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Crocidolite Asbestos Induces Apoptosis of Pleural Mesothelial Cells: Role of Reactive Oxygen Species and Poly(ADP-ribo-syl) Polymerase

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Mesothelial cells, the progenitor cells 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. Rabbit pleural mesothelial cells were exposed to crocidolite asbestos or control particles (1–10 μg/cm2) over 24 hr 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. Induction of apoptosis was dose dependent; crocidolite (5 μg/cm2) induced apoptosis (15.0 ± 1.1%, mean ± SE; n = 12) versus control particles (<4%), as measured by appearance of nuclear condensation. 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, and 3-aminobenzamide (an inhibitor of the nuclear enzyme, poly(adenosine diphosphate-ribo-syl) polymerase). We conclude that asbestos induces apoptosis in mesothelial cells via reactive oxygen species. We speculate that escape from this pathway could allow the abnormal survival of mesothelial cells with asbestos-induced mutations. — Environ Health Perspect 105(Suppl 5):1147–1152 (1997)

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 that are more consistent with apoptosis than cellular necrosis. Unlike necrosis, apoptosis is an active process under genetic control (7). Apoptosis is 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 that 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 poly (adenosine diphosphate [ADP]-ribo-syl) polymerase, a nuclear enzyme associated with DNA repair. Reactive oxygen species are produced by asbestos fibers, either alone or via ingestion by phagocytic cells (12), although it is not agreed whether asbestos-induced reactive oxygen species lead to mesothelial cell injury (13–17). Thus, it is unclear whether asbestos-induced reactive oxygen species 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. For these studies, we focused on crocidolite asbestos, the type of fiber most associated with the mesothelial-derived tumor mesothelioma (1). First, apoptosis was identified by analysis of DNA fragmentation. Apoptosis was then 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 deferoxamine, and with an inhibitor of poly (ADP-ribo-syl) polymerase.

Materials and Methods

Reagents

Crocidolite asbestos (National Institute of Health and Safety, Research Triangle Park, NC) and the control fiber wollastonite, a
relatively nonpathogenic calcium silicate fiber (Nyglo I, NYCO Minerals, Willbоро, NY), were used at comparable fiber counts. The control particles riebeckite, a non-fibrous particle of mineral content identical to crocidolite asbestos (B Mossman, University of Vermont, Burlington VT), and glass beads (mean diameter 1.6 ± 0.3 μm) (Duke Scientific, Palo Alto, CA) were used at comparable weight.

Catalase (bovine liver, thromb-free; 0.048 μg/U), superoxide dismutase ([SOD] bovine erythrocytes; 0.24 μg/U), xanthine oxidase, purine, 3-aminobenazamide (3-ABA), 3-aminobenzoic acid (3-ABOA), bovine serum albumin (IBSA fraction V), acridine orange, and propidium iodide were obtained from Sigma Chemical (St. Louis, MO). Hydrogen peroxide was from Fisher Scientific (Pittsburgh, PA). Deroxamine mesylate USP (deferoxamine B) was obtained from Ciba (Summit, NJ). Actinomycin D (Act D) was obtained from Merck (West Point, PA). Catalase and SOD were freshly dissolved in phosphate-buffered saline (PBS) 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 μM/ml) and purine (0.5 mM) and measured by the reduction of ferricytochrome C (18). Catalase was inactivated by boiling for 1 hr, and SOD by boiling for 3 hr; inactivity was confirmed by the above assays prior to use.

Cell Culture
Rabbit mesothelial cells were harvested as previously described (19). Rabbit cells were grown in standard media: iron-free medium (RPMI) 1640/Dulbecco's modified Eagle medium, HEPES (10 mM), 10% heat-inactivated fetal calf serum (Hyclone Laboratories, Logan, UT), t-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml), and studied in experimental media (standard media without fetal calf serum).

Asbestos Preparation
Fibers and control particles that had been autoclaved and found to be free of endotoxin by Limulus assay (Whittaker Bioproducts, Walkersville, MD) were sonicated (60 W for 5 sec) (Branson 450 sonifier, Danbury, CT) in cell culture medium, then added to cells at the desired surface concentration. For experiments on the role of iron, crocidolite fibers were incubated overnight with freshly prepared deferoxamin (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 prior to 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 (20).

General Experimental Procedure
Mesothelial cells were plated onto mouse laminin-coated dishes (10 μg/ml) (Gibco, Gaithersburg, MD) and allowed to adhere overnight in standard media. One hour prior to 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 hr incubation, fibers or other particles were added directly to the media for an additional incubation, usually 24 hr. 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 (21). After exposure to experimental conditions, mesothelial cells were collected, washed twice with ice-cold PBS, and the pellet containing 1 × 10⁶ viable cells was resuspended in 500 μl of lysis buffer (Tris 500 mM, pH 9.0; EDTA 2 mM; NaCl 10 mM, sodium dodecyl sulfate 1% w/v; proteinase K 1 mg/ml (Boehringer Mannheim, Indianapolis, IN)) and incubated at 48°C for 48 hr. DNA was extracted with phenol/chloroform and precipitated with ethanol. The centrifuged pellets were air dried, dissolved in Tris EDTA buffer (pH 8.0), and incubated with bovine pancreatic RNase (5 μg/ml) (Boehringer Mannheim) at room temperature for 1 to 2 hr. DNA (20–25 μg) was loaded onto an agarose gel (1.5% w/v), electrophoresed at 1.5 V/cm, stained with ethidium bromide (0.5 μg/ml, Gibco) and photographed under ultraviolet light.

Annexin V Staining
Entry into apoptosis is associated with exposure of phosphatidylserine on the outer leaflet of the plasma membrane (22), a process that can be detected by the binding of annexin V, a member of a family of proteins that bind to acidic phospholipids (23). For detection of exposed phosphatidylserine in subpopulations of cells, cells were incubated with fluorescein isothiocyanate (FITC)-labeled annexin V prepared as described (24), and analyzed using flow cytometry (25). After exposure to fibers or particles, mesothelial cells were collected and centrifuged (1500 rpm, 10 min). The cell pellet was washed and resuspended in HEPES buffer (Hank's, 15 mM HEPES, 2 mM CaCl₂), 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) was added just prior to analysis using a FACSort flow cytometer (Becton Dickinson, San Jose, CA), with acquisition and data analysis performed using CELLQuest Software (Becton Dickinson). Ten thousand 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 5 mM EDTA, 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 (26). 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 hr, 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% v/v, Sigma) 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 by Zimmerli et al. (27). At least 200 cells in each of duplicate wells were analyzed by an observer blinded to the experimental condition, then coded as either early apoptotic (bright green,
Analysis of Apoptosis at Different Oxygen Tensions
Mesothelial cells grown in plastic Falcon flasks (25 cm², phenolic cap; Corning, Corning, NY) 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, then placed in an airtight glass jar and flushed for 10 min with either nitrogen or air. After 24 hr, the mesothelial cells exposed to asbestos were collected as above and stained for apoptotic morphology. Over 24 hr, the average partial pressure of oxygen of the normoxic media was 165 ± 5 mm Hg (21.6% oxygen), while that of the hypoxic media was 61 mm Hg (8% oxygen). There were no differences between the two groups in average partial pressure of carbon dioxide (21 ± 5 mm Hg) or pH (7.65 ± 0.8).

Statistical Analysis
Data are expressed as mean ± 1 SEM, unless indicated. Statistical differences among groups were determined by one-way analysis of variance with Tukey’s test to discriminate where the differences were (28). A p value < 0.05 represented a significant difference.

Results
Crocidolite asbestos, but not control particles, induced oligonucleosomal DNA fragmentation characteristic of apoptosis in mesothelial cells at 24 hr (Figure 1A). Oligonucleosomal DNA fragmentation was seen in a dose-dependent fashion in response to asbestos (Figure 1B). Act D caused extensive apoptosis of rabbit pleural mesothelial cells and was used as a positive control.

Crocidolite asbestos induced annexin V binding to mesothelial cells that was evident by 6 hr after exposure. At 24 hr, asbestos-exposed mesothelial cells underwent significantly more early apoptosis (annexin V, positive; propidium iodide, negative) than cells not exposed to asbestos, wollastonite, or riebeckite (Table 1). At 24 hr, 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 1). When sorted, counterstained with acridine orange, and examined using fluorescent microscopy, annexin V positive cells had condensed nucleic characteristic of apoptosis.

Asbestos, but not control particles, induced apoptotic morphology in a dose-dependent fashion in rabbit pleural mesothelial cells (Figure 2). Act D (0.3 µM) induced rabbit mesothelial cells to undergo extensive apoptosis (71.0 ± 4.2% at 24 hr, n = 9). Hydrogen peroxide (30–40 µM for 24 hr) induced apoptosis (20.5 ± 2.4% of cells). At higher concentrations cells died by necrosis.

Apoptosis induced by asbestos, but not by Act D, was significantly inhibited by incubation of cells with antioxidant enzymes. When used alone, catalase (10,000 U/ml) inhibited apoptosis by 59% (Figure 3) (p < 0.04). There was no effect either of BSA at a comparable concentration (480 µg/ml) (Figure 3) or of inactive catalase (data not shown). SOD alone had no effect on apoptosis; however, when added in the presence of catalase, SOD had an additive inhibitory effect (Figure 3). When inactive SOD was added in the presence of catalase, there was no additional inhibition of apoptosis (Figure 3).

Table 1. Percentage of early apoptosis versus late apoptosis/necrosis in asbestos-exposed pleural mesothelial cells at 24 hr as determined by annexin V binding.

| Rabbit pleural cells | Concentration, µg/cm² | Early apoptosis | Necrosis or late apoptosis (annexin V+, propidium iodide+) |
|----------------------|-----------------------|-----------------|---------------------------------------------------------|
|                      |                       | annexin V+, total gated cells, % | annexin V+, total gated 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        | 50                    | 53.2 ± 8.0**    | 24.5 ± 3.6**                                           |

*0.3 µM. Cells were exposed to fibers, particles, or actinomycin D for 24 hr 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; 10,000 cells analyzed in each condition.
A hypoxic environment inhibited crocidolite asbestos-induced apoptosis (Figure 4). Incubation of crocidolite fibers overnight with deferoxamine (5 mM) to inactivate iron-catalyzed oxygen radical production also significantly decreased asbestos-induced apoptosis (saline-coated crocidolite [5 µg/cm²], 13.8 ± 0.9% apoptosis; deferoxamine-coated crocidolite, 5.3 ± 1.2%; p < 0.01, n = 3). This inhibition was observed only in RPMI and was eliminated by addition of iron (FeCl₃, 5 mM) or by the use of iron-containing media (data not shown). 3-ABA, an inhibitor of a poly(ADP-ribosyl) polymerase, significantly inhibited asbestos-induced apoptosis compared to 3-ABOA, a structural analogue with no blocking activity (29) (Figure 5). 3-ABA did not inhibit Act D-induced apoptosis.

Discussion

Asbestos has a myriad of effects in cultured cells, including mesothelial cells, such as induction of gene expression (30), production of growth factors and cytokines (19), inhibition of growth (16), induction of damage to chromosomes (2,31) and DNA (16), transformation (32), and necrosis (5). In this and in other recent reports (33,34), apoptosis has been identified as a new and potentially important mechanism by which pleural mesothelial cells respond to asbestos. We suspect that apoptosis 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, we found that apoptosis involved a large percentage of cells (15–25%) of cells exposed to crocidolite 5 µg/cm²), which exceeded the percentage of cells undergoing necrosis. Apoptosis may be an important response of mesothelial cells and one that should be considered in future studies of asbestos pathogenesis.

In these studies active oxygen species played a key role in the induction of asbestos-induced apoptosis of 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. Although it is possible that the catalase may have penetrated intracellularly, as was recently shown for vascular smooth muscle cells (35), it is more likely that the antioxidant enzymes functioned extracellularly, perhaps adjacent to fibers that were internalized while still enveloped in a cellular membrane (6). In the moderately hypoxic environment of 8% oxygen, asbestos-mediated apoptosis was reduced by 60%. This level of oxygen has been associated with lower levels of reactive oxygen species (36) and inhibits certain types of oxygen-dependent apoptosis (37). The action of oxygen species in mesothelial cell apoptosis was specific to asbestos because antioxidant enzymes had no effect on apoptosis induced by Act D (38). Deferoxamine coating, known to decrease hydroxyl radical production from asbestos (39), also decreased apoptosis significantly. Reactive oxygen species mediate toxicity from asbestos in many in vitro and in vivo models (12,16,40), although their role in injury to mesothelial cells is unclear (13–15). In these studies, we identified a novel and important biologic effect from asbestos-dependent reactive oxygen species on mesothelial cells.

Inhibition of poly(ADP-ribosyl) polymerase inhibited asbestos-induced apoptosis. This nuclear enzyme, which is activated by DNA strand breaks and utilizes cellular nicotinamide adenine dinucleotide (NAD) in a possible repair function, has been implicated as a central mediator of cellular injury in response to oxidants (41). As a sensor of DNA injury, it may function to signal apoptosis in the face of extensive...
DNA damage, presumably by depleting NAD (42). 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 (43) and to inhibit the enzyme specifically (44) and
which did not alter fiber uptake. 3-ABA, but not a closely related structural analogue
3-ABOA, significantly reduced apoptosis due to asbestos (29). The reduction of
apoptosis by inhibition of this enzyme supports a role for asbestos-induced DNA
damage in mediating apoptosis. Indeed, asbestos induces DNA strand breaks as
early as 2 hr after exposure (45) and induces unscheduled DNA synthesis within
24 hr (46). Asbestos also induces production of poly(ADP-ribosyl) polymerase (46)
and blocks the enzyme that has been protective in some studies of asbestos-
induced injury (47-49). Thus, our results implicate poly(ADP-ribosyl) polymerase as a
link between the DNA damage that results from asbestos to 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 the poly(ADP-ribosyl) polymerase enzyme
as important mediators of this response. We speculate that apoptosis represents a mechana-
ism by which mesothelial cells possessing DNA damaged by asbestos are deleted. If
so, escape from the normal apoptotic pathway may be an important step in the mul-
tistep process leading to the development of asbestos-induced neoplasia.

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