11-1-1996

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Abstract

Mesothelial cells, the progenitor cell of the asbestos-induced tumor mesothelioma, are particularly sensitive to the toxic effects of asbestos, although the molecular mechanisms by which asbestos induces injury in mesothelial cells are not known. We asked whether asbestos induced apoptosis in mesothelial cells and whether reactive oxygen species were important. Pleural mesothelial cells (rabbit or human) were exposed to asbestos (crocidolite, amosite, or chrysotile) or control particles at moderate doses (1–10 $\mu g/cm^2$) over 24 h and evaluated for oligonucleosomal DNA fragmentation, loss of membrane phospholipid asymmetry, and nuclear condensation. Asbestos fibers, not control particles, induced apoptosis in mesothelial cells by all assays and induction of apoptosis was dose dependent for all types of asbestos, with crocidolite (5 $\mu g/cm^2$) inducing 15.0±1.1% (mean±SE; n = 12) apoptosis versus control particles < 4%. Apoptosis induced by asbestos, but not by actinomycin D, was inhibited by extracellular catalase, superoxide dismutase in the presence of catalase, hypoxia (8% oxygen), deferoxamine, 3-aminobenzamide [an inhibitor of poly(ADP-ribosyl) polymerase], and cytochalasin B. Only catalase and cytochalasin B decreased fiber uptake. We conclude that asbestos induces apoptosis in mesothelial cells via reactive oxygen species. Escape from this pathway could allow the abnormal survival of mesothelial cells with asbestos-induced mutations. (*J. Clin. Invest.* 1996. 98:2050–2059.) Key words: oxygen radicals • annexin V • flow cytometry • deferoxamine • internalization

Introduction

Asbestos fibers produce neoplasms, inflammation, and fibrosis of the lung and pleura, although the molecular mechanisms by which asbestos induces these biologic effects have not been established (1). Asbestos is particularly toxic to mesothelial cells, the progenitor of the asbestos-induced tumor mesothelioma (2). In in vitro studies with mesothelial cells, asbestos leads to inhibition of growth (3), disruption of mitosis (4), induction of DNA and chromosomal damage (2), and disruption of the cell membrane consistent with necrotic cell death (5). However, we observed that rabbit pleural mesothelial cells exposed to crocidolite asbestos become small and shrunken (6), features more consistent with apoptosis than cellular necrosis. Unlike necrosis, apoptosis is an active process under genetic control (7). Apoptosis has been identified as important for the elimination of injured cells such as those injured by viruses, irradiation, or oxygen radicals (7). The loss of normal apoptotic responses has been implicated in oncogenesis because cells with DNA damage that would normally die can survive as one step in the multistep process leading to neoplastic transformation (8, 9). Because of the importance of apoptosis in the regulation of cell populations and the deletion of damaged cells, we asked whether asbestos fibers induce apoptosis of pleural mesothelial cells.

Of the diverse stimuli shown to induce apoptosis, reactive oxygen species may play a central role; not only do they induce apoptosis when directly added to cells, but they may be a common pathway for action of many seemingly unrelated stimuli (10, 11). One of the ways that reactive oxygen species may initiate apoptosis is via their damage to DNA. In some models, DNA strand breaks may induce apoptosis by the intermediate activation of a nuclear enzyme associated with DNA repair, poly(ADP-ribosyl) polymerase. Reactive oxygen species are known to be produced by asbestos fibers, either when alone or via ingestion by phagocytic cells (12), although it is not agreed whether asbestos-induced reactive oxygen species lead to mesothelial cell injury (13–15). Even in studies in which antioxidant enzymes have protected mesothelial cells, the role of reactive oxygen species in that injury is still in doubt because antioxidant enzymes may also interfere with fiber uptake (16, 17). Thus, it is unclear whether asbestos-induced reactive oxygen species injure mesothelial cells and would therefore play a role in mesothelial cell apoptosis.

We therefore asked whether asbestos induces apoptosis in mesothelial cells and, if so, if this induction of apoptosis is mediated by reactive oxygen species independent of effects on fiber uptake. For these studies, we focused on crocidolite asbestos because it is the type of fiber most associated with the mesothelial-derived tumor, mesothelioma (1). First, apoptosis was identified by analysis of DNA fragmentation. Then, apoptosis was quantified by analysis of the loss of membrane phospholipid asymmetry using annexin V binding and by morphologic evaluation of acridine orange–stained, condensed nuclei. Finally, the role of reactive oxygen species was evaluated by exposure of cells to asbestos in the presence of antioxidant enzymes, in a hypoxic environment, with chelation of iron by de-

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Received for publication 9 April 1996 and accepted in revised form 19 August 1996.

The Journal of Clinical Investigation
Volume 98, Number 9, November 1996, 2050–2059

feroxamine and with an inhibitor of poly(ADP-ribosyl) polymerase. Fiber uptake was quantified by microscopic inspection by dark field of mesothelial cells treated with trypsin to remove adherent fibers (6).

Methods

Reagents. The two major types of asbestos were used: amphibole (crocidolite and amosite) and serpentine (chrysotile). Crocidolite asbestos (National Institute of Health and Safety, Research Triangle Park, NC), amosite asbestos (gift of Dr. A. Churg, University of British Columbia, Vancouver, British Columbia), and chrysotile asbestos (gift of Dr. B. Mossman, University of Vermont, Burlington, VT) were used at comparable numbers of fibers per square centimeter. The control fiber, wollastonite, a relatively nonpathogenic calcium silicate fiber (Nyglos I; NYCO Minerals, Willsboro, NY), was also used at comparable fiber counts. The control particles, riebeckite (gift of Dr. B. Mossman), a nonfibrous particle of mineral content identical to crocidolite asbestos, and glass beads (mean diameter 1.6±0.3 μm; Duke Scientific Corp., Palo Alto, CA) were used at comparable weight.

Catalase (bovine liver, thymol-free; 0.048 μg/U), SOD (bovine erythrocytes; 0.24 μg/U), xanthine oxidase, purine, 3-aminobenzamide (3-ABA),[1] 3-aminobenzoic acid (3-ABOA), BSA (fraction V), acridine orange, propidium iodide, and cytochalasin B were obtained from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide was from Fisher Scientific (Pittsburgh, PA). Deferoxamine mesylate USP (desferrioxamine B) was obtained from CIBA (Summit, NJ). Actinomycin D was obtained from Merck and Co. (West Point, PA). Catalase and SOD were dissolved in PBS fresh for each experiment. Enzyme activity was confirmed by the ability of catalase to degrade hydrogen peroxide, as measured in an assay of horseradish peroxidase–dependent oxidation of phenol red (18), and the ability of SOD to remove the superoxide generated by the mixture of xanthine oxidase (0.2 U/ml) and purine (0.5 mM) and measured by the reduction of ferricytochrome *c* (18). Catalase was inactivated by boiling for 1 h, and SOD by boiling for 3 h; inactivity was confirmed by the above assays before use.

Cell culture. Rabbit mesothelial cells were harvested as described previously (19) and human mesothelial cells were harvested from samples of parietal pleura obtained at thoracotomy of patients with benign lung lesions, in accordance with the UCSF Human Research Committee. Rabbit cells were grown in standard media: RPMI 1640/DMEM, Hepes (10 mM), 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) and studied in experimental media: standard media without fetal calf serum. Human cells were grown and studied in LHC-MM medium (Biofluids, Inc., Rockville, MD), L-Glutamax II (2 mM, Gibco Laboratories, Grand Island, NY), and penicillin/streptomycin. In addition to standard tests as described (19), cells were confirmed to be mesothelial by positive nuclear staining for the Wilms' tumor antigen (20) (anti-WT ascites HC17 provided by Dr. Steve Albelda and Dr. Ulrich Rodeck, The Wistar Institute, Philadelphia, PA). For all experiments, cells were used between passages 2 and 6.

Asbestos preparation. Fibers and control particles were sonicated (power 60 W for 5 s) (450 sonifier; Branson Ultrasonics Corp., Danbury, CT) in cell culture medium and then added to the cells at the desired surface concentration. For some experiments, crocidolite fibers were incubated overnight with catalase (5,000 U/ml; 240 μg/ml), SOD (600 U/ml; 144 μg/ml), or BSA at comparable protein concentrations in experimental media, washed, and sonicated before addition to cell monolayers. For experiments on the role of iron, crocidolite fibers were incubated overnight with freshly prepared deferoxamine (5 mM) in 50 mM NaCl (Chelex-100–treated; Bio-Rad, Hercules, CA) or in Chelex-treated NaCl alone, washed in Chelex-treated saline, and sonicated before addition to cell monolayers. Experiments using deferoxamine-treated fibers were performed in RPMI, an iron-free medium, and in the dark to minimize reduction of iron (21).

Proliferation assays. For analysis of proliferation, mesothelial cells (5,000 cells/well) were seeded onto laminin-coated (10 μg/ml) 96-well flat-bottom microtiter plates (Fisher Scientific), incubated overnight in standard media, and then exposed to fibers in triplicate wells in experimental media. At 18 h, [^{3}H]thymidine (1 μCi/well) (Amersham Corp., Arlington Heights, IL) was added for the final 6 h of incubation. Free floating cells were combined with adherent cells detached with trypsin (0.25%, wt/vol) and EDTA (0.5 mM), counted by hemocytometer, and transferred to glass fiber filters using a PHD cell harvester (Cambridge Technology Inc., Cambridge, MA). Cells were fixed with ethanol (100%) followed by TCA precipitation (7%, vol/vol), and radioactivity was measured with a scintillation counter (Beckman Instruments, Palo Alto, CA).

General experimental procedure. Mesothelial cells were plated onto mouse laminin-coated dishes (10 μg/ml) (Gibco Laboratories) and allowed to adhere overnight in standard media. 1 h before the experiment, the cells were incubated in experimental media containing various blockers (e.g., catalase, SOD, 3-ABA) or their negative controls (e.g., inactive catalase, inactive SOD, 3-ABOA). After the 1-h incubation, fibers or other particles were added directly to the media for an additional incubation, usually of 24 h. In all experiments unless otherwise stated, after exposure to fibers, floating cells were collected and combined with adherent cells detached with trypsin (0.25%) and EDTA (0.5 mM) before processing for the following assays.

Analysis of DNA fragmentation. DNA fragmentation into nucleosomal bands was detected by agarose gel electrophoresis with slight modifications (22). After exposure to experimental conditions, mesothelial cells were collected, washed twice with ice-cold PBS and the pellet containing 1–4 × 10^6 cells was resuspended in 500 μl of lysis buffer (Tris 500 mM, pH 9.0, EDTA 2 mM, NaCl 10 mM, SDS 1% wt/vol, proteinase K [1 mg/ml, Boehringer Mannheim, Indianapolis, IN]) and incubated at 48°C for 48 h. DNA was extracted with phenol/chloroform and precipitated with ethanol. The centrifuged pellets were air-dried, dissolved in TE buffer, and incubated with bovine pancreatic RNase (5 μg/ml) (Boehringer Mannheim) at room temperature for 1–2 h. DNA (20–25 μg) was loaded onto an agarose gel (1.5%, wt/vol), electrophoresed at 1.5 V/cm, stained with ethidium bromide (0.5 μg/ml, Gibco Laboratories), and photographed under ultraviolet light.

Annexin V staining. Entry into apoptosis has been shown to be associated with exposure of phosphatidylserine on the outer leaflet of the plasma membrane (23), a process that can be detected by the binding of annexin V, a member of a family of proteins that binds to acidic phospholipids (24). For detection of exposed phosphatidylserine in subpopulations of cells, cells were incubated with FITC-labeled annexin V prepared as described (25) and analyzed using flow cytometry (26). After exposure to fibers or particles, mesothelial cells were collected and centrifuged (1,500 rpm, 10 min). The cell pellet was washed and resuspended in Hepes buffer (Hanks', 15 mM Hepes, 2 mM $CaCl_2$), stained with FITC-labeled annexin V (3 μg/ml in Hepes buffer) for 10 min on ice, and washed. Propidium iodide (15 μg/ml, Sigma Chemical Co.) was added just before analysis using a FACSort® flow cytometer (Becton Dickinson, San Jose, CA), with acquisition and data analysis performed using CELLQuest Software (Becton Dickinson). 10,000 events per sample were acquired to ensure adequate mean data. The specificity of annexin V staining was determined by a lack of binding in calcium-free buffer, reversibility of binding after addition of EDTA (5 mM), and lack of binding in face of an excess of unlabeled annexin V (40 μg/ml).

Morphological analysis of apoptosis. For quantification of apoptosis by morphologic criteria, cells were stained with both acridine orange and propidium iodide, in a modification of a standard assay (27).

1. *Abbreviations used in this paper:* 3-ABA, 3-aminobenzamide; 3-ABOA, 3-aminobenzoic acid.

Apoptotic cells are characterized by a highly condensed nucleus that stains vividly with DNA dyes; in early apoptosis, acridine orange enters the cell but propidium iodide is excluded and the nucleus is stained green; in late apoptosis with loss of membrane integrity, both dyes enter the cell and the nucleus is stained orange-red. After exposure to fibers for 24 h, mesothelial cells were collected, centrifuged, and washed with PBS before staining with acridine orange (10 μg/ml) and propidium iodide (25 μg/ml) for 4 min. Cells were then washed in PBS, fixed in glutaraldehyde (2.5%, vol/vol; Sigma Chemical Co.) for 30 min in the dark, and pipetted on glass slides. Dual stained cells were viewed using a fluorescence microscope equipped to detect each probe separately, as described (28). At least 200 cells in each of duplicate wells were analyzed by an observer blinded to the experimental condition and were coded as either early apoptotic (bright green, highly condensed chromatin), late apoptotic (bright orange, highly condensed chromatin), or necrotic cells (bright orange nucleus without condensed chromatin) (27). Data are expressed as the total number of apoptotic cells (early plus late) as a percentage of the total.

Analysis of apoptosis at different oxygen tensions. Mesothelial cells, grown in plastic Falcon flasks, were exposed to asbestos fibers in hypoxic or normoxic conditions. The flasks were filled completely with experimental media and bubbled either with 100% nitrogen (0% oxygen) to deplete oxygen or with air (21% oxygen) as a control. After media were aspirated for analysis of oxygen content, the flasks were capped and placed in an airtight glass jar which was also flushed at high flows for 10 min with either nitrogen or air. After 24 h, a sample of media was again analyzed, and the mesothelial cells were collected as above and stained for apoptotic morphology. Over the 24 h, the average P_{O2} of the normoxic media was 165±5 mmHg (21.6% oxygen), while that of the hypoxic media was 61 mmHg (8% oxygen). There were no differences between the two groups in average P_{CO2} (21±5 mmHg) or pH (7.65±0.08).

Fiber counting. An assay of fiber uptake over 24 h was modified from an assay described by us for 2-h studies in which incubation of mesothelial cells with trypsin/EDTA releases adherent fibers and gives internalized fiber counts matching those by confocal microscopy (6). Cells with more than four fibers per cell seen on dark field were counted and used as an assay for fiber uptake. Mesothelial cells exposed to asbestos fibers for 24 h were processed as above for apoptotic morphology, with the exception that free floating as well as adherent cells were exposed to trypsin (0.25%, wt/vol) and EDTA (0.5 mM) for 5 min at room temperature. After analysis by fluorescence microscopy for apoptosis, the cells were examined in a blinded fashion under dark-field microscopy.

Statistical analysis. Data are expressed as mean±1 SEM, unless indicated. Statistical differences among groups were determined by one-way ANOVA with Tukey's test to discriminate where the difference lay (29). $P < 0.05$ was accepted as representing a significant difference.

Results

Crocidolite asbestos, at concentrations of 3 and 5 μg/cm^2, was shown to be toxic to rabbit pleural mesothelial cells by inhibition of thymidine uptake over 24 h (Fig. 1 *A*). At the same concentrations, crocidolite asbestos decreased cell numbers when compared with no asbestos and with a control particle, wollastonite (Fig. 1 *B*).

Crocidolite asbestos, but not control particles, induced oli-

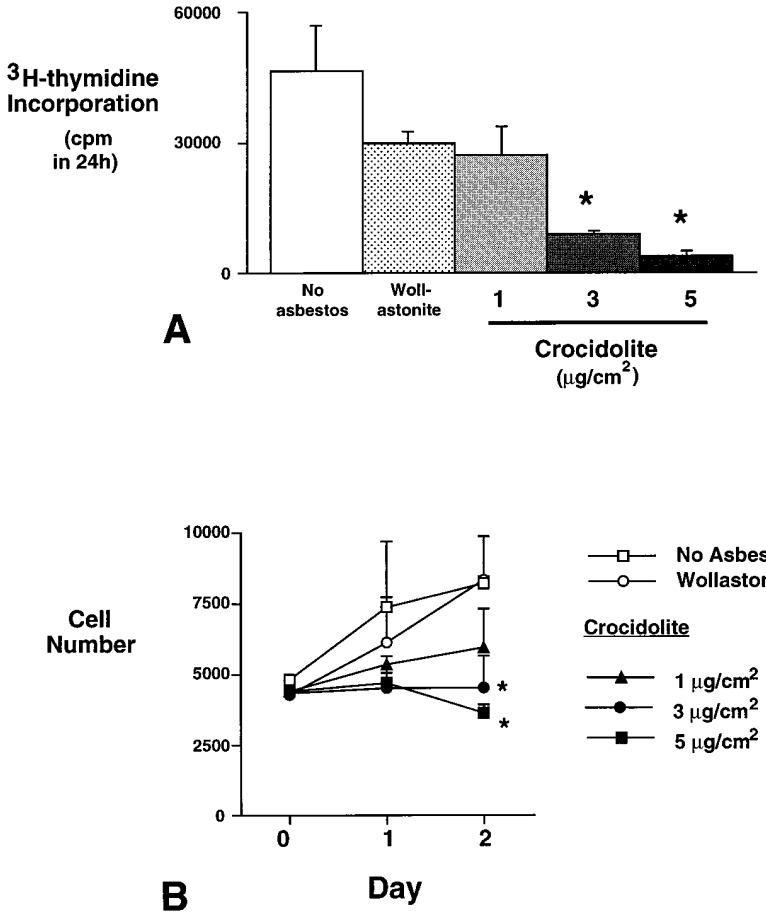

Figure 1. Crocidolite asbestos inhibits proliferation of rabbit pleural mesothelial cells. Mesothelial cells were incubated with crocidolite asbestos, wollastonite (10 μg/cm^2), or experimental media (serum-free). (*A*) Thymidine uptake at 24 h. After 18 h of a 24-h incubation, [^{3}H]thymidine was added to cells for 6 h. Cells (free floating and adherent) were harvested. Incorporation of thymidine was determined by precipitating ^{3}H-labeled DNA on glass fiber filters followed by liquid scintillation counting. (*B*) Cell numbers of asbestos-exposed mesothelial cells. Cells (free floating and adherent) were exposed to fibers or experimental media alone for 24 or 48 h, harvested, and counted by hemacytometer. Data are means±SE; *different from wollastonite, $P = 0.03$, $n = 3$ experiments.

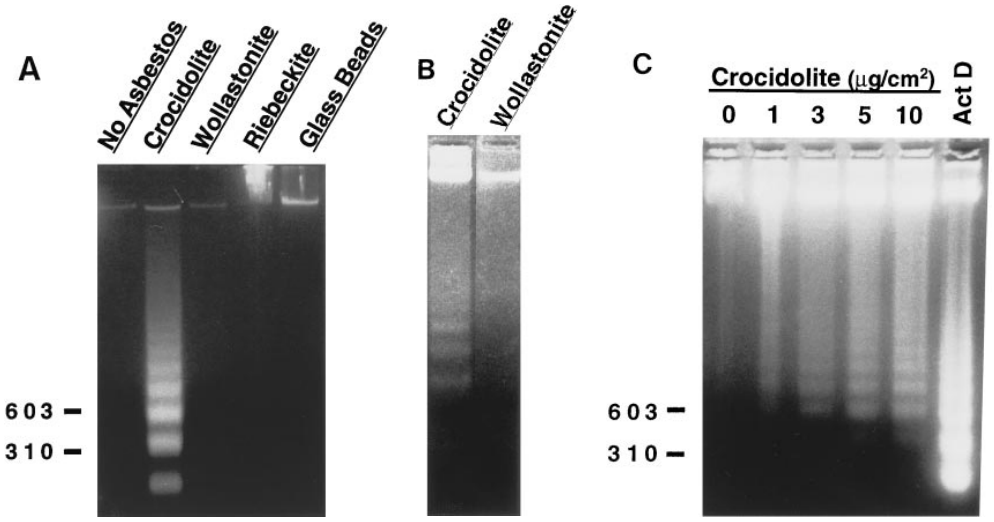

Figure 2. Crocidolite asbestos, not control fibers or particles, induces oligonucleosomal DNA fragmentation in mesothelial cells in a dose-dependent fashion. After exposures of 24 h, pleural mesothelial cells (free floating and adherent) were harvested for isolation of DNA. DNA was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. (*A*) Rabbit mesothelial cells were exposed to medium alone, crocidolite (3 μg/cm^2), wollastonite (6 μg/cm^2), riebeckite (3 μg/cm^2), or glass beads (3 μg/cm^2). (*B*) Human mesothelial cells were exposed to crocidolite (10 μg/cm^2) or to wollastonite (20 μg/cm^2). (*C*) Rabbit mesothelial cells were exposed to experimental medium alone, crocidolite asbestos at increasing concentrations, or actinomycin D (0.3 μM). Molecular weight markers indicated in base pairs.

gonucleosomal DNA fragmentation characteristic of apoptosis in rabbit (Fig. 2 *A*) and human mesothelial cells (Fig. 2 *B*) at 24 h. Oligonucleosomal DNA fragmentation was seen in a dose-dependent fashion in response to asbestos (Fig. 2 *C*). DNA fragmentation was not evident after 2 h of exposure to asbestos but was evident by 6 h (data not shown). Actinomycin D caused extensive apoptosis of rabbit pleural mesothelial cells and was used as a positive control.

Crocidolite asbestos induced annexin V binding to rabbit (Fig. 3) and human mesothelial cells that was evident by 6 h after exposure. At 24 h, asbestos-exposed mesothelial cells underwent significantly more early apoptosis (annexin V positive; propidium iodide negative) than cells exposed to either no asbestos, wollastonite, or riebeckite (Table I). At 24 h, of the cells exposed to asbestos, the percentage of cells with early apoptosis was significantly greater than the percentage with either necrosis or late apoptosis (propidium iodide positive; Table I). When sorted, counterstained with acridine orange, and examined using fluorescent microscopy, annexin V–positive cells were confirmed to have condensed nuclei characteristic of apoptosis.

Asbestos, but not control particles, induced apoptotic mor-

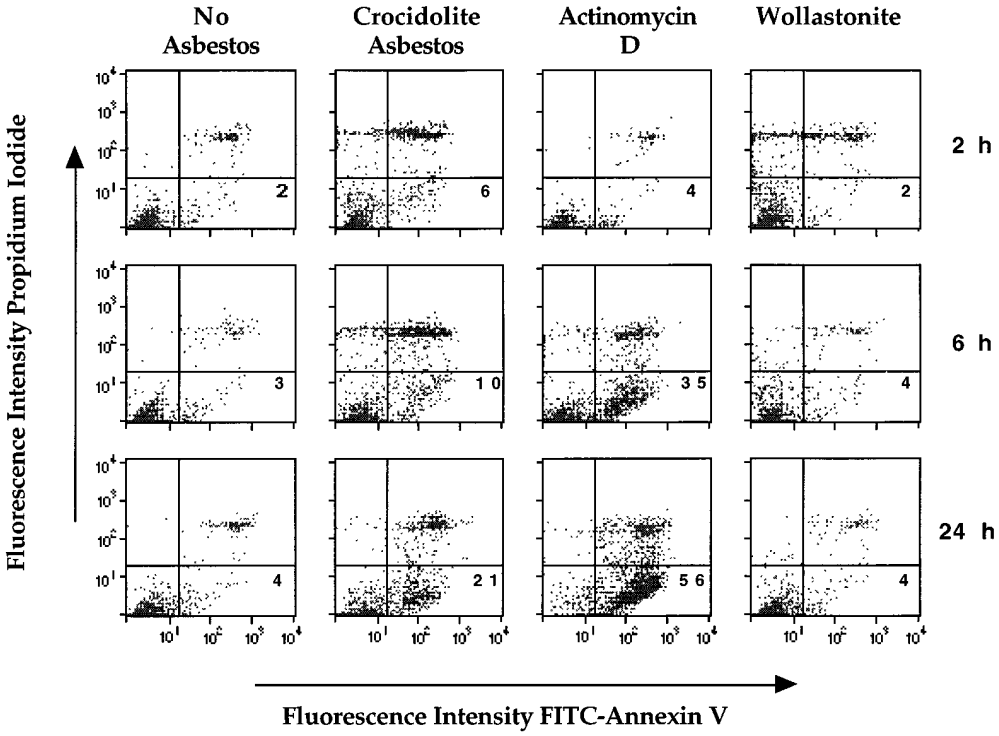

Figure 3. Crocidolite asbestos induces early apoptosis in mesothelial cells as detected by binding with FITC-annexin V. After exposure to experimental media, crocidolite (3 μg/cm^2), actinomycin D (0.3 μM), or wollastonite (6 μg/cm^2) for 2, 6, or 24 h, rabbit pleural mesothelial cells were harvested and stained with FITC-annexin V and propidium iodide for analysis by flow cytometry. Normal cells are in the left lower quadrant, early apoptotic in the right lower quadrant, late apoptotic/necrotic in the right upper quadrant, and necrotic in the left upper quadrant. The percentage of gated cells in early apoptosis (annexin V positive, propidium iodide negative) in this representative experiment is indicated.

phology in a dose-dependent fashion in rabbit pleural mesothelial cells (Fig. 4 and 5), as well as in human pleural mesothelial cells. In the human mesothelial cells, crocidolite asbestos caused 10.8±1.4% apoptosis (at 5 μg/cm^2) and 17.5±3.9% (at 10 μg/cm^2) while wollastonite caused 1.9±0.6% (at 10 μg/cm^2) ($P < 0.02$ each different from each other, $n = 9$). At equivalent fiber concentrations, crocidolite asbestos induced more apoptosis than did chrysotile asbestos ($P < 0.05$) (Fig. 5). Of the cells that were apoptotic, > 95% were in the early apoptotic stage (condensed, green nuclei). Of the total number of cells, < 5% were necrotic (not condensed, red nuclei). The dose response was evident in either the total cell population or the adherent population but, in the free-floating cells, apoptosis was not different over a range of concentrations of crocidolite between 1 and 10 μg/cm^2 (28.0±3.5% apoptosis; $n = 3$). Asbestos-induced apoptosis was significantly greater than that of controls whether in serum-free conditions (Fig. 5) or in 10% serum (11.1±2.2% crocidolite [5 μg/cm^2], 2.5±1.2% wollastonite [10 μg/cm^2]; $P < 0.03$, $n = 3$). Actinomycin D (0.3 μM) induced rabbit mesothelial cells to undergo extensive apoptosis (71.0±4.2% at 24 h, $n = 9$). Hydrogen peroxide (30–40 μM for 24 h) induced apoptosis (20.3±2.4% of cells); at higher concentrations, cells died by necrosis.

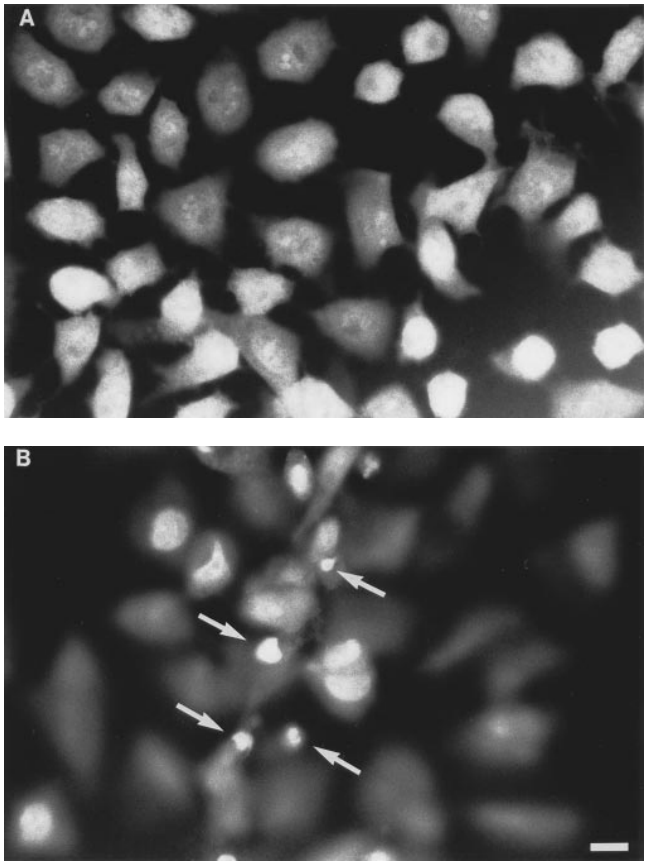

Figure 4. Mesothelial cells exposed to crocidolite asbestos develop an apoptotic morphology. After exposure to medium alone or crocidolite asbestos (5 μg/cm^2) for 24 h, adherent rabbit pleural mesothelial cells were stained with acridine orange and propidium iodide and photographed using fluorescence microscopy. (*A*) Cells exposed to medium alone exhibit normal morphology. (*B*) Crocidolite-exposed cells exhibit features typical of apoptosis including dense nuclear condensation and cell shrinkage (*arrows*). Bar, 5 μm.

Table I. Percentage of Early Apoptosis vs. Late Apoptosis/ Necrosis in Asbestos-exposed Pleural Mesothelial Cells at 24 h as Determined by Annexin V Binding

Concentration (μg/cm^2)	Early apoptosis (annexin V +, propidium iodide −) (% of total gated cells)	Necrosis or late apoptosis (annexin V +, propidium iodide +) (% of total gated cells)
Rabbit pleural cells		
Crocidolite 5	25.0±6.0*	18.0±4.3‡
Wollastonite 10	5.7±0.9	5.3±1.2
Riebeckite 5	3.5±1.5	5.0±2.0
Glass beads 5	4.5±1.5	3.0±1.0
No asbestos	3.1±0.5	4.8±1.1
Actinomycin D	53.8±8.0*	24.5±3.6‡
Human pleural cells		
Crocidolite 5	18.3±3.0*	10.5±3.7‡
Wollastonite 10	7.5±1.5	6.8±2.0
No asbestos	4.6±0.6	5.0±1.9

Cells were exposed to fibers, particles, or actinomycin D (0.3 μM) for 24 h before harvesting both free floating and adherent cells for labeling with FITC-labeled annexin V and propidium iodide. Labeled cells were analyzed by flow cytometry and gated to exclude signals with low forward scatter. ‡Different from control fibers and particles; *different from control fibers and the propidium iodide positive group, $P < 0.03$. Data represent mean±SE from three experiments, with 10,000 cells analyzed in each condition.

Apoptosis induced by asbestos, but not by actinomycin D, was significantly inhibited by incubation of cells with antioxidant enzymes. When used alone, catalase (10,000 U/ml) inhibited apoptosis by 59% (Fig. 6) ($P < 0.04$). There was no effect either of BSA at a comparable concentration (480 μg/ml) (Fig. 6) or of inactive catalase (data not shown). SOD alone had no effect on apoptosis, however, when added in the presence of catalase, SOD, but not inactive SOD, had an additive inhibitory effect (Fig. 6). Catalase alone and together with SOD also inhibited apoptosis induced by amosite and chrysotile asbestos, but not by actinomycin D. Increasing the preincubation time increased the effectiveness of catalase but not of SOD; at a preincubation time of 3 h, catalase (5,000 U/ml) now significantly decreased asbestos-induced apoptosis (by 59%, to 7.0±0.1%), and catalase (10,000 U/ml) decreased apoptosis by 95% (to 4±0.9%). A coating of antioxidant enzymes on the fibers did not inhibit apoptosis (14.2±2.3% apoptosis, crocidolite 5 μg/cm^2 coated with catalase, SOD or catalase plus SOD; 13.5±3% apoptosis, crocidolite coated with no protein or BSA).

A hypoxic environment inhibited crocidolite asbestos-induced apoptosis (Fig. 7). Incubating crocidolite or chrysotile fibers overnight with deferoxamine (5 mM) to inactivate iron-catalyzed oxygen radical production also significantly decreased asbestos-induced apoptosis (saline-coated crocidolite [5 μg/cm^2], 13.8±0.9% apoptosis; deferoxamine-coated crocidolite, 5.3±1.2%; $P < 0.01$, $n = 3$; saline-coated chrysotile [10 μg/cm^2], 9.6±1.1%; deferoxamine-coated chrysotile, 4.1±0.5%; $P < 0.02$, $n = 6$). This inhibition was observed only in iron-free medium (RPMI) and was eliminated by addition of iron ($FeCl_3$ 5 mM) or by the use of iron-containing media (data not shown). Incubation of the mesothelial cells in deferoxamine (0.1–5 mM) overnight before addition of fibers had no effect

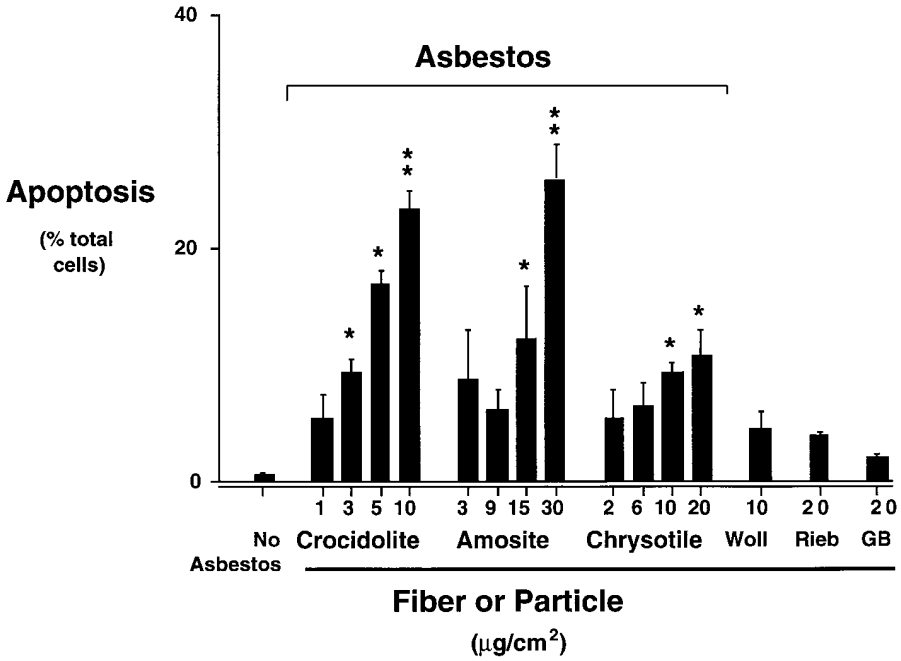

Figure 5. Asbestos induces apoptosis of mesothelial cells in a dose-dependent fashion. Rabbit mesothelial cells were exposed to media containing asbestos fibers including crocidolite, amosite, or chrysotile at comparable numbers of fibers or to experimental media, wollastonite (*Woll*), riebeckite (*Rieb*), or glass beads (*GB*). Cells were stained with acridine orange and propidium iodide and examined using fluorescence microscopy for the percentage of cells with apoptotic nuclei. Data are means±SE; *different from apoptosis due to wollastonite, **different from apoptosis at lower fiber concentrations; $P = 0.03$, $n = 5$ experiments, 200 cells counted per condition in each experiment.

on asbestos-induced apoptosis. An inhibitor of a poly(ADP-ribosyl) polymerase, 3-ABA, significantly inhibited asbestos-induced apoptosis compared with a structural analogue with no blocking activity, 3-ABOA (30) (Fig. 8). In addition, cytochalasin B (5 μg/ml) inhibited apoptosis by 84% (crocidolite [5 μg/cm^2], 14.8±1.2% apoptotic cells; crocidolite plus cytochalasin B, 5.7±0.9%; cytochalasin B alone, 4.0±1.0% apoptosis; $P < 0.01$, $n = 3$). For chrysotile asbestos, 3-ABA and cytochalasin B were also effective inhibitors of apoptosis (data not shown). Neither 3-ABA nor cytochalasin B inhibited actinomycin D–induced apoptosis.

Extracellular catalase, but not BSA or a coating with catalase, decreased fiber uptake (Table II). However, other measures, such as SOD (in the presence of catalase), hypoxia, deferoxamine, and 3-ABA (at 2.5 mM) inhibited apoptosis without any effect on fiber uptake (Table II). As expected, cytochalasin B significantly inhibited fiber uptake.

Discussion

In this study, we have identified apoptosis as a new and potentially important mechanism by which pleural mesothelial cells respond to asbestos. Under these experimental conditions, apoptosis developed in a clear dose response relationship to all types of asbestos but was more effectively induced by crocidolite, the fiber most closely associated with asbestos-induced pleural disease, than by chrysotile. Apoptosis involved more mesothelial cells than did necrosis, and the appearance of apop-

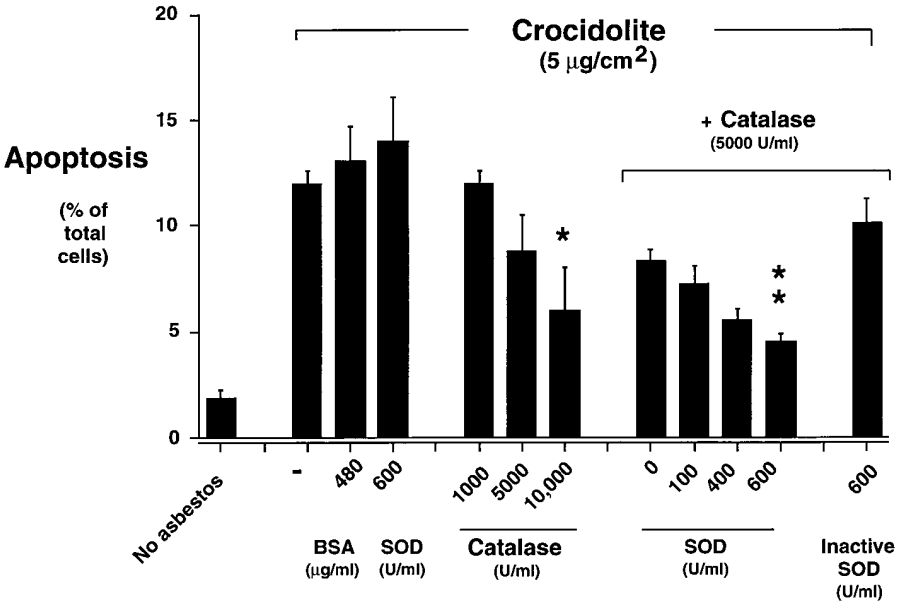

Figure 6. Catalase and SOD (in the presence of catalase), but not SOD alone, inhibit crocidolite-induced apoptosis in mesothelial cells. After rabbit pleural mesothelial cells were incubated for 1 h with catalase, SOD alone, SOD in the presence of catalase (5,000 U/ml), or various controls, crocidolite asbestos (5 μg/cm^2) was added for an additional 24 h. Cells were harvested, stained with acridine orange and propidium iodide, and examined for the percentage of cells with apoptotic nuclei using fluorescence microscopy. Data are means±SE; *different from asbestos alone or asbestos plus BSA, **different from catalase (5,000 U/ml) without SOD or with inactive SOD; $P = 0.03$, $n = 5$ experiments; 200 cells counted per condition in each experiment.

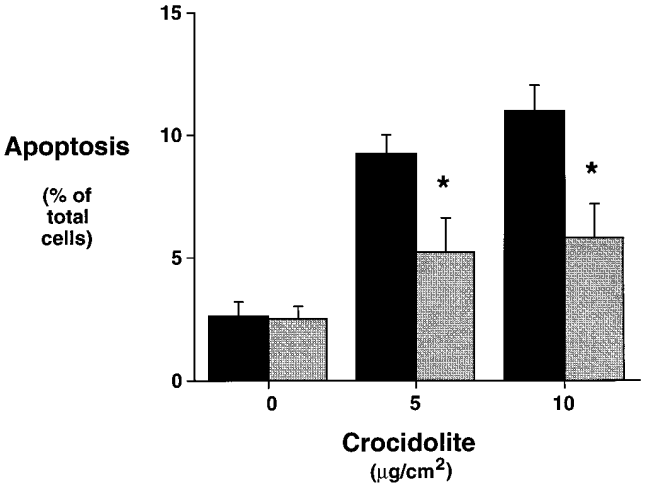

Figure 7. Moderate hypoxia inhibits asbestos-induced apoptosis in rabbit mesothelial cells. Mesothelial cells were exposed to crocidolite asbestos (5 or 10 μg/cm^2) or media alone in the presence of normoxia (21% oxygen; *black bars*) or hypoxia (8% oxygen; *shaded bars*). After 24 h, cells were stained using acridine orange and propidium iodide and examined for the percentage of apoptotic cells using fluorescence microscopy. Data are means±SE; *different from apoptosis in cells exposed to an equivalent concentration of asbestos under normoxic conditions; $P = 0.03$, $n = 3$ experiments; 200 cells counted per condition in each experiment.

tosis correlated with the appearance of the asbestos-induced inhibition in cell proliferation and decrease in cell numbers. In addition, we found that the apoptotic response to asbestos was dependent on reactive oxygen species and poly(ADP-ribosyl) polymerase. These mechanisms were independent of fiber uptake, an additional important factor in asbestos-induced apoptosis as shown by the specific inhibitory effect of cytochalasin. Understanding the role of apoptosis may provide new insights into the interaction of asbestos and its target cell, the mesothelial cell.

Asbestos has been shown to have a myriad of effects in cultured cells, including mesothelial cells, such as induction of gene expression (31), production of growth factors and cytokines (19), inhibition of growth (16), induction of damage to chromosomes (2, 32) and DNA (16), transformation (33), and necrosis (5). We now report a novel asbestos-induced effect, apoptosis, a process that may not have been recognized previously because of its inapparent nature. Cells dying by apoptosis become small, shrunken, and, if in the early stage of apoptosis or if phagocytosed by neighboring cells, never release cellular contents such as lactate dehydrogenase to the extracellular environment. By two different quantitative assays, apoptosis involved a large percentage of cells (between 15 and 25% of cells exposed to crocidolite 5 μg/cm^2), which exceeded the percentage of cells undergoing necrosis. Apoptosis would thus have been a large contributor to the decrease in cell number observed in asbestos-exposed mesothelial cells. Apoptosis may be an important response of mesothelial cells and one, we believe, that should be considered in future studies of asbestos pathogenesis.

Active oxygen species played a key role in the induction of asbestos-induced apoptosis in mesothelial cells. The extracellular antioxidant enzymes, catalase and SOD in the presence of catalase, but not inactivated enzymes or BSA, inhibited almost all asbestos-induced apoptosis. It is possible that the enzymes, particularly catalase, may have penetrated intracellularly, as has been shown recently for vascular smooth muscle cells (34). The increased effectiveness of the longer incubation times is consistent with a time-dependent movement of enzyme intracellularly. However, it is more likely that the antioxidant enzymes functioned extracellularly, perhaps adjacent to fibers that have been internalized while still enveloped in a cellular membrane (6). Of course, the interpretation of the catalase effect is confounded by its inhibition of fiber uptake. Therefore, it was important to show by other means that reactive oxygen species mediated asbestos-induced apoptosis independent of fiber uptake. In the moderately hypoxic environment of 8% oxygen, asbestos-mediated apoptosis was reduced

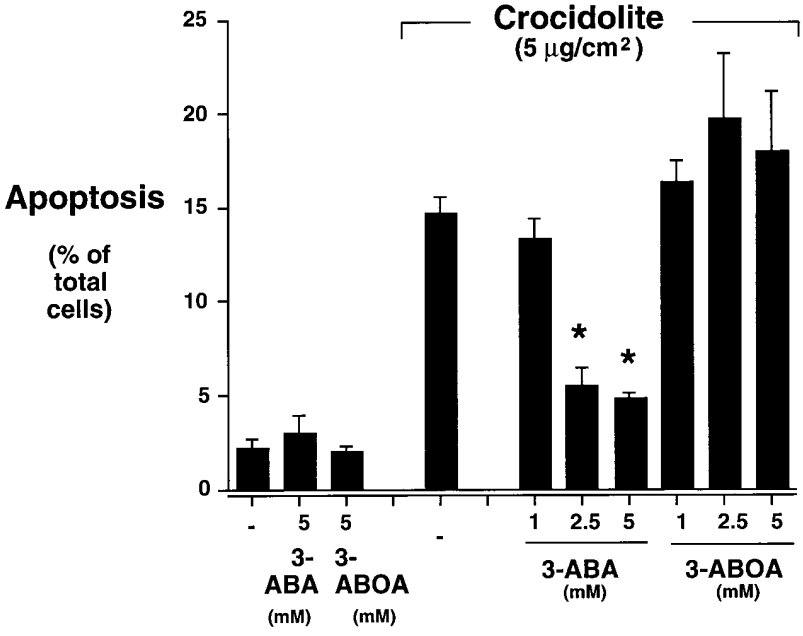

Figure 8. Inhibition of poly(ADP-ribosyl) polymerase by 3-ABA inhibits crocidolite-induced apoptosis of rabbit pleural mesothelial cells. Mesothelial cells were incubated for 1 h with either 3-ABA, an inhibitor of poly(ADP-ribosyl) polymerase, or 3-ABOA, an inactive structural analogue, before addition of either crocidolite (5 μg/cm^2) or media for an additional 24 h. Mesothelial cells were harvested, stained with acridine orange and propidium iodide, and examined for the percentage of apoptotic cells using fluorescence microscopy. Data are means±SE; *different from cells exposed to crocidolite in the presence of 3-ABOA; $P = 0.03$, $n = 3$ experiments; 200 cells counted per condition in each experiment.

Table II. The Effect of Experimental Conditions on Fiber Internalization

Condition	Cells with > 4 fibers per cell (% of total cells)
Crocidolite ($\mu g/cm^2$)	
1	16.7±9.2
3	50.4±6.8
5	68.4±3.8
10	74.6±3.0
10 at 4°C	3.0±1.5
Crocidolite (5 $\mu g/cm^2$) plus	
Catalase 500 U/ml	67.0±3.8
Catalase 1,000 U/ml	70.5±4.2
Catalase 5,000 U/ml	55.3±3.2
Catalase 10,000 U/ml	46.7±4.8*
BSA 480 μg/ml	65.5±4.5
Catalase 5,000 U/ml × 1 h	63.0±0
Catalase 5,000 U/ml × 2 h	65.5±1.5
Catalase 5,000 U/ml × 3 h	52.5±6.5*
SOD 600 U/ml	62.7±12.9
Catalase 5,000 U/ml + SOD 600 U/ml	61.0±3.9
Normoxia	62.5±3.5
Hypoxia	59.7±3.5
Deferoxamine-coated (5 mM)	62.7±2.5
Saline-coated	60.4±3.1
3-ABA 1.0 mM	70.0±5.2
3-ABA 2.5 mM	59.3±6.9
3-ABA 5.0 mM	52.5±6.8*
Cytochalasin B 2.5 μg/ml	19.0±2.0*

Rabbit pleural mesothelial cells were exposed to crocidolite asbestos for 24 h. If blockers were used, they were added to the cells 1 h before the addition of fibers (unless otherwise stated). Both free floating and adherent cells were exposed to trypsin/EDTA to remove adherent fibers, and fixed and examined under dark field for the percentage of cells with more than four fibers per cell. Deferoxamine-coated and saline-coated asbestos were studied in RPMI. *Different from percentage of cells with > 4 intracellular fibers at crocidolite 5 $\mu g/cm^2$, $P < 0.05$. Data represent mean±SE from three experiments, 200 cells counted per condition.

by 60% with no effect on fiber uptake. This level of oxygen has been associated with lower levels of reactive oxygen species (35) and has been shown to inhibit certain types of oxygen-dependent apoptosis (36). The action of oxygen species in mesothelial cell apoptosis was specific to asbestos because antioxidant enzymes had no effect on apoptosis induced by actinomycin D (37). Deferoxamine coating, known to decrease hydroxyl radical production from asbestos (38), also decreased apoptosis significantly with no effect on fiber uptake. Of interest, iron chelation by deferoxamine coating was effective for asbestos types that contained either a high percentage of iron (crocidolite, 27% iron) or a low percentage of iron (chrysotile, 0.7% iron) (38), suggesting that even a small amount of iron on a fiber is sufficient to induce significant toxicity. Efforts to chelate iron intracellularly had no effect on asbestos-induced apoptosis, identifying the fiber as the origin of the reactive iron. Reactive oxygen species have been shown to mediate toxicity from asbestos in many in vitro and in vivo models (12, 16, 39) although their role in injury to mesothelial cells has been unclear (13–15). In these studies, we have identified a novel and important biologic effect from asbestos-dependent reactive oxygen species on mesothelial cells.

There may be more than one reactive oxygen species involved in the production of apoptosis in these studies. Because of the inhibition by deferoxamine, one important reactive oxygen species is likely to be the hydroxyl radical, a highly reactive product of the interaction of oxygen and the iron on the surface of asbestos. In ambient oxygen, the reduced iron, Fe (II), can initially catalyze the production of superoxide radical which can then dismute to hydrogen peroxide (38). Then, the hydrogen peroxide can be metabolized to hydroxyl radical by the participation of Fe (II) in the Fenton reaction. Our data support this sequence of events because either removal of hydrogen peroxide by catalase or the chelation of the iron by deferoxamine inhibits the asbestos-induced injury. However, SOD alone did not inhibit apoptosis, perhaps because superoxide is dismuted by SOD to hydrogen peroxide, which could continue to form hydroxyl radical. However, in the presence of catalase which could detoxify the hydrogen peroxide, SOD did have an additional effect suggesting an independent role for superoxide, as has been shown in other models of apoptosis (40, 41).

The inhibition of poly(ADP-ribosyl) polymerase inhibited asbestos-induced apoptosis. This nuclear enzyme, which is activated by DNA strand breaks and uses cellular NAD in a possible repair function, has been implicated as a central mediator of cellular injury in response to oxidants (42). As a sensor of DNA injury, it may function to signal apoptosis in the face of extensive DNA damage, presumably by depleting NAD (43). To inhibit poly(ADP-ribosyl) polymerase, we used 3-ABA at low concentrations (1, 2.5 mM) reported to have minimal effects on cell metabolism (44) and to inhibit the enzyme specifically (45), and which did not alter fiber uptake. The 3-ABA, but not a closely related structural analogue 3-ABOA, significantly reduced the apoptosis due to asbestos (30). The reduction of apoptosis by inhibition of this enzyme supports a role for asbestos-induced DNA damage in mediating apoptosis. Indeed, asbestos has been shown to induce DNA strand breaks as early as 2 h (46) and unscheduled DNA synthesis within 24 h (46a). Asbestos has also been shown previously to induce production of poly(ADP-ribosyl) polymerase (47) and blockade of the enzyme has been protective in some studies of asbestos-induced injury (48–50). Thus, our results implicate the poly(ADP-ribosyl) polymerase as a link between the DNA damage known to result from asbestos to the finding in this study of asbestos-induced apoptosis.

Because the internalization of fibers may be important for asbestos toxicity (6, 51), it was necessary to evaluate our interventions for their effect on fiber uptake. Once internalized, for example, asbestos may induce injury by interfering mechanically with the mitotic spindle or allowing generation of reactive oxygen species closer to the nucleus. Interestingly, catalase, commonly used to identify a role of reactive oxygen species in asbestos studies, was shown to inhibit fiber uptake. The effect of catalase may have been nonspecific (16), although we found that neither the addition of BSA, a similar sized and charged protein, nor a coating of catalase on the fibers inhibited fiber uptake. Alternatively, the effect of catalase may have been specific, as has been reported for epithelial cell uptake of asbestos (17). Nonetheless, the finding was troublesome and required the use of additional antioxidant experiments, using hypoxia and deferoxamine, to show that inhibition of apoptosis

was due to a reduction in oxygen species and not due indirectly to an inhibition of fiber uptake. An independent effect of fiber uptake is best shown by the inhibition of asbestos-induced apoptosis by cytochalasin B, an inhibitor of actin assembly that decreases fiber uptake (6) without interfering with the detection of apoptosis via nuclear condensation (52) or the apoptosis induced by actinomycin D. Therefore, both the generation of reactive oxygen species and the internalization of fibers are shown to be important for asbestos-induced apoptosis.

In conclusion, we have shown that a significant percentage of pleural mesothelial cells undergo apoptosis after exposure to asbestos, a clinically relevant stimulus. Our studies identify reactive oxygen species and fiber uptake as important mechanisms in mediating this response. We speculate that apoptosis represents a mechanism by which mesothelial cells possessing DNA damaged by asbestos are deleted. If so, escape from the normal apoptotic pathway may be one important step in the multistep process leading to the development of asbestos-induced neoplasia.

Note added in proof. During the final review of this manuscript, it was reported that crocidolite asbestos can induce apoptosis in rat pleural mesothelial cells (53).

Acknowledgments

We acknowledge Robert Ertsey, Dr. Bob Cameron, and Dr. David Jablons for their assistance.

This work was supported in part by National Institutes of Health grants RO1 ESO6331 (to V.C. Broaddus) and PPG HL24075 (to L.M. Scavo), by American Heart Association-California Affiliate grant 93-209 (to J.D. Ernst), and by National Institute of Environmental Health Sciences Clinical Investigator Award KO8 ES00253 and an American Lung Association Research Grant (to A.M. Boylan).

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