Mesothelial cell apoptosis is confirmed in vivo by morphological change in cytokeratin distribution

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Although apoptosis or programmed cell death can be easily detected in vitro by a multitude of assays, in vivo detection, quantification and identification of the cells undergoing apoptosis are more difficult. Detection and quantification of apoptotic parenchymal cells in vivo may be difficult because the cells of interest constitute a small percentage of the total cell population, may be diluted by infiltrating inflammatory cells that themselves undergo apoptosis, and, if they cannot be separated from the tissue, must be analyzed within a three-dimensional tissue architecture. In addition, quantification can be underestimated because the apoptotic cells of interest are rapidly ingested by phagocytes (13). Identification of cells undergoing apoptosis may be difficult as well because, as a consequence of apoptosis, cells will change their appearance, may become detached from their usual location, and may lose their identifying biomarkers. In the lung, several studies have shown the existence of apoptosis in lung parenchymal cells either in normal developmental (10, 31) or pathological states (2, 20, 30). In most of these studies, the identity of the cell was presumed because of its location in the tissue. No study has identified apoptosis of the pleural mesothelial cell in vivo, perhaps because the cell has few unique biomarkers and exists as a thin surface layer that is difficult to study morphologically.

Many in vitro studies (3, 9, 22, 26) have identified mesothelial cell apoptosis, in particular after exposure to asbestos fibers. Asbestos fibers are known to induce many pleural diseases, particularly the tumor mesothelioma (29). The mesothelial cell is likely to be a primary target of asbestos fibers, whether by direct action of the fiber or by the indirect action of other asbestos-exposed cells. In in vitro studies, mesothelial cells appear to be highly sensitive to the toxic effects of asbestos, perhaps more so than are other cells (18, 24). Asbestos is phagocytosed, and then via oxidant or mechanical means, the fibers induce DNA and chromosomal damage (17, 24, 37). Apoptosis of the mesothelial cells may be related to this asbestos-induced genotoxic or oxidant damage (7, 9).

A major interest in mesothelial cell apoptosis lies in understanding the role of asbestos-induced injury in the production of malignancy (7). Apoptosis, a genetically controlled means of cell death, may be important for the deletion of unwanted or abnormal cells and may thus protect against malignancy. In fact, defects in apoptosis may be a necessary precondition for the development of malignancy (15, 36). Also, mesothelial cell apoptosis could alter the response of the pleura to other toxic insults, including pleurodesis agents.

Therefore, we asked if mesothelial cells in vivo undergo apoptosis in response to known in vitro apoptotic stimuli such as crocidolite asbestos and actinomycin D plus murine tumor necrosis factor-α (mTNF-α). To study apoptosis in vivo, we chose to use cytokeratin as a means of identifying the apoptotic cells as mesothelial. We discovered that apoptotic mesothelial cells developed a striking morphological change in their cytokeratin distribution. We then aimed to confirm in vitro that this morphological change was associated with apoptosis. We then studied pleural cells and pleural tissue for evidence of apoptosis in mesothelial cells stained for cytokeratin.

**METHODS**

Reagents. Crocidolite asbestos (National Institute of Health and Safety, Research Triangle Park, NC) has been described

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previously (11) and has a mean length of 19 µm and a width of 0.25 µm. The other agents instilled in the pleural space were RPMI 1640 phenol red-free medium (RPMI; Gibco BRL, Life Technologies, Grand Island, NY); wollastonite, a control fiber (Nyglas I, NYCO Minerals, Willboro, NY); actinomycin D (Merck, West Point, PA) or mTNF-α (R&D Systems, Minneapolis, MN). Antibodies used for anti-cytokeratin staining were, for mouse cells and tissues, monoclonal rabbit anti-cow cytokeratin (wide-spectrum screening; Dako, Carpinteria, CA) and goat anti-rabbit FITC-conjugated antibody (Zymed, San Francisco, CA) and, for rabbit and human cells, mouse anti-human AE1/AE3 (Dako) and sheep anti-mouse FITC-conjugated antibody (Sigma, St. Louis, MO).

Cells and cell culture. Rabbit pleural mesothelial cells were harvested by trypsinization of the pleural space as described (6), and the same technique was used in this study to harvest mouse pleural mesothelial cells. Animal use was in accordance with the University of California, San Francisco Committee on Animal Research. Human mesothelial cells were harvested from pleural effusions or ascites in accordance with the University of California, San Francisco Committee on Animal Research. The liquid was collected in a sterile manner and centrifuged (Corning, Corning, NY), and the pellet was resuspended in medium. The cells were plated onto 75-cm² dishes and allowed to grow until near confluence before use or before being frozen and stored in liquid nitrogen for later use. Rabbit and mouse cells were grown in RPMI-DMEM (1:1 vol/vol), HEPES (10 mM), heat-inactivated FCS (10%; HyClone Laboratories, Logan, UT), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 µg/ml). Human cells were grown in LHC-MM medium (Biofluids, Rockville, MD), FCS (10%, total), L-Glutamax II (2 mM; Gibco BRL), and penicillin-streptomycin. Cells were used between passages 2 and 6.

In vitro experiments. For experiments, mesothelial cells were plated onto mouse laminin-coated dishes (10 µg/ml for 1 h; Gibco BRL) and allowed to adhere overnight in standard growth medium. Cells at 70% confluence were studied the following day. Crocidolite asbestos fibers were dispersed in PBS and added to the cells for 24 h in experimental medium (standard growth medium without serum). Actinomycin D (0.3 µM) and mTNF-α (20 ng/ml) were added for 12 h. Both floating cells and adherent cells detached with trypsin-EDTA (0.25%-0.5 mM) were collected for further analysis.

Hydrogen peroxide (100–300 µM for 12–24 h; Sigma) was added in some experiments to induce necrosis. These cells were later stained with propidium iodide (15 µg/ml; Sigma) and 1 µg/ml of the nuclear stain 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, OR). Necrosis was confirmed by positive staining with propidium iodide and by a lack of nuclear condensation.

In vivo instillates. Instillates were chosen in an attempt to induce apoptosis in vivo. RPMI alone or wollastonite in RPMI was used as a negative control and crocidolite asbestos or actinomycin D plus mTNF-α in RPMI as the experimental instillate. Broadus et al. (8) had used RPMI previously as a benign noninflammatory pleural instillate in rabbits. We chose wollastonite, a relatively nonpathogenic calcium silicate fiber, as a control fiber; at comparable fiber counts requiring twice the mass of material, wollastonite induced significantly less mesothelial cell apoptosis in vitro than crocidolite did (9). Crocidolite asbestos was instilled at 25 µg; if dispersed evenly in the pleural space (surface area 5 cm², visceral plus parietal in a mouse) (1), this amount of asbestos would approximate 5 µg/cm², a concentration that induces ~15% apoptosis of rabbit and human pleural mesothelial cells in vitro in 24 h (9). Actinomycin D is effective at inducing apoptosis in in vitro studies of rabbit and human mesothelial cells, and in the presence of transcriptional arrest, TNF-α can act synergistically to induce apoptosis (9, 25). Actinomycin D can also inhibit neutrophil influx in animal models (33). We chose concentrations four- to fivefold higher than those used in vitro assuming dilution and diffusion and selected 12 h for maximal effect based on in vitro experience. Therefore, experimental pleural instillates contained either crocidolite asbestos (25 µg), wollastonite (50 µg), or actinomycin D (1.0 µM) plus mTNF-α (100 ng/ml) in RPMI in a total volume of 0.5 ml. All solutions for in vivo use were tested for endotoxin by Limulus assay and found to be negative.

After the instillate was drawn into a 1-ml tuberculin syringe, the instillate was pushed through an attached 3-way stopcock into the tubing of a 25-gauge butterfly needle (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) to the tip of the needle. The stopcock was closed, and the plunger was removed. The syringe was held vertically by a clamp in preparation for the instillation.

In vivo pleural instillation. This minimally invasive intrapleural instillation technique has been described briefly for rabbits (27). Healthy male C57BL/6 mice, 6–8 wk old, were anesthetized by continuous inhalation of methoxyflurane (Metofane, Mallinkrodt Veterinary, Mundelein, IL) and taped in a supine position. The chest was shaved and cleaned with Betadine solution. With scissors, a 0.5-cm transverse incision was performed in the anterior right chest wall, and the muscles were separated with forceps until two ribs and the intervening intercostal muscles were exposed. Through the muscles of the intercostal space, the 25-gauge butterfly needle was pointed toward the head and inserted at a shallow angle underneath a rib until the needle tip was within the tissue. The attached three-way stopcock was opened, and the needle was slowly advanced under the rib until a free flow of the instillate demonstrated entry into the subatmospheric pleural space. After the instillate flowed completely into the pleural space (30 s), with no welling of liquid seen, the needle was removed. The skin was sutured with silk suture 6-0, and the mouse was rotated to promote uniform distribution of the instillate. Mice woke rapidly and moved normally within minutes of the procedure. In mice examined immediately after injection or after 10–24 h, there was no evidence of air or blood in the pleural space. Inspection of the lung revealed either no identifiable mark or, in some cases, a reddish mark on the surface adjacent to the injection site.

After 24 h for the RPMI, wollastonite, and crocidolite asbestos groups and 12 h for the actinomycin D plus mTNF-α group, the mice were deeply anesthetized with methoxyflurane and exsanguinated by aortic transection. Pleural cells were harvested by inserting a 22-gauge angiocath through the right diaphragm and lavaging five times with 1.5 ml of PBS (calcium and magnesium free) each time. For the 30 min to 1 h until fixation, harvested cells were maintained at 4°C to minimize apoptosis ex vivo. In six mice, an additional attempt was made to harvest apoptotic mesothelial cells by instillation of warmed trypsin-EDTA (0.25%-0.5 mM; 1 ml) for 10 min followed by inactivation of trypsin with serum and then pleural lavage as above. Total cells recovered by lavage and trypsinization plus lavage were centrifuged, resuspended in 0.5 ml, counted with a hemacytometer, and used for other assays. For analysis of cell differentials, 1–2 drops of cell suspension were cytospun onto slides at 500 rpm for 5 min (Shandon, Pittsburgh, PA) and stained with Diff-Quik (Dade Behring, Newark, DE).
For pleural tissue studies, a different set of mice (n = 4) underwent pleural instillation as above. After 24 h for RPMI and crocidolite and 12 h for actinomycin D plus mTNF-α, the tissues were harvested as follows. To avoid dislodging apoptotic cells from the surface, no pleural lavage was performed. After exsanguination, fresh cold 2% paraformaldehyde was injected into the pleural space and kept for 10 min to fix the tissues. The fixative was withdrawn, and the pleural space was washed gently three times with 1 ml of PBS. Bilateral parasternal and paravertebral incisions were made to remove the right (experimental) and left (uninjected, control) chest walls. The diaphragm was also removed by incisions adjacent to the ribs. To provide a better visualization of the tissues, the chest wall was flattened by cutting the ribs externally in three different places without damaging the mesothelial layer.

Immunocytochemistry for cytokeratin. Cells were stained for cytokeratin initially to identify cells as mesothelial and later to indicate apoptosis. Cells from pleural liquid and lavage fluid were dropped onto the wells of three-well slides (SPI Supplies, Structure Probe, West Chester, PA), air-dried, fixed with fresh cold 2% paraformaldehyde for 10 min, and washed three times with PBS. The fixed cells were permeabilized with 0.1% Triton X-100 for 5 min, washed three times with PBS and blocked with 3% BSA (fraction V, Sigma) for 20 min.

Mouse cells from either in vivo or in vitro studies were incubated with the primary antibody rabbit anti-cow cytokeratin (DAKO) diluted 1:250 in 3% BSA for 30 min at room temperature (RT) and washed three times with PBS. The cells were then incubated with goat anti-rabbit FITC-conjugated antibody (Zymed, South San Francisco, CA) diluted 1:100 in 3% BSA for 30 min. For counting all cells in the in vivo studies and for analysis of nuclear morphology, the cells were also stained with 1 µg/ml of DAPI for 30 min at RT. The samples were washed again with PBS three times, mounted with Slowfade reagent (Molecular Probes), and placed under coverslips for examination with a fluorescence microscope (Leica). Rabbit and human mesothelial cells from in vitro studies were stained identically except that the primary antibody was mouse anti-human AE1/AE3 (DAKO) used at 1:150 for 1 h and the secondary antibody was FITC-labeled sheep anti-mouse (Sigma) used at 1:100 for 1 h. Both anti-cytokeratin antibodies are reactive with a wide range of keratins.

Mouse chest wall and diaphragm tissues were stained for cytokeratin essentially as were the cells, except for a longer permeabilization step. Tissues were permeabilized with 0.1% Triton for 25 min, washed, and blocked with 3% BSA for 20 min. The pleural surface was incubated with the rabbit anti-cow anti-cytokeratin antibody diluted 1:250 in 3% BSA for 30 min at RT. The samples of tissue were then washed three times with PBS and the pleural surface was incubated with goat anti-rabbit FITC-conjugated antibody diluted 1:100 in 3% BSA for 30 min at RT in the dark. After being washed with PBS, the samples were kept in Slowfade antifade equilibrium buffer for 1–3 h before examination. For fluorescence microscopy, the tissues were affixed to a slide with a water-insoluble glue. The pleural surface was kept immersed in Slowfade glycerol buffer, and the microscope lens was lowered until it contacted the liquid. Because of the uneven surface, no coverslip was used. The entire tissue was scanned while the focus was continuously changed to keep the mesothelial surface in view.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling staining for apoptosis. For some in vivo and in vitro experiments, cells were processed for terminal deoxy-

Fig. 1. Cytokeratin distribution of rabbit pleural mesothelial cells grown in culture and exposed to crocidolite asbestos for 24 h. Most cells demonstrated a normal filamentous network of cytokeratin (arrowheads), although 2 cells demonstrated an abnormal, aggregated distribution of cytokeratin (arrows). Bar, 5 µm.
and human mesothelial cells exposed to either asbestos or actinomycin D plus mTNF-α (data not shown). The change in cytokeratin was correlated with classic findings of apoptosis, namely condensed nuclear morphology and in situ end labeling of DNA (TUNEL). Almost all mesothelial cells with aggregated cytokeratin were found to have either a condensed nucleus (Fig. 2) or an extremely pyknotic or no nucleus. For example, in cells with aggregated cytokeratin, 76.7 ± 6.6% (SD; n = 4 animals) had a condensed nucleus, 21.4 ± 7.9% had an extremely pyknotic or absent nucleus, and 2.0 ± 2.0% had a normal nucleus. Similarly, all mesothelial cells with aggregated cytokeratin were found to have a positive TUNEL stain or no visible nucleus (n = 2 animals). Alternatively, of mesothelial cells with TUNEL-positive nuclei, most but not all cells had cytokeratin aggregation [69.0 ± 13.5% (SD); n = 3 animals]. Necrotic cells exposed to high concentrations of hydrogen peroxide showed absent staining or faint staining of fragmented, linear cytokeratin, easily distinguished from the brightly staining aggregated cytokeratin pattern of apoptosis (data not shown).

For the in vivo experiments in mice, we then confirmed the presence of this cytokeratin staining pattern and determined the best method of harvesting apoptotic mesothelial cells. From asbestos-exposed mice, we examined cells obtained from pleural liquid, from pleural lavage fluid, from trypsinization, and from examination of pleural tissue itself. In the recovered pleural liquid (0.02–0.1 ml), few mesothelial cells were present. In pleural lavage fluid, mesothelial cells with aggregated or normal cytokeratin could be easily seen among the majority of nonstaining cells. The cells with aggregated cytokeratin could be shown to stain positively with TUNEL (Fig. 3) or to have no apparent nucleus. Occasionally, fragments of brightly staining, aggregated cytokeratin were seen within the cytoplasm of phagocytes. Trypsinization of the pleural space increased the percentage of mesothelial cells recovered but failed to recover significant numbers of additional apoptotic cells (Table 1). Finally, examination of the intact parietal pleura revealed no apoptotic cells attached to the surface. Whereas the parietal pleura from a contralateral, a control, or an RPMI-instilled pleural space demonstrated a continuous mesothelial cell layer (Fig. 4A), the parietal pleura exposed to asbestos for 24 h showed gaps in the cell layer (Fig. 4B). On phase contrast, asbestos fibers were seen to accumulate in intercostal areas and were not found over most of the mesothelial surface. At the sites of accumulated asbestos, gaps in the continuous mesothelial layer were most prominent. Fragmented linear cytokeratin, similar to that seen in frankly necrotic cells in vitro could be seen around these sites. After actinomycin D plus mTNF-α instillation, gaps in the mesothelial cell layer could be seen but appeared scattered over the surface and not localized to intercostal sites. No abnormalities were found on the diaphragmatic mesothelial surface. Non-adherent cells with aggregated cytokeratin could some-
times be found floating over the pleural surface (Fig. 4C). We concluded that apoptotic mesothelial cells were loosely adherent or detached from the surface and thus were best harvested by lavaging the pleural space.

We then used aggregated cytokeratin as a marker for quantitating mesothelial cell apoptosis within the pleural space. After instillation of crocidolite asbestos or actinomycin D plus mTNF-α, an increased number of apoptotic mesothelial cells were recovered than after instillation of RPMI alone or wollastonite (Fig. 5; Table 2).

The instillations induced a variable inflammatory response. The percentage and total number of neutrophils were greater after wollastonite (49 ± 6% polymorphonuclear neutrophils, 5.4 × 10^6 total cells) and crocidolite (62 ± 7%, 11.4 × 10^6 total cells) than after RPMI (13 ± 9%, 0.2 × 10^6 total cells) and actinomycin D plus mTNF-α [19 ± 14% (SE), 1.9 × 10^6 total cells; P < 0.05; n = 2 animals]. The percentages of macrophages were not different among the groups (24%,

| Table 1. Yield of apoptotic mesothelial cells after intrapleural crocidolite asbestos |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                | Total Cells, 10^6 | Mesothelial Cells, %total | Apoptotic Mesothelial Cells, 10^3 | %mesothelial   |
| Lavage                         | 6 20.1±2.5 0.6±0.1 | 120.6±23.0 5.6±0.5      |                             |
| Lavage after trypsin           | 6 4.6±0.6 2.0±0.3* | 88.4±16.3 0.3±0.1*      |                             |

Values are means ± SE; n, no. of mice. Crocidolite (25 µg) was instilled in pleural space for 24 h. Cells were lavaged from pleural space. In each mouse, after standard lavage, warm trypsin was instilled for 10 min, followed by a second lavage. Total cells were counted with a hemacytometer. Cells were dried on slides and stained for cytokeratin as described in METHODS. Mesothelial cells, identified by positive cytokeratin staining, were counted with fluorescent microscopy as a percentage of total cells identified by nuclear staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), and total number was calculated. Apoptotic cells, those with aggregated cytokeratin, were counted as percentage of total mesothelial cells. *Significantly different from lavage, P < 0.01.

![Fig. 4. Parietal pleura of a mouse exposed to either intrapleural RPMI 1640 medium (RPMI; A) as a control or 25 µg of crocidolite asbestos (B and C) for 24 h. Pleural space was opened, and fixative was instilled without lavage to fix pleura in situ. After fixation, chest wall was removed, and pleural surface was permeabilized and stained for cytokeratin. A: after exposure to RPMI, mesothelial cells with characteristic flat as well as cuboidal morphology lie in a continuous, single cell layer. B: after exposure to asbestos, mesothelial cells (comparable in size to flat mesothelial cells in A) show a gap between otherwise normal cells. C: a mesothelial cell with aggregated cytokeratin overlies pleural surface at a site where mesothelial surface was denuded. Cell is in contact with mesothelial cells with normal cytokeratin. Bar, 10 µm.](http://ajplung.physiology.org/)
Murine tumor necrosis factor—cytokeratin as described in METHODS. Mesothelial cells, identified by positive cytokeratin staining, were counted with fluorescent microscopy. Cells were recovered by lavage, and total cells were counted with a hemacytometer. Cells were dried on slides and stained for D

Aggregation appeared to develop later than the nuclear change. This pattern suggests that cytokeratin reorganization may be developing during the time of DNA cleavage, although persisting beyond the final stages of nuclear condensation, fragmentation, and loss of DNA. If used as the sole marker of apoptosis, cytokeratin aggregation may not detect some cells with early nuclear changes of apoptosis, but because it appears to persist later than the nuclear changes, cytokeratin aggregation may allow detection of cells as apoptotic for which nuclei are extremely pyknotic or no longer present.

Mesothelial cells express a certain pattern of cytokeratins, that of the simple, nonkeratinizing, nonstratified epithelium (16, 35). Cytokeratins, one of the major families of intermediate filaments, constitute a family of >20 proteins, divided into type I (relatively acidic, K9–K20) and type II (relatively basic, K1–K8) (16). Each type of epithelium expresses these keratins in a pairwise fashion, with a member of type I and of type II forming noncovalent heteropolymers in a cell-specific pattern. Mesothelial cells, as representative of simple epithelium, express mostly K18 and K19 (type I) as well as K8 (type II) (16, 35). During apoptosis, it is now recognized that there is cleavage of K18 and K19 into stable fragments, although K8 and other type II keratins may remain intact (23). This cleavage is associated with the aggregated cytokeratin pattern. Therefore, our findings may be able to be generalized to other epithelium expressing these keratins, and, if cleavage of all type I keratins is characteristic of apoptosis, then these findings could be applicable to all cytokeratin-expressing cells.

The degree of apoptosis we identified was lower than that found in vitro to the same stimuli. Whereas in vitro, crocidolite induces 15% mesothelial cell apoptosis (9), in the in vivo setting, crocidolite at the same relative dose induced apoptosis in 5% of recovered mesothelial cells. Similarly, actinomycin D plus mTNF-α will induce 40–60% mesothelial cell apoptosis in vitro, whereas in vivo, this stimulus induced 22% apoptosis in the recovered mesothelial cells. It must be emphasized

### DISCUSSION

In this study, we have shown that mesothelial cells undergo apoptosis in vivo. The mesothelial cell, the only cell in the pleural space to express keratin intermediate filaments, was able to be identified and determined to be apoptotic by its pattern of cytokeratin staining. With in vitro studies, we were able to show that the change in cytokeratin distribution was induced by stimuli known to induce apoptosis in vitro and was correlated with apoptotic nuclear morphological change in the mesothelial cells. The cytokeratin staining pattern persisted during apoptosis beyond the late nuclear changes. Cytokeratin had a different appearance after necrosis, enabling us to distinguish the two modes of death. Although this cytokeratin aggregation has been described in vitro in epithelial cell lines (12, 14, 32, 34), this study represents the first time that such a change has been described in primary cells in vitro and, most importantly, in vivo.

Apoptosis involves drastic morphological changes such as cytoplasmic blebbing and cell shrinking, indicating a widespread alteration of the cytoskeletal framework. Cytoskeletal elements known to be cleaved or reorganized during apoptosis now include members of the three major cytoskeletal families: actin, microtubules, and the intermediate filaments such as nuclear lamins, vimentin, and cytokeratin (21). Cytokeratin aggregation has now been described in epithelial cell lines undergoing apoptosis, associated with changes in nuclear morphology and phosphatidylserine exposure on the cell surface (32, 34). Indeed, efforts to disrupt cytokeratin, such as with transient expression of filaggrin, a filament-associated protein, have induced apoptosis, suggesting that cytoskeletal disruption can cause as well as result from apoptosis (14). More recently, both cleavage and phosphorylation of keratin 18 were identified during apoptosis in epithelial cell lines, with the presumption that proteolytic cleavage, phosphorylation, or both led to the aggregation of cytokeratin (12).

In our primary cells, cytokeratin aggregation was shown to identify a cell as apoptotic. Cytokeratin aggregation appeared to develop later than the nuclear changes because all the cells with aggregated cytokeratin had nuclear condensation and positive TUNEL staining or had lost their nuclei, whereas cells with TUNEL positivity did not all have the cytokeratin change. This pattern suggests that cytokeratin reorganization may be developing during the time of DNA cleavage, although persisting beyond the final stages of nuclear condensation, fragmentation, and loss of DNA.

### Table 2. Total cells, mesothelial cells, and apoptotic mesothelial cells recovered from pleural space

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Total Cells, $\times 10^6$</th>
<th>Mesothelial Cells, % of Total</th>
<th>Mesothelial Cells, $\times 10^4$</th>
<th>Apoptotic Mesothelial Cells, % of Mesothelial</th>
<th>Apoptotic Mesothelial Cells, $\times 10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI</td>
<td>6</td>
<td>14.0 ± 0.3</td>
<td>0.3 ± 0.0</td>
<td>4.3 ± 0.7</td>
<td>0.1 ± 0.1</td>
<td>0.01 ± 0.0</td>
</tr>
<tr>
<td>Woll</td>
<td>3</td>
<td>10.2 ± 2.2</td>
<td>1.2 ± 0.6</td>
<td>107.3 ± 38.9</td>
<td>1.0 ± 0.7</td>
<td>0.7 ± 0.6</td>
</tr>
<tr>
<td>Crocidolite</td>
<td>12</td>
<td>137.3 ± 23.7</td>
<td>0.5 ± 0.1</td>
<td>74.8 ± 17.7</td>
<td>5.1 ± 0.5*</td>
<td>4.0 ± 1.0*</td>
</tr>
<tr>
<td>Act D + TNF</td>
<td>6</td>
<td>6.1 ± 1.5</td>
<td>1.3 ± 0.4</td>
<td>96.2 ± 46.5</td>
<td>22.4 ± 4.5*</td>
<td>28.1 ± 16.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of mice. RPMI, RPMI 1640 medium; Woll, wollastonite (50 µg); Act D + TNF, actinomycin D (1.0 µM) plus murine tumor necrosis factor-α (100 ng/ml). Instillates were instilled either for 24 h (RPMI, Woll, or crocidolite asbestos (25 µg)) or for 12 h (Act D + TNF). Cells were recovered by lavage, and total cells were counted with a hemacytometer. Cells were dried on slides and stained for cytokeratin as described in METHODS. Mesothelial cells, identified by positive cytokeratin staining, were counted with fluorescent microscopy as a percentage of total cells identified by nuclear staining with DAPI. Apoptotic cells, those with aggregated cytokeratin, were counted as percentage of total mesothelial cells. Total numbers of mesothelial cells and apoptotic mesothelial cells were calculated. *Significantly different from RPMI and Woll control experiments, P < 0.05.

RPMI; 24%, actinomycin D-mTNF-α; 33%, wollastonite; 24%, crocidolite).
that these percentages are of recovered mesothelial cells that themselves are only a fraction of the total mesothelial cell population. Thus we know the percentages of apoptosis are much lower in the pleural space than in vitro to similar stimuli. Differences between in vitro and in vivo results may be expected given the cytoprotective environment found in vivo, with appropriate growth factors, matrix substrates and cell-cell interactions. The apoptotic stimuli are also altered in vivo: crocidolite asbestos accumulates in certain locations, especially in intercostal spaces, leading to an uneven distribution, and actinomycin D plus mTNF-α is diluted in the pleural liquid, may be inactivated, and is able to diffuse out of the pleural space. In the in vivo setting, apoptotic cells will also be phagocytosed by macrophages. Indeed, we saw phagocytes (staining positively for CD18; data not shown) with small amounts of intracellular material with dotlike aggregates of cytokeratin, most likely representing ingested apoptotic mesothelial cells. Despite the difficulties in comparing in vitro and in vivo conditions, the relative potency of asbestos and actinomycin D plus mTNF-α for induction of apoptosis was maintained.

Quantitation of apoptosis at a single time may seriously underestimate the total impact of apoptosis both in vitro (4) and in vivo (13). It has been calculated that apoptotic cells are ingested and cleared within 1–2 h by neighboring parenchymal or phagocytic cells (13), making the total amount of apoptosis in a tissue difficult to assess. A small percentage of cells found in apoptosis at a single time may signify a large accumulated effect of apoptosis over time. An estimate given by Scavo et al. (31) is that, assuming a window of detection of an apoptotic cell of 1 h, a finding of 0.2% apoptosis at a single time would equate to a 19% loss of cells over 4 days. The degree of apoptosis we have seen in vivo may thus have significance for pleural mesothelial cell turnover and response to toxic insults. A small degree of apoptosis may have a particularly large impact in localized areas of injury, such as locations of asbestos fiber accumulation.

Asbestos effects on the mesothelium in vivo may be important for understanding asbestos-induced pleural diseases. Although in vitro studies of mesothelial cells have been extensive, there are fewer in vivo studies of the asbestos-exposed mesothelium. We saw that asbestos concentrated in areas of the parietal pleura associated with sites of lymphatic stomata as described in humans exposed to asbestos (5). At these sites, loss of the mesothelial layer was seen along with evidence of fragmented cytokeratin as seen in vitro with necrosis. These findings suggest the presence of both necrosis and apoptosis in vivo. Indeed, in an earlier in vivo study of mice instilled intraperitoneally with asbestos, Moalli et al. (28) described trypan blue-positive cells around the site of asbestos deposition in the diaphragm, likely representing necrotic cells. Also, with scanning electron microscopy of the peritoneal diaphragmatic surface 12 h after an injection of 200 µg of crocidolite asbestos, mesothelial cells were shown to have surface blebs and loss of microvilli, changes now known to be characteristic of apoptosis (28). Although we have now identified mesothelial cell apoptosis in vivo, we cannot conclude from these studies whether asbestos induces apoptosis by direct interaction with the mesothelial cell, as in vitro, or by secondary effects such as via asbestos-induced inflammation. Additional experiments will be necessary to identify those features of asbestos important in generating mesothelial cell apoptosis in vivo.

Although apoptosis was found after pleural instillation of asbestos, it is not known if mesothelial cell apoptosis would be found after inhalational exposure to asbestos. Clearly, the instillation of asbestos into the pleural space leads to higher acute exposure than inhalation of asbestos followed by the gradual migration of fibers from the lung to the pleural space. Recognizing that asbestos accumulates and concentrates at discrete locations in the pleura after inhalational exposure, it is possible that clinically relevant apoptosis, necrosis, and proliferation may be induced in these locations. Based on our findings, apoptosis of mesothelial cells in such locations may be missed by histological approaches that depend on cell attachment and may better be detected by analysis of lavaged cells. The mesothelial surface was viewed in a new way by the use of cytokeratin staining. Whereas other cell stains such as DAPI or acridine orange stained a multitude of cells both on and underneath the mesothelial layer, cytokeratin staining revealed the single cell layer of the mesothelium. Gaps and other abnormalities in the mesothelial layer could be detected, even when otherwise obscured by overlying inflammatory cells. This approach provides a technique for a more detailed study of mesothelial cell responses to various toxins and could be applied to a quantitative study of mesothelial cell loss.

The identification of mesothelial cell apoptosis in vivo establishes an approach to the study of this cell and its interaction with asbestos and other toxic stimuli. The recognition that cytokeratin staining can both identify the cell of origin and determine its apoptotic state has led to a simple assay for this elusive cell. The approach lends itself to the study of mesothelial cells and possibly other cytokeratin-expressing cells in vivo.

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