

Induction of secondary apoptosis, inflammation, and lung fibrosis after intratracheal instillation of apoptotic cells in rats

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Wang, Liying, James F. Scabilloni, James M. Antonini, Yon Rojanasakul, Vincent Castranova, and Robert R. Mercer. Induction of secondary apoptosis, inflammation, and lung fibrosis after intratracheal instillation of apoptotic cells in rats. *Am J Physiol Lung Cell Mol Physiol* 290: L695–L702, 2006. First published November 18, 2005; doi:10.1152/ajplung.00245.2005.—Uncontrolled apoptosis has been associated with several pulmonary disorders; however, the molecular mechanism underlying this process and the fate of apoptotic cells in vivo are unclear. Here we show that direct administration of apoptotic cells to the lungs of rats caused pulmonary inflammation and fibrosis, as indicated by emigration of inflammatory cells to the air spaces, TNF- α immunoreactivity, and connective tissue accumulation, indicating a direct relationship between apoptotic cells and the observed lung pathologies. To determine how the lungs process the accumulated apoptotic cells, normal or apoptotic cells from autologous donor rats were labeled with fluorescent nanobeads and intratracheally instilled into the lungs of rats. Probe distribution and lung cell apoptosis were determined at various times over a 28-day period by confocal fluorescence microscopy and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling, respectively. Labeled apoptotic cells were cleared by lung macrophages within 1 wk after the treatment. However, the total number of apoptotic cells in the lung remained high at 28 days posttreatment. The results indicate a continuous induction of secondary apoptosis by apoptotic cell instillation, which may contribute to the observed lung pathology. Analysis of lung cell apoptosis by caspase assays showed an elevation of caspase-8 but not caspase-9 in the treatment group at 28 days posttreatment, indicating involvement of the death receptor-mediated pathway in the apoptotic process. Together, our results demonstrate a direct effect of apoptotic cell accumulation on inflammatory and fibrotic pulmonary responses and the continuous induction of lung cell apoptosis by apoptotic cell instillation.

clearance; Brown Norway (BN/CrlBR) rat; pulmonary disorders; caspase-8

APOPTOSIS IS AN ACTIVE FORM of cell death that requires the induction of specific enzymes and death signaling pathways. Cell death by apoptosis has a fundamental significance in both normal tissue homeostasis and disease pathogenesis. Apoptosis encompasses a highly conserved series of molecular events termed the apoptotic cascade, leading to cell shrinkage, chromatin condensation, and DNA fragmentation and ending with cellular disintegration into numerous membrane-enclosed apoptotic bodies. These apoptotic bodies become rapidly phagocytosed by neighboring cells and/or professional phagocytes, such as macrophages, thereby preventing the eventual disintegration of apoptotic bodies and further tissue injury via leakage

of their noxious contents. In view of its physiological role, apoptosis is a two-stage process with an intracellular component representing molecular signal transduction events leading to an intercellular component, characterized by prompt phagocytic clearance of apoptotic bodies. Dysregulation of either the intracellular or intercellular component of the apoptotic process is an established causative and/or contributing factor in various pathological disorders, including neurodegenerative disorders (17, 24), cancer (16, 26, 29, 33), and inflammatory diseases (1, 5, 6, 15).

Apoptosis has also been implicated in several pulmonary disorders caused by a variety of agents. The induction of apoptosis by pneumotoxic agents, such as bleomycin (13, 14), silica (11), immune complexes (22), and endotoxin (3), has been associated with lung pathologies, such as acute lung injury, pulmonary inflammation, and fibrosis. Despite the demonstrated relationship between apoptosis and pulmonary disorders and the fact that various apoptosis-inducing agents can cause lung diseases, the direct role of apoptosis in the pathogenesis of these pulmonary diseases has not been clearly established. Furthermore, the fate of apoptotic cells during the disease process and their effects on lung pathogenesis have not been thoroughly investigated.

Apoptosis is generally thought to provide a clearance mechanism for unwanted or dying cells, thereby preventing tissue injury and inflammation (10, 28). Phagocytic clearance of apoptotic cells by macrophages has also been reported to have a suppressive effect on proinflammatory cytokine and chemokine production (4, 19). Thus the observations that apoptosis induced by a variety of agents can cause pulmonary inflammatory disorders are surprising and raise a question that other apoptosis-related events or nonapoptotic mechanisms may be involved. This study was undertaken to determine whether direct pulmonary administration of apoptotic cells in the absence of exogenous stimulating agents can cause lung inflammation and fibrosis. Because failed clearance of apoptotic lung cells is believed to contribute to the pathological processes, we also investigated how apoptotic cells are processed and cleared in the lung and how this process affects the pulmonary responses. To study lung clearance of apoptotic cells, we instilled fluorescently labeled apoptotic or nonapoptotic cells into the lung of rats and studied their fate and resultant pulmonary responses. Our findings support the importance of phagocytic clearance on the induction of apoptosis and pulmonary malfunctions.

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MATERIALS AND METHODS

Experimental animal model. Inbred male Brown Norway (BN/CrlBR) rats (200–250 g) monitored to be free of specific viral, mycoplasmal, and bacterial pathogens and parasites but cultured positive for *Staphylococcus aureus* were used throughout. The animals were housed in an American Association for Accreditation of Laboratory Animal Care-approved facility under temperature-, humidity-, and light-controlled conditions. All methods involving animals were conducted under protocols approved by the Institutional Animal Care and Use Committee. Food and water were available ad libitum. For intratracheal instillation studies, animals were lightly anesthetized by an intraperitoneal injection of 0.6 ml of 1% sodium methohexital (Brevital; Eli Lilly, Indianapolis, IN); instillations were performed via a ball-tipped 18-gauge animal feeding needle. Normal or apoptotic cells from autologous donor rats (1×10^6 cells) were instilled. Polybeads (15 μ m diameter) (Polysciences, Warrington, PA) were washed and resuspended in PBS. Mixture of the beads with 0.5 μ m of Bright Blue fluorescent beads were instilled to rat lung by the same method above. Five rats per group were killed at 4 h and 1, 7, and 28 days after the instillations. The right lungs were lavaged to collect cellular contents, and the left lungs were fixed for histology.

Lung histology and Sirius Red stain. The lungs were airway fixed by intratracheal instillation with 10% buffered formalin at 20 cmH₂O. After measurements of fixed lung volume, the lungs were embedded in paraffin and sectioned at 5 μ m. The sections were then mounted on glass slides, deparaffinized in xylene, rehydrated, and stained with hematoxylin and Sirius Red (12) to assess lung inflammation and fibrosis.

Bronchoalveolar lavage. To obtain cells for pulmonary instillations, we anesthetized a donor group of rats. Rats were killed by exsanguinations under anesthesia with pentobarbital sodium (Pentotol; Med-Pharmex, Pomona, CA). A tracheotomy tube was inserted surgically. The lungs were lavaged by alternate instillation and withdrawal of 8 ml of ice-cold Ca²⁺/Mg²⁺-free PBS. The lung was lavaged until 80 ml were collected to obtain cells. The lavages were combined and centrifuged, and the cell pellets were resuspended in PBS for apoptotic and nonapoptotic cell preparations. Lavaged cell counts were performed with a Coulter Multisizer II (Coulter Electronics, Hialeah, FL).

Preparation of labeled apoptotic and nonapoptotic cells for instillations. Lavaged cells were counted and divided into two cell preparations. We induced apoptosis in one of the cell preparations by exposing it to ultraviolet (UVB) irradiation (245 nm) for 20 min in PBS. After 12 h, the cells were washed three times by alternate centrifugation and resuspension to remove any cellular mediators in the supernatants. The cells were then analyzed for apoptosis by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) and confirmed by DNA ladder as previously described (32). Cell necrosis was also determined by trypan blue exclusion assay. Virtually all cells in the preparations showed negative trypan blue staining, indicating that the cell preparations were relatively free of necrotic cells. For cell labeling, both preparations were labeled with fluorescent beads by incubation with 0.5 μ m of Bright Blue fluorescent beads (5×10^6 beads/million cells) for 1 h at 37°C. The cell preparations were then washed three times by alternate centrifugation and resuspension with Ca²⁺/Mg²⁺-free PBS to remove free beads. Samples were taken to verify that essentially all cells were labeled with fluorescent beads. Apoptosis was induced in one of the cell groups as described above.

TUNEL and caspase activity assays. Identification of lung cell apoptosis was performed with a TUNEL kit (Promega, Madison, WI) and caspase assays (Biovision, Mountain View, CA), according to the manufacturer's instructions. In brief, cytopspins of the bronchoalveolar lavage (BAL) cells were fixed in 4% paraformaldehyde at 4°C for 30 min, washed with PBS, and incubated with 1% Triton X for 10 min. Lung tissue sections were deparaffinized, rehydrated, and incubated

with 100 μ g/ml proteinase K at 37°C for 30 min. Thereafter, the following steps were the same for both cytopsin and lung tissue section preparations. After 10-min incubation at room temperature with equilibration buffer provided by the manufacturer, the slides were immersed in TdT and fluorescein-dUTP, which were diluted in equilibration buffer and allowed to incubate for 60 min at 37°C. After being washed with 2% SSC washing buffer provided in the kit, the slides were counterstained with propidium iodide (Molecular Probes, Eugene, OR). After staining, the slides were examined with an Olympus AX-70 fluorescence microscope with an Olympus U-MWIB filter or with a confocal microscope (see below). A bright green-yellow fluorescence signal in the nucleus indicated TUNEL-positive apoptotic cells, whereas normal cells exhibited a red nuclear fluorescence. For quantitation of cell apoptosis, a minimum of 20 random fields was analyzed for each sample at a magnification of $\times 200$.

Caspase activities were determined fluorometrically using two caspase substrates, isoleucine-glutamic acid-threonine-aspartic acid (IETD)-amino-4-methyl coumarin (AMC) for caspase-8 and leucine-glutamic acid-histidine-aspartic acid (LEHD)-AMC for caspase-9. The substrates are specifically cleaved by the respective enzymes at the Asp residue to release the fluorescent leaving group, AMC. Lung tissue homogenates containing 50 μ g of protein were prepared and incubated with 100 mM HEPES, pH 7.4, containing 10% sucrose, 0.1% 3-[(3-cholamidopropyl)-1] propane sulfonate, 10 mM dithiothreitol, and 50 μ M caspase substrate in a total reaction volume of 0.25 ml. The reaction mixture was incubated for 60 min at 37°C. At the end of incubation, the liberated fluorescent group AMC was determined fluorometrically at the excitation and emission wavelengths of 380 and 460 nm, respectively.

Confocal microscopy of labeled and lavaged cell preparations. A three-channel mode was used for confocal fluorescence microscopy of labeled cell preparations and imaging of lavaged lung cells. For this purpose a Carl Zeiss laser scanning confocal microscope system (LSM 510) with an Axioplan 2 upright microscope and a $\times 63$ Apochromat water immersion objective were used. A Coherent UV laser with excitation of 364 nm and a 385- to 470-nm band-pass filter was used to image the UV fluorescent submicron Bright Blue beads. An Argon laser with an excitation of 488 nm and a 505- to 530-nm band-pass filter was used to image the yellow green of TUNEL-positive apoptotic cells. A helium-neon light source with the excitation wavelength of 543 nm and a long-pass filter of 560 nm was used to image the red nuclear fluorescence of propidium iodide.

Measurements of the fate of instilled apoptotic and normal cells. Analysis of cells from BAL at 4 h and 1, 7, and 28 days after instillation was performed to determine the fate of instilled-labeled apoptotic or normal cells. Five animals per group were studied with data expressed as means \pm SE. After lavage, cells from each animal were centrifuged and resuspended to 0.1 ml in PBS. Two cytopspins of the resuspended lavages were made, and the slides were prepared for TUNEL assay as described. Twenty fields of the cytopspins from each animal were then examined under a fluorescence microscope through a $\times 40$ objective. In each field cells containing the submicron Bright Blue beads were identified. Each cell was then examined to determine whether it contained a TUNEL-positive and/or propidium iodide-positive nucleus. The presence of submicron Bright Blue beads was determined with an Olympus U-MNU filter cube with 360- to 370-nm excitation and 430- to 485-nm band-pass emission filter. A Triple cube (4',6-diamidino-2-phenylindole/FITC/propidium iodide) was used to identify TUNEL-positive nuclei and propidium iodide-positive nuclei (filter set 61001; Chroma Technology, Rockingham, VT). The results were expressed as the percentage of lavaged cells that contained a TUNEL-positive nucleus with Bright Blue bead label and TUNEL-negative nucleus with Bright Blue bead label. Cells not containing Bright Blue beads were not included in this analysis. Examination of lavage from animals instilled with Bright Blue bead-labeled normal cells was also conducted with the filters described

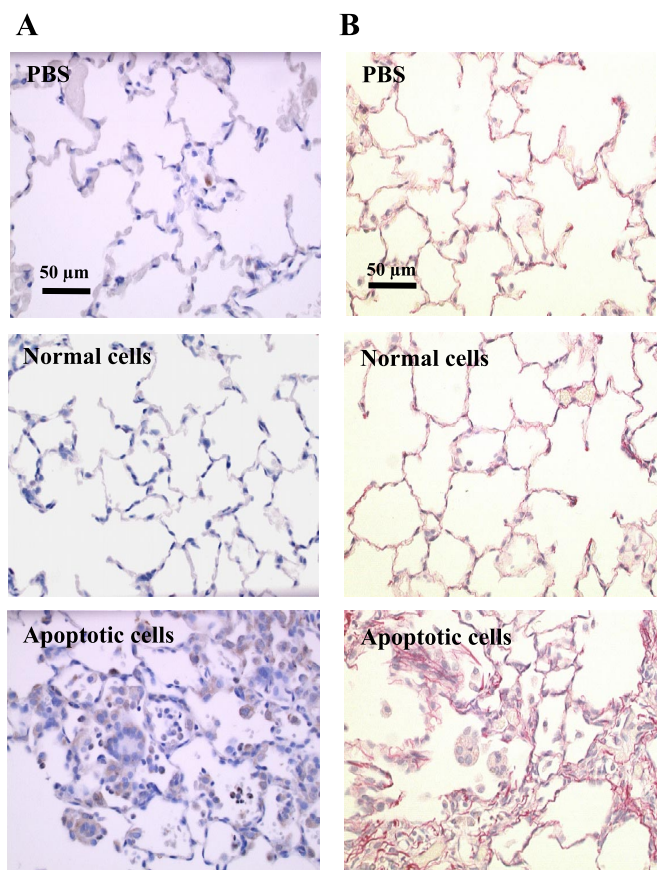


Fig. 1. Induction of lung inflammation and fibrosis by apoptotic cell instillation. Brown Norway (BN) rats were treated with normal or apoptotic cells (1×10^6 cells/rat) by intratracheal instillations. Twenty-eight days after the treatment, rats were killed, and lung sections were prepared for analysis of TNF- α and collagen content by immunohistochemistry and Sirius Red staining, respectively. *A*: TNF- α protein stained brown as was shown in lung cells from rats exposed to apoptotic cells, but not to normal cells. Rats receiving apoptotic cells also showed increased infiltration of inflammatory cells in the air spaces compared with the control rats. *B*: increased collagen deposition and lung wall thickening were observed in apoptotic cell instilled lungs but not in control lungs.

above. Phagocytosis of neither the labeled normal cells nor the TUNEL-positive, Bright Blue bead-labeled cells was identified.

Immunohistochemistry. Immunohistochemical analysis of lung tissue samples was performed according to Sequenza protocol (Thermo Shandon, Pittsburgh, PA) using Dako peroxidase kit for rat (Dako, Carpinteria, CA) and liquid 3,3'-diaminobenzidine substrate kit (ZYMED Laboratories, South San Francisco, CA). In brief, tissue sections were deparaffinized in xylene, rehydrated, and microwaved in citrate buffer, pH 6.0, for antigen retrieval. After the sections were treated with 3% H_2O_2 -methanol (1:1) to block endogenous peroxidases, they were incubated overnight at 4°C with primary antibodies at the following concentrations: mouse anti-rat TNF- α (Biosource, Camarillo, CA), 1:100 dilution; rabbit anti-rat caspase-8 and caspase-9 (R&D Systems, Minneapolis, MN), 1:1,500 dilution.

ELISA assays. Supernatants from the first BAL were assayed for TNF- α and transforming growth factor (TGF)- β using ELISA kits (R&D Systems) according to the manufacturer's instructions. The sensitivity of the assays was 15–30 pg/ml, and the coefficient of variation for the assays was <10%.

Statistical analysis. The numeric data are presented as means \pm SE of four to six separate experiments. The difference between data groups and controls was determined by a Student's *t*-test. A *P* value <0.05 was considered statistically significant.

RESULTS

Pulmonary responses to apoptotic cell instillation. To study the direct role of lung cell apoptosis in pulmonary inflammation and fibrosis, rats were intratracheally instilled with apoptotic lung cells from autologous donor rats (1×10^6 cells/rat). At 28 days postexposure, lung tissue sections were prepared and examined for lung morphology as well as TNF- α and collagen content. Control rats received an equal number of nonapoptotic lung cells or PBS. Figure 1*A* shows that pulmonary instillation of apoptotic lung cells caused a marked increase in inflammatory cell influx and TNF- α protein production. TNF- α immunostaining was observed mainly in infiltrating cells that were identified to be alveolar macrophages. In contrast, administration of nonapoptotic cells to the lungs of rats caused no detectable effects on infiltrating cells and TNF- α expression compared with the PBS-treated control. Pulmonary instillation of apoptotic lung cells also caused an increase in lung collagen content as demonstrated by Sirius Red staining of lung tissue sections (Fig. 1*B*). No significant change in Sirius Red staining was observed in rat lungs receiving normal nonapoptotic cells. These results indicate that accumulation of apoptotic cells in rat lungs can induce pulmonary inflammation and fibrosis.

To provide a more quantitative analysis of pulmonary cell infiltration, we performed BAL cell counts in animals treated with PBS, normal cells, or apoptotic cells. Figure 2*A* shows that administration of apoptotic cells caused a time-dependent increase in lavaged cell number over control level. This increase was attributed to pulmonary cell infiltration since instillation of an equal number of normal nonapoptotic cells had no significant effect on the cell count. Figure 2*B* shows the dose

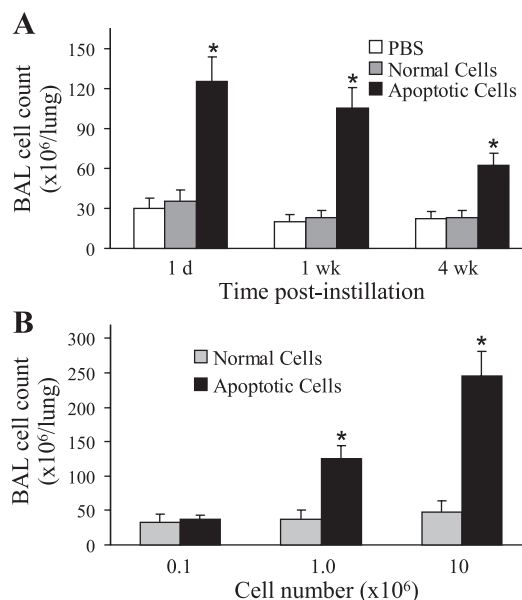


Fig. 2. Bronchoalveolar lavage (BAL) cell count following apoptotic cell instillation. *A*: BN rats were instilled intratracheally with PBS, normal cells, or apoptotic cells (1×10^6 cells/rat). At indicated times after the treatment, rats were killed, and the lungs were lavaged for analysis of cell number. *B*: rats were similarly instilled with varying doses of normal or apoptotic cells (0.1 – 10×10^6 cells/rat). One day (d) after the treatment, lung lavages were examined for cell number. Values are means \pm SE, $n = 4$ rats/group. **P* < 0.05 vs. PBS control.

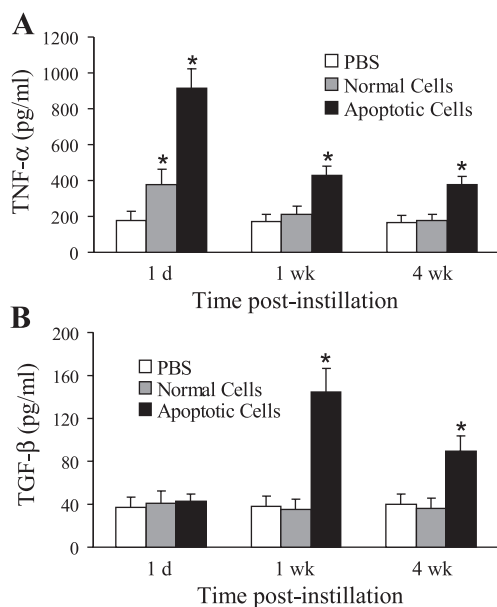


Fig. 3. Cytokine induction following apoptotic cell instillation. BN rats were instilled intratracheally with PBS, normal cells, or apoptotic cells (1×10^6 cells/rat). The rats were killed at various times after the treatment, and the lungs were lavaged for analysis of TNF- α (A) and transforming growth factor (TGF)- β (B) by ELISA. Values are means \pm SE, $n = 4$ rats/group. * $P < 0.05$ vs. PBS control.

effect of apoptotic cell instillation on lavaged cell count. At a low dose of 0.1×10^6 cells, no significant increase in the cell count was observed. At higher doses (1 million and 10 million apoptotic cells), a significant and dose response increase in the cell number was observed. These results indicate that instillation of sufficient numbers of apoptotic cells into the rat lungs can induce pulmonary infiltration of inflammatory cells.

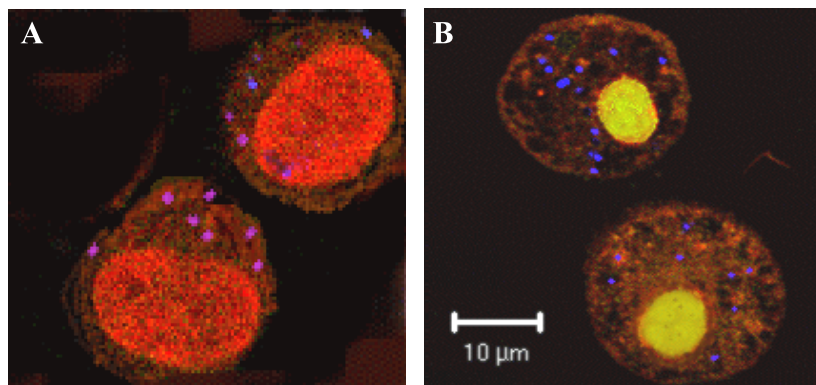
We also analyzed pulmonary cytokine responses to apoptotic cell instillation by an ELISA assay. TNF- α and TGF- β were analyzed in this study since they are known mediators of lung inflammation and fibrosis. Figure 3 shows that the levels of TNF- α and TGF- β in the BAL fluids were both elevated after apoptotic cell instillation. The increase in TNF- α level was rapid and peaked at ~ 1 day posttreatment, where it subsequently declined (Fig. 3A). Administration of nonapoptotic cells to the lung of rats had no significant effect on the TNF- α level at all time points, except at 1 day posttreatment where a moderate increase in the cytokine level was observed. In contrast to TNF- α , the TGF- β response to apoptotic cell

instillation was more delayed with a peak response time of ~ 1 wk (Fig. 3B). Similar to TNF- α , however, the level of TGF- β remained elevated over control level at 4 wk posttreatment. Administration of nonapoptotic cells had no significant effect on the cytokine level at all time points. These results indicate that accumulation of apoptotic cells in the rat lungs can induce pulmonary cytokine production, which may contribute to the observed lung inflammation and fibrosis.

Characterization of normal and apoptotic cell preparations used for instillations. To study the fate of instilled apoptotic cells following pulmonary administration, normal and apoptotic cells were prepared and labeled with submicron fluorescent beads. Figure 4 shows intracellular distribution of the fluorescent beads in normal and apoptotic cells analyzed by confocal fluorescence microscopy. Apoptosis was induced in apoptotic cell preparations by UVB radiation as described in MATERIALS AND METHODS. Over 90% of the irradiated cells become apoptotic as indicated by TUNEL-positive nuclear fluorescence and condensation, whereas essentially no apoptotic cells were detected in nontreated cell preparations.

Phagocytosis of apoptotic cells by lung macrophages. Figure 5 shows confocal fluorescent micrographs of lavaged lung cells harvested from rats after instillation with labeled apoptotic cells. Free TUNEL-positive cells with fluorescent bead labels can be seen at 4 h postinstillation (Fig. 5A). The indicated cell is likely to be one of the instilled apoptotic cells since it exhibited TUNEL-positive nucleus with DNA condensation and the presence of the labeling fluorescent beads in the cell. At 1 day posttreatment, the majority of apoptotic cells had been engulfed by resident macrophages. Figure 5B shows that an instilled apoptotic cell has been ingested by a resident macrophage. The phagocytosing macrophage exhibited normal nuclear morphology and TUNEL-negative nucleus with engulfed apoptotic cell TUNEL-positive nucleus and labeling blue fluorescent beads in its cytoplasm. Over 85% of the instilled apoptotic cells were phagocytosed by lung macrophages after 1 day of instillation. Figure 5C shows time course of lung clearance of labeled apoptotic cells after instillation. The number of labeled apoptotic cells with TUNEL-positive nucleus rapidly declined over time. By 7 days, almost none of the instilled, labeled cells could be detected in the lavage fluid. In contrast, the number of resident macrophages ingesting apoptotic bodies, i.e., those exhibiting both a TUNEL-negative nucleus and TUNEL-positive apoptotic bodies in the cytoplasm, increased after 4 h, peaked at 1 day postexposure, and subsequently declined to near basal level after 7 days. No

Fig. 4. Analysis of labeled apoptotic and nonapoptotic cell preparations by confocal fluorescence microscopy. Lung macrophages from autologous donor rats were isolated by BAL and labeled with fluorescent beads (5×10^7 beads/million cells) for 1 h at 37°C . Apoptosis was induced in apoptotic cell preparations by exposing the cells to UVB for 20 min in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS. After 12 h, the cells were washed and analyzed for apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). A: confocal fluorescent micrograph of normal nonapoptotic cell preparation showing submicron fluorescent bead labels (purple blue). B: apoptotic cell preparation showing TUNEL-positive nucleus (yellow) and purple blue fluorescent bead labels.



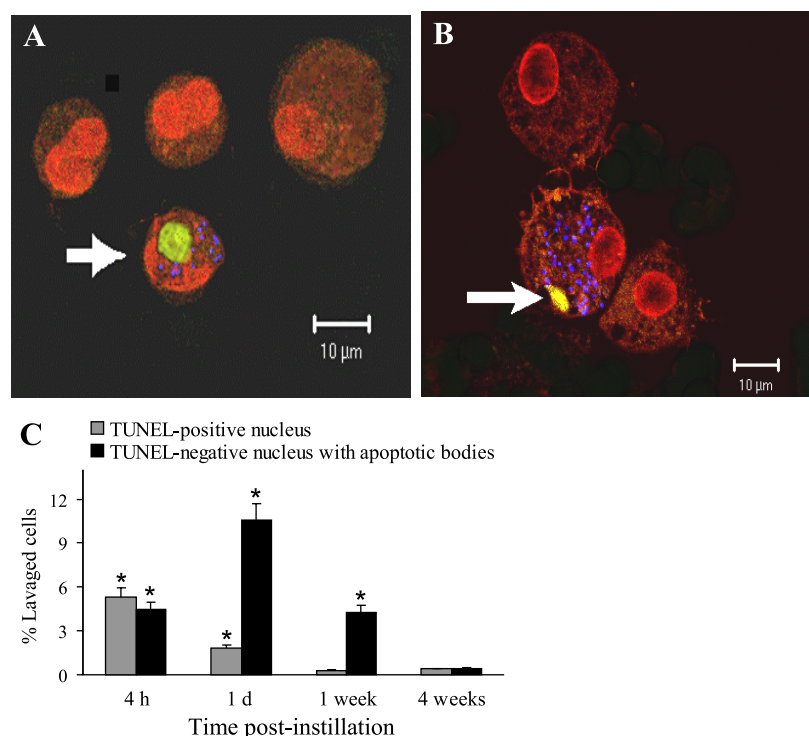


Fig. 5. Confocal fluorescent micrographs of lavaged lung cells harvested from rats after instillation with labeled apoptotic cells and the clearance of labeled apoptotic cells in rat lungs. *A*: samples obtained at 4 h postinstillation. Arrow indicates an instilled apoptotic cell containing TUNEL-positive nucleus with fluorescent bead labels. *B*: samples obtained at 1 d postinstillation. Arrow indicates TUNEL-positive nuclear remains of an instilled apoptotic cell that has been ingested by a resident macrophage with TUNEL-negative nucleus. Fluorescent beads, which were originally in the instilled apoptotic cells, have been released into the cytoplasm of resident macrophage. *C*: lavaged cells from rats after instillation with labeled apoptotic cells (1×10^6 cells/rat) were analyzed by TUNEL. Instilled apoptotic cells were indicated as TUNEL-positive nucleus with purple blue microdots (as shown in *A*, arrow). Normal cells ingesting apoptotic cells were indicated as a cell with a normal nucleus, which stained by propidium iodide, with TUNEL-positive nuclear remains and purple blue beads (as shown in *B*, arrow) in the cytoplasm. Values are means \pm SE, $n = 6$ rats/group. * $P < 0.05$ vs. values at 4 wk.

labeled nonapoptotic cells were found in macrophages at any time points (data not shown). These results indicate that instilled apoptotic cells were rapidly cleared by resident macrophages, while labeled normal cells were not. Intratracheally instilled 15- μ m-diameter beads, which are of comparable size as apoptotic cells, plus 0.5- μ m fluorescent tracer beads were ingested by macrophages within 1 day (Fig. 6). These data confirmed the efficiency and the clearance capability of rat lung.

Induction of apoptosis of resident cells following apoptotic cell instillation. To determine the potential apoptosis-inducing effect of apoptotic cell instillation in rat lungs, animals were

exposed to PBS or normal or apoptotic cells intratracheally. At various times after the instillations, total lung cell apoptosis was determined by TUNEL. Figure 7 shows that administration of normal nonapoptotic cells or PBS, as expected, had no effect on lung cell apoptosis at any time point. In contrast, administration of apoptotic cells resulted in an increase in lung cell apoptosis at all time points even after 28 days postinstillation. Because our earlier study indicated that labeled apoptotic cells were cleared from the lungs within 7 days (Fig. 5C), the observation that apoptosis remained high in the rat lungs at 28 days posttreatment suggests a new induction of apoptosis of the resident cell subsequent to the apoptotic cell instillation.

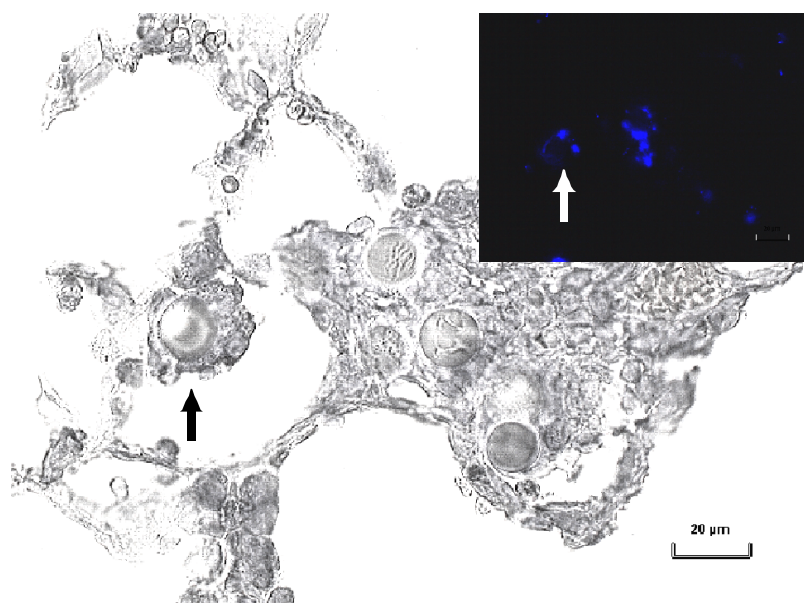


Fig. 6. Differential interference contrast image of lung section 1 d after intratracheal instillation of 15- μ m-diameter beads plus 0.5 μ m of fluorescent tracer beads. The fluorescent micrograph inset of the same area demonstrates that the macrophages that ingested the large beads also ingested the smaller fluorescent tracer beads. The arrows (black and white) identify the same macrophage in the 2 images.

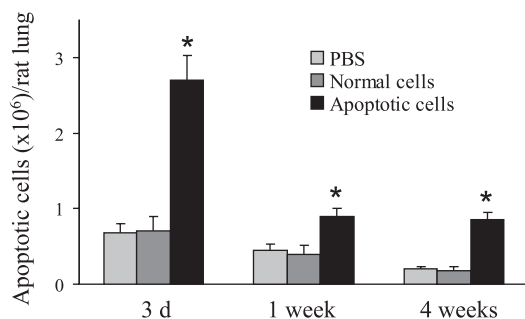


Fig. 7. Time course of lung cell apoptosis following apoptotic cell instillation. Rats were treated with PBS or normal or apoptotic cells (1×10^6 cells/rat). At indicated times after the treatment, lung tissue sections were prepared and analyzed for apoptosis by TUNEL. Values are means \pm SE, $n = 6$ rats/group. * $P < 0.05$ vs. PBS control.

To further confirm our results, we performed caspase-8 and -9 immunohistochemical localization of apoptosis on 28 days postinstillation. We also performed caspase activity assays using fluorogenic caspase substrates, IETD-AMC for caspase-8 and LEHD-AMC for caspase-9. Rats were instilled intratracheally with PBS or normal or apoptotic cells, and lung tissue samples were prepared and analyzed for caspases. Figure 8, A and B, shows that administration of apoptotic cells caused an increase in caspase-8 activity and protein expression compared with PBS- or normal cell-treated controls. TUNEL assays yielded consistent results (Fig. 8C). Unlike caspase-8, the activity of caspase-9 was minimally affected by the administration of either apoptotic or normal cells (Fig. 8A). Its expression level was also low and not different among the treatment groups (data not shown). Because caspase-8 is involved in death receptor-mediated apoptosis (2), our results suggest the involvement of this death signaling pathway in the induction of programmed cell death following apoptotic cell instillation.

DISCUSSION

Apoptosis has been implicated in several pulmonary disorders induced by a variety of agents; however, its direct role in the disease process is unclear. The results of this study demonstrate that direct *in vivo* administration of apoptotic cells to the lungs of rats can induce pulmonary inflammation and fibrosis as indicated by inflammatory cell influx, TNF- α and TGF- β production, and collagen accumulation (Figs. 1–3). Control rats receiving nonapoptotic cells showed no lung pathologies, indicating a direct relationship between apoptosis and lung disorders. Previous studies by our group and others (3, 10, 20, 21) indicate that apoptotic cells are rapidly cleared from the lung by macrophages, and impairment of this clearance mechanism, i.e., by the excessive presence of apoptotic signals, can lead to the development of secondary necrosis. The results of this study demonstrate the clearance of instilled apoptotic cells by resident lung macrophages (Fig. 5). However, the number of apoptotic cells in the lung remained high even after 28 days posttreatment compared with the control (Fig. 7). This result suggests that the phagocytic clearance mechanism of the lung is compromised, probably due to the overwhelming and prolonged presence of apoptotic cells. Because apoptotic cells are known to be rapidly cleared *in vivo*, it seems unlikely that the apoptotic cells observed at 28 days posttreatment are the same cells initially instilled.

To test whether the apoptotic cells observed at late time points were those originally instilled or subsequently induced, we labeled apoptotic cells with fluorescent nanobeads and instilled them into the lungs of rats. Their fate was then determined at various times postinstillation. Our results showed that $\sim 50\%$ of the instilled apoptotic cells were taken up by macrophages after 4 h and $>85\%$ of the instilled cells were cleared after 1 day (Fig. 5C). As shown in Fig. 6, $\sim 90\%$ of 15- μm beads were ingested by macrophages 1 day after instillation, suggesting instilled apoptotic macrophages are phagocytized as efficiently as inert beads of comparable size.

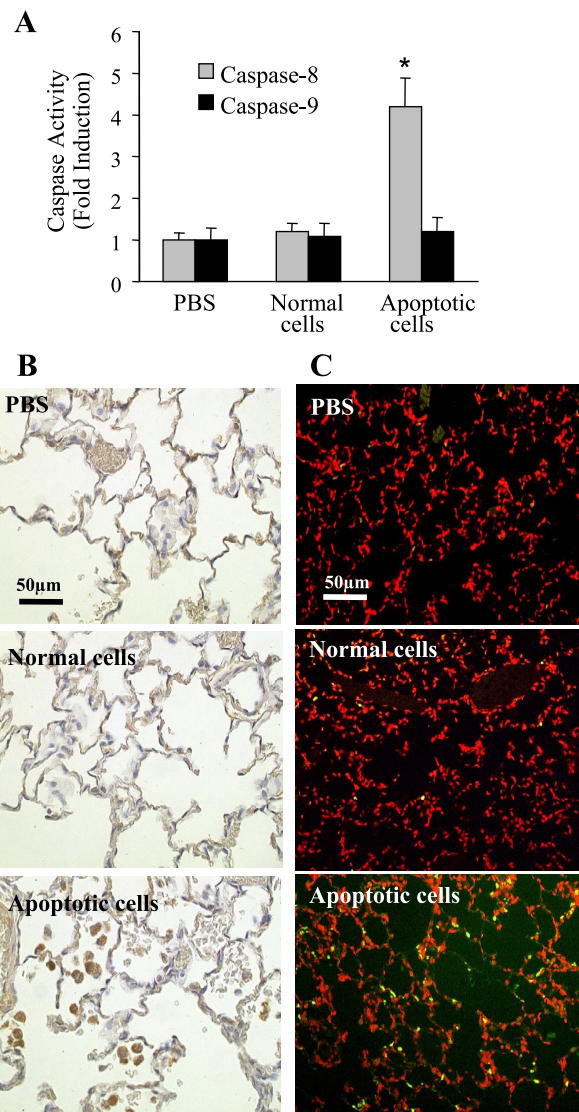


Fig. 8. Caspase and TUNEL detection of apoptosis in rat lungs at 28 d posttreatment with PBS, normal cells, or apoptotic cells (1×10^6 cells/rat). A: fluorometric assays of caspase-8 and caspase-9 activities in rat lungs after pulmonary cell instillation. Tissue homogenates (50 μg of protein) were prepared and determined for enzyme activities using the fluorometric substrates IETD-AMC and LEHD-AMC. Data are means \pm SE ($n = 4$ rats/group). * $P < 0.05$ vs. PBS-treated control. B: lung tissue sections were stained with caspase-8 antibody and horseradish peroxidase-conjugated secondary antibody as described in MATERIALS AND METHODS. Positive caspase-8 protein is indicated by dark brown color. C: fluorescent micrographs of lung tissue sections stained with TUNEL to show apoptotic cells (yellow) and counterstained with propidium iodide for nonapoptotic cells (red).

Consistent with the removal of the labeled apoptotic cells, an increase in the number of macrophages ingesting labeled apoptotic cells was also observed at 1 day postinstillation, and this number declined thereafter. These results demonstrate that apoptotic cells are rapidly ingested and processed by resident alveolar macrophages in the normal rat lung.

Although the number of labeled apoptotic cells is near zero by 7 days, the number of unlabeled apoptotic cells remained at a high level at 28 days postinstillation (Fig. 7). These results indicate that new apoptotic cells were induced at later times following the apoptotic cell instillation. The mechanism by which instilled apoptotic cells induce new rounds of apoptosis is unclear but may involve death receptor-mediated apoptosis signaling since both the expression and activity of caspase-8, a key enzyme governing this death pathway (2, 26), were elevated 28 days after the apoptotic cell instillation (Fig. 8). It is generally accepted that apoptosis is executed along two major pathways. One of these centers on death receptors, such as TNF- α receptor-1 and Fas (CD95/APO-1), which use caspase-8 activation as a signaling mechanism to induce apoptosis (27, 31, 34). The other pathway involves the participation of mitochondria, which release caspase-activating proteins into the cytosol that trigger apoptosis via caspase-9 activation (8, 25). The observation that caspase-8 but not caspase-9 was elevated and activated in this study suggests that the death receptor pathway is the dominant pathway induced by apoptotic cell instillation.

The role of death receptor-mediated apoptosis in pulmonary disorders has been well demonstrated. Activation of death receptors by death ligands or agonistic antibodies causes lung cell apoptosis and inflammation, which subsequently leads to pulmonary fibrosis (7, 9, 13, 18). Inhibition of death receptor activation by neutralizing antibodies, on the other hand, prevents the development of lung disorders caused by several pneumotoxic agents (13, 23, 24). The induction of secondary apoptosis observed in this study would likely affect the recruitment of new phagocytic macrophages into the air spaces, which was also observed at 28 days posttreatment (Fig. 1). Macrophages are a primary source for proinflammatory cytokines, such as TNF- α . Increased TNF- α production was observed in the lung after apoptotic cell instillation. TNF- α has been reported to contribute to the observed lung fibrosis (7, 23) and to cause inflammatory lung injury by damaging cells and tissues and by inducing the upregulation of adhesion molecules and emigration of inflammatory cells (23, 24). Although TNF- α is likely to play a role in the observed lung pathologies, other cellular mediators, such as TGF- β and matrix metalloproteinases, may also be involved, and this needs further investigation. In particular, TGF- β is a known mediator of lung fibrosis, and its increased production was also observed in lung tissues following apoptotic cell instillation (Fig. 3).

In summary, we have demonstrated that pulmonary administration of apoptotic cells resulted in an induction of secondary apoptosis and the development of pulmonary inflammation and fibrosis. This apoptotic process appears to be mediated via the death receptor pathway, possibly via TNF receptor activation. Such induction of apoptosis is likely to overwhelm or impair the phagocytic clearance function of the lung, which is necessary to maintain homeostasis. This condition promotes pulmonary infiltration of inflammatory cells, which further contributes to lung inflammation and fibrosis. Impairment of the

clearance function of the lung would also lead to the development of secondary necrosis, which exacerbates the lung injury. Because excessive apoptosis is directly linked to pulmonary disorders, strategies aimed at controlling apoptosis or promoting lung clearance function may provide logical and effective means for the treatment of inflammatory and fibrotic lung diseases.

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