetate buffer, pH 4.5, and extracted with chloroform/methanol (1:1, v/v) (*12*). The radioactivity associated with the chloroform and aqueous methanol phases was measured by scintillation counting. BaP metabolites in the culture medium also were analyzed by high pressure liquid chromatography (HPLC) on an Altex 5 μm Ultrasphere ODS column attached to a Varian Model 5000 instrument. Metabolites were eluted initially with a linear 0-100% methanol water gradient over 50 min and later with 100% methanol for 15 min at a flow rate of 1 mL/min. The radioactivity in 0.5 mL fractions was measured.

Alkaline Elution

To determine whether asbestos, alone or coated with BaP, caused single-strand breakage of DNA, HTE-B cells were plated at 5×10^5 cells/60 mm Petri dish and incubated for 48 hr in 0.1 μCi/mL ^{3}H-thymidine (43 Ci/mmole, Amersham, Arlington Heights, IL). After 48 hr, the radiolabeled medium was replaced with fresh medium, and BaP (10^{-5}, $10^{-6}M$), chrysotile (1-3 μg/mL medium), crocidolite (1-10 μg/mL medium) or BaP-coated chrysotile and crocidolite at identical amounts (1-10 μg/mL medium) were added for various time periods (i.e., 1-96 hr). These amounts of asbestos did not inhibit cell growth. BaP was solubilized in medium using DMSO (0.33% final concentration), and untreated cultures received DMSO alone.

Cells were harvested with a rubber policeman and aliquots analyzed by alkaline elution as reported previously (*13*). The flow rate in tetrapropylammonium hydroxide (pH 12.1) was adjusted to 3 mL/hr over a 24-hr period. The radioactivity of 3-mL fractions was then determined. Positive elution controls were obtained after 1 hr incubation of cells with methyl methanesulfonate (20 μg/mL) (*14*).

Results

Cellular Uptake and Metabolism of ^{3}H-BaP

Examination of ^{3}H-BaP-coated crocidolite in serumless medium before its addition to cultures showed association of the label with individual fibers (Fig. 1). In medium without serum, the ^{3}H-BaP remained associated with the asbestos indefinitely. In contrast, dissociation of ^{3}H-BaP from fibers occurred when fibers came in contact with cells, i.e.,

as early as 15 min after addition of radiolabeled asbestos to cultures (Fig. 2).

When introduced into cultures on asbestos, the cellular uptake and retention of ^{3}H-BaP was increased (Table 1). Approximately 70% of the total BaP added (ca. $2 \times 10^{-7}M$) was incorporated into cells at 1 hr, and 50% was retained after an 8-hr period. In contrast, if BaP was solubilized in medium, an initial influx of 20% was observed at 1 hr and only 5% remained at 8 hr.

Water-soluble metabolites of BaP, which include glucuronides, were almost undetectable at 1 hr, but increased to between 40 and 50% of the total amount of BaP applied by 8 hr. Organic-soluble metabolites were minimal at all times. Amounts of metabolites appearing after introduction of both types of BaP-coated asbestos were consistently but not markedly decreased (Table 1).

Single Strand Breakage of DNA

Addition of crocidolite and chrysotile at nontoxic concentrations over a range of time periods did not cause breakage of DNA as detected by the sensitive alkaline elution technique (Fig. 3). Negative results were obtained with use of both UICC crocidolite, which is known to contain small amounts of organic matter including PAH (*15*) and asbestos extracted with ethyl acetate to remove organic contaminants.

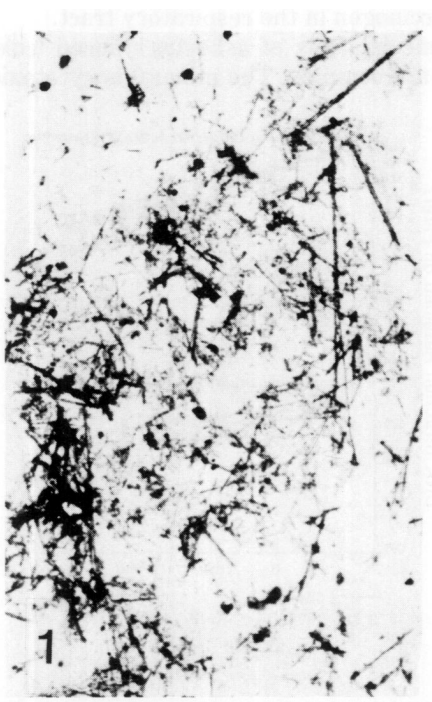

FIGURE 1. Autoradiograph of UICC crocidolite after adsorbance of ^{3}H-BaP. When suspended in serumless medium before addition to cultures, fibers are beaded with label. × 480.

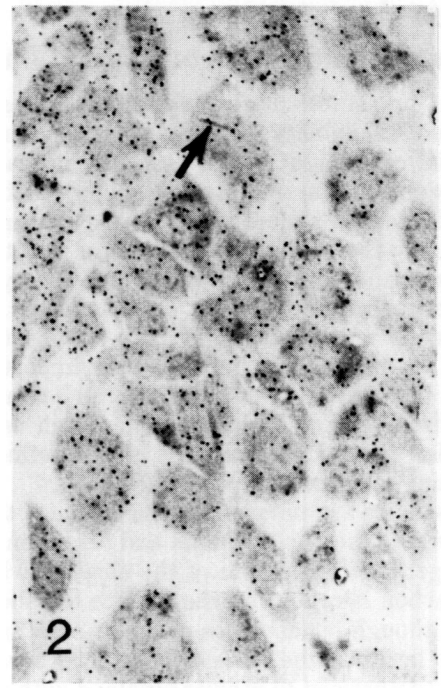

FIGURE 2. Autoradiograph of HTE-B cells 1 hr after exposure to ^{3}H-BaP-coated crocidolite. Note the concentration of ^{3}H-BaP in the cells. In contrast, ^{3}H-BaP has dissociated from the fibers (arrow). × 1275.

We showed recently a slight but consistent increase in the breakage of DNA when $10^{-5}M$ BaP, a concentration causing 50% inhibition of cell growth, was added to HTE-B (*14*). An increase in elution was observed between 2 and 8 hr after addition at BaP but was no longer detectable at 15 hr. No increase in breakage was observed when 2 to 3 × $10^{-6}M$ of BaP was used alone or adsorbed to asbestos (Fig. 4). These amounts of BaP were the maximum that could be coated on nontoxic amounts of asbestos.

Table 1. Uptake and retention of ^{3}H-benzo(a)pyrene (BaP) by tracheal epithelial cells.

	% of ^{3}H-BaP introduced[a]			
	1 hr	2 hr	4 hr	8 hr
Cellular content				
BaP alone	22[a]	18	6	5
BaP-coated crocidolite	68	68	48	42
BaP-coated chrysotile	70	70	50	38
Water-soluble metabolites in medium	2	12	38	50
BaP-coated crocidolite	1	3	25	40
BaP-coated chrysotile	1	2	23	38

[a]Percentage of total amounts of ^{3}H-benzo(a)pyrene introduced.

Discussion

Cellular Uptake and Metabolism of BaP

Data presented here suggest that asbestos-enhanced availability of BaP to tracheobronchial epithelial cells might be a significant mechanism of carcinogenesis. Our results are supported by studies of Lakowicz and colleagues who document by fluorimetry the increased transport of BaP to artificial membranes and microsomes when the hydrocarbon is adsorbed on a variety of particulates (*16-18*). Dispersions and particles of BaP do not transfer readily to membranes indicating an important, although undefined, role of the "carrier" mineral. In comparison to nonfibrous materials such as hematite, silica and carbon, asbestos is more effective in facilitating membrane uptake of BaP.

Our studies are unique because they are the first to show both increased uptake and retention of BaP by "target" epithelial cells of the airways when the hydrocarbon is coated on the surface of asbestos fibers. Although nonasbestos particles such as hematite also facilitate the initial cellular incorporation of BaP (in comparison to results obtained with solubilization of BaP in medium), their effect on retention of the hydrocarbon are not as striking as those observed with use of asbestos (Eastman, Mossman and Bresnick, unpublished).

In subsequent experiments, asbestos has been added 1 hr before ^{3}H-BaP to cultures of tracheal epithelial cells. Under these circumstances, increased uptake and retention of cellular BaP are not observed. This suggests that the fiber once inside the cell does not concentrate BaP. Because asbestos enhances both the rate of BaP uptake and its retention, the cells are effectively exposed to a much greater level of BaP for longer periods. This provides the potential for an enhanced interaction with DNA, the presumed target molecule for BaP-induced transformation.

Interaction of BaP and Asbestos with DNA

Asbestos inconsistently causes chromosomal aberrations in various cell types (*19, 20*) and is negative in the Ames test (*21*). The observation that many carcinogens cause DNA strand breakage and repair prompted us to examine the effects of both chrysotile and crocidolite, alone and coated with BaP, on breakage of DNA in tracheal epithelial cells. Our results show that neither asbestos nor BaP-coated asbestos cause alkali-sensitive lesions in DNA. These data suggest that the role of asbestos in bronchogenic carcinoma is epigenetic and is supported further by our studies with tracheal explants (*22, 23*). After implantation into syngeneic animals, carcinomas develop from tissues exposed to asbestos coated with PAH, whereas neoplasms fail to evolve from tissues exposed to asbestos alone. These observations support the concept of asbestos acting as a (co)carcinogen and not as a complete carcinogen in the respiratory tract.

The mechanisms of asbestos-induced (co)carcinogenesis are complex. The mineral is cytotoxic, caus-

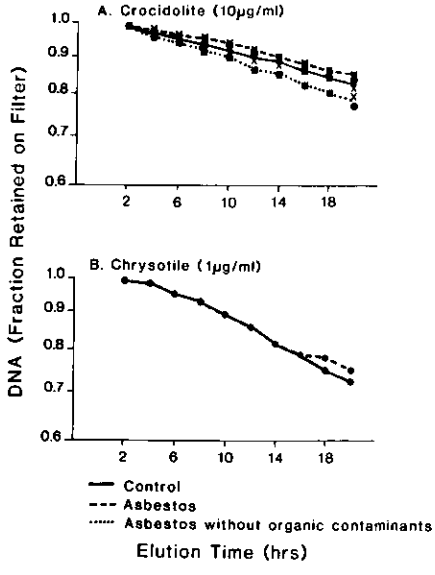

FIGURE 3. Alkaline elution of DNA after exposure of HTE-B cells for 24 hr to (*A*) crocidolite and (*B*) chrysotile at nontoxic amounts.

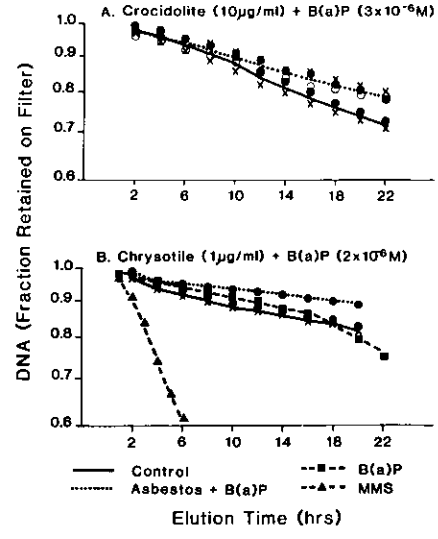

FIGURE 4. Alkaline elution of DNA after exposure of HTE-B cells for 2 hr to (*A*) BaP-coated crocidolite and (*B*) chrysotile. Methyl methanesulfonate (20 μg/mL for 1 hr) was used as a positive control.

ing proliferation of epithelial cells and squamous metaplasia, a lesion which appears to inhibit mucociliary clearance of asbestos (24). In addition, we document here the capacity of the mineral to adsorb PAH and facilitate their transport to and retention by epithelial cells of the airways.

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