Apoptosis in the lung: induction, clearance and detection

P. M. Henson and R. M. Tuder
Department of Pediatrics, National Jewish Medical and Research Center, and Division of Pulmonary and Critical Care Medicine, Departments of Medicine and Pathology, University of Colorado at Denver and Health Sciences Center, Denver, Colorado

Henson PM, Tuder RM. Apoptosis in the lung: induction, clearance and detection. Am J Physiol Lung Cell Mol Physiol 294: L601–L611, 2008.—Apoptosis and other forms of programmed cell death are important contributors to lung pathophysiology. In this brief review, we discuss some of the implications of finding apoptotic cells in the lung and methods for their detection. The balance between induction of apoptosis and the normally highly efficient clearance of such cells shows that these are highly dynamic processes and suggests that abnormalities of apoptotic cell clearance may be an alternative explanation for their detection. Because recognition of apoptotic cells by other lung cells has additional effects on inflammation, immunity, and tissue repair, local responses to the dying cells may also have important consequences in addition to the cell death itself.

efferocytosis; inflammation

PRESENTLY, THERE IS A GREAT DEAL of interest in the participation of apoptosis in various forms of lung diseases or, for that matter, in normal lung development, structure, and function. In this short review, we will discuss apoptosis in the lung in the context of its potential role as a process in health and disease and outline methods, with caveats, for its detection. Because most lung diseases have been examined in one way or another for the presence of apoptotic cells, we will not provide a catalog of such studies but rather attempt to address the implications of finding apoptotic cells in the lung for the progression or resolution of disease processes.

Apoptotic cells are usually removed from tissues by a highly efficient recognition and phagocytic clearance mechanism involving either professional phagocytes, macrophages, and dendritic cells or nonprofessional phagocytes, including endothelial cells, epithelial cells, smooth muscle cells, and fibroblasts. We have termed this clearance process efferocytosis (6, 23). Recognition and engulfment of apoptotic cells also modulate local inflammatory and immunologic responses and may therefore contribute to replenishment of normal cells and restoration of tissue structure to maintain homeostasis (22). Disruption of any of these responses to apoptotic cells may contribute to loss of tissue homeostasis, increased inflammation, fibrosis, or even autoimmunity.

PROGRAMMED CELL DEATH, APOPTOSIS, AND NECROSIS

Programmed cell death (PCD), of which apoptosis is one manifestation, is a mechanism for deletion of unwanted or damaged cells in the maintenance of normal tissue homeostasis. Consequently, it is particularly manifest during development, tissue remodeling, inflammation, and tissue injury. In fact, PCD is occurring all the time throughout most of the mammalian body, particularly evident for the constant clearance (with replacement) of cells within the hematopoietic compartment. The term apoptosis was coined in 1972 by Kerr et al. (34) to describe a particular morphological manifestation of cell death involving condensation of the nucleus. Since that time, the name has often become used superficially to equate with PCD in general. The distinction of PCD is that the cell itself participates in the processes of its death, in contrast with externally induced death, for example, by physical processes such as acid, heat, or chemicals or by the effects of some potent toxins. PCD itself has been divided into a number of categories, including a more strictly defined apoptosis, and also encompasses cell autonomous necrosis, cell death resulting from autophagy, cell senescence, and so-called mitotic catastrophe (see reviews in Refs. 16, 21, 52). The problem is that there are few clear-cut differentiating features for these different forms of cell death. Nuclear condensation, intranucleosomal DNA cleavage, membrane blebbing, cell shrinkage, involvement of caspases, exposure of cell surface phosphatidylserine, and more are all features of classically defined apoptosis. However, exceptions can be found for any one of these, and a number of these features have also been reported during processes, such as frank necrosis, that are not normally considered to be true apoptosis. An additional complexity in attempts to pigeonhole the different processes of PCD is that they also are often not temporally discreet. Thus, if an apoptotic cell is not removed by the normally efficient clearance mechanisms, it undergoes secondary changes, often called secondary necrosis or cytolysis, which eventually result in liberation of cell contents into the environment (13, 35). In the context of this review and the discussion of apoptotic cell detection in the lung, we will focus primarily on caspase-mediated apoptosis. However, as other markers become readily available for different forms of cell death, it may become important to explore these in different lung diseases.

Induction of apoptosis. The mechanisms by which apoptosis is induced and regulated in different cells have received a great deal of investigation (see for example reviews in Refs. 1, 7, 11,
In simple terms, extrinsic (from membrane receptor signaling and mediated through caspase 8) or intrinsic (involving the mitochondria and mediated through caspase 9) pathways lead to activation of executioner caspases (caspases 3 and 7) that initiate the multiple changes in the cell, including the alterations to the nucleus. Stimuli for extrinsic pathways involve activation of TNF family receptors, including Fas and TNF receptors themselves (68). Intrinsic pathway activation can result from genotoxic effects, oxidants, ultraviolet and gamma irradiation, and alteration of the homeostatic interactions between pro- and anti-apoptotic Bcl-2 family members (7). Assays to detect active caspases, or their effects, represent some of the methods used to identify apoptotic cells. However, it should be noted that noncaspase modes of PCD (which have also sometimes been called apoptosis) exist (e.g., see Ref. 51). Other changes seen in “apoptosing” cells are alterations to the cell surface. These are critical to recognition of the dying cell, leading both to its removal and to its effects on macrophages and surrounding cells. Some of these changes are outlined in recent reviews (22, 37, 63) and include the exposure of phosphatidylserine on the outer membrane leaflet, which is also sometimes been called apoptosis (actually anti-inflammatory), whereas necrotic cells induce inflammation (and in some models, constitute active “danger signals” to initiate local tissue and immunologic responses) (20, 21). An example of this difference between cells is the relative persistence of intact apoptotic neutrophils compared with that shown in lymphocytes (a more rapid loss of cell integrity after apoptosis; Henson, unpublished observations). A teleological explanation could be that cells such as neutrophils with higher content of proinflammatory and injurious molecules should be rapidly cleared before disruption.

**Apoptosis vs. necrosis.** A distinction is often made between apoptosis and necrosis, in terms of mechanism and morphological characteristics of cell death on the one hand and in the tissue response to the dying cell on the other (see Table 1 and Refs. 11, 52). A key separation between these two endpoints is the integrity of the cell structure, i.e., the disruption of cell membrane and loss of cell contents in necrotic cells. However, this also is imprecise, particularly in the context of differentiation between necrosis and apoptosis in tissue sections of pathological specimens. In this setting, although necrotic cells have lost their cellular structure, their outlines may be relatively preserved. On the other side, apoptotic cells may initially retain their plasma membrane integrity, but most cell types do liberate membrane blebs (so-called apoptotic bodies), leading to decreased cell size (11). With time, apoptotic cell membranes become permeable by increasing degrees, although the cell itself may not disintegrate. Accordingly, during apoptosis, each cell type may exhibit a different window of time wherein the membrane remains impermeable and the cell remains essentially intact before undergoing secondary necrosis and loss of contents and structure. This window is thought to be important for effective phagocytic removal of the dying cell before the release of potentially injurious, proinflammatory, and proimmunogenic contents into the tissues. These concepts are intimately linked to the perhaps oversimplified but common concept that apoptotic cells are noninflammatory (actually anti-inflammatory), whereas necrotic cells induce inflammation (and in some models, constitute active “danger signals” to initiate local tissue and immunologic responses) (20, 21). A related complexity in terminology is the distinction often seen in the literature between “early” and “late” apoptotic cells. This usually refers to cells in culture rather than in tissues and often reflects different effects on recognition of, and response to, the dying cells by macrophages. Sometimes, the distinction is merely a temporal one, but other investigators have distinguished late apoptotic cells as those that begin to show permeability of their plasma membranes to dyes such as propidium iodide (see below), while still retaining overall integrity of cell structure. Others might consider these as examples of early secondary necrosis. The clear-cut implication of these comments is to draw attention to the need to define the characteristics of the cell undergoing apoptosis or other forms of PCD in any studies of the processes involved or the responses to such cells, certainly in vitro and wherever possible in vivo as well.

**Table 1. Comparisons between apoptosis and necrosis**

<table>
<thead>
<tr>
<th>Type of Cell Death</th>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear changes</td>
<td>Chromatin condensation</td>
<td>Random fragmentation and degradation of DNA</td>
</tr>
<tr>
<td></td>
<td>Internucleosomal DNA cleavage</td>
<td>No DNA laddering</td>
</tr>
<tr>
<td></td>
<td>DNA laddering</td>
<td></td>
</tr>
<tr>
<td>Morphological changes</td>
<td>Often membrane blebbing</td>
<td>Cell swelling and rupture</td>
</tr>
<tr>
<td></td>
<td>Formation of apoptotic bodies</td>
<td>Vacuolation</td>
</tr>
<tr>
<td>Membrane integrity</td>
<td>Retention of membrane integrity (to PI penetration) in early apoptotic cells. Late apoptotic cells may become PI positive</td>
<td>Loss of membrane integrity, becoming PI and Trypan blue positive</td>
</tr>
<tr>
<td>Surface changes</td>
<td>Development of apoptotic cell recognition ligands including phosphatidylserine and calreticulin</td>
<td>May also express similar surface changes, in part because of the loss of membrane integrity and access to intracellular structures; however, as noted below, these may be less effective as stimuli</td>
</tr>
<tr>
<td>Effect on inflammatory responses</td>
<td>Generally anti-inflammatory, inhibiting the production of proinflammatory mediators and stimulating nearby cells to produce anti-inflammatory molecules</td>
<td>Generally proinflammatory, stimulating the production of proinflammatory mediators</td>
</tr>
</tbody>
</table>

**Assays**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUNEL</td>
<td>Positive</td>
<td>Can be positive</td>
</tr>
<tr>
<td>Annexin V</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Active caspase</td>
<td>Positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>

PI, propidium iodide; TUNEL, transferase-mediated dUTP nick end labeling.
Apoptosis and necrosis are dynamic processes, and histological observations serve only as a snapshot. As will be discussed below, a critical issue here is that detection of increased numbers of apoptotic cells in a tissue may indeed mean increased rates of apoptosis; however, it is equally possible that there is decreased clearance of apoptotic cells or that both of these processes together contribute to increased detection (Fig. 1). Necrotic cells are also usually cleared from tissues (even if the inflammatory and immunologic responses to recognition of necrotic cells differ from those of apoptotic cells), so that detection of necrotic cells in tissues may lead to the same sets of questions. Larger areas of necrosis, for example after ischemia, may suffer from delays in clearance because scavenger cells (usually macrophages) are insufficient in numbers and/or cannot penetrate the damaged tissue. Similar issues of (temporary?) overload of the clearance mechanisms may also explain detection of apoptotic cells.

DETECTION OF INCREASED NUMBERS OF APOPTOTIC CELLS IN THE LUNG

Examination of tissues from many different types of lung disease has revealed the presence of apoptotic cells, often in excess of those seen in control “normal” tissue. One example of this is in chronic obstructive pulmonary disease (COPD) (28, 32, 42). These types of observation bring up a number of key questions. One is the issue of appropriate controls. There is a reasonable expectation of increased PCD with age, so matching for this is critical. However, obtaining normal human lung tissue is difficult and emphasizes the need for shared tissue banks. Apoptotic cells have been detected in bronchiolar lavage or induced sputum, which may indeed indicate enhanced PCD and/or decreased clearance. However, the clearance question here may indicate different issues from those seen in the tissues themselves. For example, apoptotic neutrophils in cystic fibrosis may be caught up in the mucus and not be available to macrophages for easy removal (77). The inefficiency of sampling from such sources limits quantitative assessment and again brings up the question of controls. In all cases, it behooves the investigator to include in the examination the presence of apoptotic cells that may be located within the vacuoles of phagocytes. In fact, substantial information can be gleaned from the proportions of free vs. ingested apoptotic cells that can help address the issue of defective clearance (because digestion is the ultimate fate of the ingested cell, the detection of phagocytosed apoptotic cells could also reflect defective digestion). The identity of the cell that is apoptotic will also be critical. Is this a generalized observation; for example, in the alveolus does apoptosis extend to all cells of the alveolar structure or is it limited to only epithelium, endothelium, or interstitial cells? This distinction is likely to be important in determining cause, implications, and effects. The type of cell is often hard to distinguish by light microscopy and requires cell-specific markers that might, unfortunately, be lost during the apoptotic process. Because the form of PCD will also be important for both cause and effect of the dying cells, this too constitutes a question that could reasonably be asked when dead cells are detected. However, this may be very difficult to ascertain in tissues because markers for the different types of cell death are not always specific and, as noted, may for any given cell, depend on time from initiation of the PCD processes.

METHODS OF APOPTOTIC CELL DETECTION

The list of pathobiological settings associated with apoptosis is growing and includes almost all of the relevant lung diseases, such as acute lung injury (41, 44), emphysema (8, 28), pulmonary hypertension (4, 71), lung cancer, and fibrotic lung processes (40, 72). The documentation of apoptosis in lung cells has been critical in such studies. The lung has more than 40 native cells, highlighting the potential challenge to elucidate the role of apoptosis in a particular lung cell population. The dynamic nature of apoptosis involving complex cellular signaling events taking place within a cell and among different cells of an organ can hardly be captured by a snapshot assay or measurement (Fig. 2). The decision of how best to document apoptosis rests on the understanding of some critical biological and molecular aspects of this process, which can be divided into three stages: 1) initiation or trigger stage, 2) decision or initial stage, and 3) execution stage (75, 76) (Fig. 2). The initiation stage involves signaling pathways related to preparations for the cell to die (76), including altered expression of Bcl-2 family members (Table 2), overexpression of c-myc, or localized hypoxia (61). Although highly relevant to the understanding on how pathological triggers lead to activation of apoptotic process, the identification of these signaling mechanisms does not provide documentation per se that apoptosis is indeed happening or is important for the observed lung pathology. The decision process for the intrinsic pathway involves the interaction between the mitochondria and Bcl-2 family of molecules (76). Progressive depolarization of mitochondrial transmembrane potential and opening of the mitochondrial...
3. Execution

2. Decision

duration, lasting a few hours (69). Available to document lung apoptosis rely on late features of the apoptotic process. In summary, most of the assays presently aimed at characterizing execution stages involve global cellular and molecular alterations, all of which can be used to document the different stages of cell death, and, to a limited extent, to lung tissue sections or fluorescence-activated cell sorting (FACS) preparations (Table 2). The decision process can be applied at the isolated cell level and, for example, by aggregation of partly cleaved keratin 18 and 19 in apoptotic lung cells. PS, phosphatidylserine; \( \Psi_m \), mitochondrial transmembrane potential.

As mentioned above, the value of an endpoint of apoptosis will depend on the rate of apoptosis, the clearance rate of apoptotic cells, and the length of the specific process being probed. For example, cytochrome \( c \) release occurs rapidly and is complete by 5 min of an apoptotic stimulus, before the exteriorization of phosphatidylserine and the loss of plasma membrane integrity (24). Further important considerations consist of the specificity of the particular assay, since necrotic and actively transcribing cells share some of the endpoints used for apoptosis. Each of these criteria may be better suited for different cell preparations, i.e., lung sections, dispersed lung cells, or lung cultures. As apparent throughout the present discussion, all of these preparations provide complementary documentation of apoptosis in the lung.

As summarized in Table 1, the characterization of apoptosis can be done morphologically (62), by assessment of cell membrane changes, by documentation of DNA fragmentation, and by measurement of enzyme processes involved in DNA fragmentation and protein degradation. Furthermore, several assays that measure cell and nuclear permeability with cell-impermeable dyes aid significantly in complementing the morphological documentation of apoptosis (Table 2). Examples are shown in Figs. 3 and 4.

**Apoptotic cell morphology.** Although cell morphology (for example, nuclear condensation) at the light or electron microscopic levels is considered the gold standard of apoptosis (76), it is of limited use in lung tissue sections. Apoptotic cell recognition by light microscopy is difficult in the lung compared with in organs that have a large number of apoptotic cells with high cell turnover, such as lymphoid follicles or gastrointestinal epithelium. Another morphological aspect of an apoptotic cell is the characteristic appearance of cell markers, such as aggregation of partly cleaved keratin 18 and 19 in mesothelial cells (43) and fibroblasts (47). Enhanced accuracy of cell morphology can be achieved by flow cytometric analysis of cultured or dispersed lung cell preparation using forward scattering (measuring size) and side scattering (measuring intensity).

Table 2. Comparison of different methods to analyze apoptosis in different experimental samples pertinent to the study of apoptosis in the lung.

<table>
<thead>
<tr>
<th>Stages of Apoptosis</th>
<th>Cell Culture</th>
<th>FACS</th>
<th>Lung Lysates</th>
<th>Histological Lung Sections</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Trigger</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Cell signaling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Decision</td>
<td>+</td>
<td>++</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Mitochondrial transmembrane depolarization</td>
<td>+ +</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bcl-2 family member assays</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Opening of mitochondrial pore</td>
<td>+ +</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Annexin V, low expression</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3. Execution</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphology</td>
<td>+</td>
<td>++</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Cell membrane function</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Annexin V</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td>PI/7-AAD</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DNA fragmentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphology</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Comet assay</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TUNEL</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>In situ ligation of labeled DNA fragment</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Single stranded DNA IHC</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Protease activation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active caspase 3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Protein targets of caspase 3 (lamin, PARP, cytokeratin 18)</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Cell signaling events involve signaling cascades that may lead to activation of the decision stage leading to apoptosis, such as c-myc overexpression or localized hypoxia. 7-AAD, 7 amino-actinomycin D; IHC, immunohistochemistry; PARP, poly-ADP-ribose polymerase. *Not tried but potentially useful; "potential useful; ++ useful; − not useful.

Invited Review

L604

Fig. 2. Stages of apoptosis: outline of cellular and molecular events that take place in the cytoplasm, nucleus, cell membrane, and mitochondria throughout the process of decision and execution. Effecrocytosis leads to removal of apoptotic cells. The final balance between engagement of the apoptotic process and apoptotic cell clearance will dictate the signal of specific assays aimed at identifying and quantifying apoptotic lung cells. PS, phosphatidylserine; \( \Psi_m \), mitochondrial transmembrane potential.
double labeling with cell-specific markers. Proper gating is required to segregate particular cell populations or dispersed lung cells by FACS. However, dispersed lung cells uniform cell populations, such as in cell culture, and to disperse granularity (39). These parameters can be easily applied to document in lung sections. The most popular methods to map morphologically in different organs, is difficult to reliably apoptosis (59, 80). DNA fragmentation, which can be observed and, conversely, pronounced DNA damage may not represent can occur in the absence of significant DNA fragmentation, of apoptosis. However, it should be pointed out that apoptosis early and late stages of lung cell apoptosis in vivo. This is a characteristic but late feature DNA fragmentation.

Cell membrane changes. Most assays to measure cell membrane changes rely on functional tests using live cells (generally in culture). Binding exposed on the outer face of the cell membrane allows the recognition of most if not all apoptotic cells throughout the execution stage and potentially throughout the decision process (75). Because phosphatidylserine is normally present on the inner leaflet of the membrane, any disruption of the membrane can lead to annexin V positivity. The assay is therefore usually accompanied by a probe (propidium iodide) or 7-amino actinomycin D for membrane permeability, and true apoptotic cells are taken to be annexin V positive and propidium iodide negative. Unfortunately, although useful, this assay is not completely specific because viable endothelial cells primed by inflammatory stimuli can also transiently expose phosphatidylserine (18). Although annexin V assay has been mostly used for cell cultures and dispersed lung cells by FACS, membrane exteriorization of phosphatidylserine can be potentially monitored in vivo with intravenously administered biotinylated annexin V, as documented with whole chicken embryos (66, 74) and mouse hearts subjected to ischemia-reperfusion (76). A similar approach may be suited to the study of cell membrane alterations in the early and late stages of lung cell apoptosis in vivo.

DNA fragmentation. This is a characteristic but late feature of apoptosis. However, it should be pointed out that apoptosis can occur in the absence of significant DNA fragmentation, and, conversely, pronounced DNA damage may not represent apoptosis (59, 80). DNA fragmentation, which can be observed morphologically in different organs, is difficult to reliably document in lung sections. The most popular methods to map DNA fragmentation are DNA labeling [transferase-mediated dUTP nick end labeling (TUNEL), in situ end labeling, or DNA nick translation] and DNA laddering. TUNEL, which is based on the incorporation of a labeled deoxyuridine on free 3’ DNA ends by terminal deoxynucleotidyl transferase, provides the most sensitivity and specificity compared with the other two labeling methods, in both cell cultures (62) and FACS (56). It offers the advantage of double labeling with cell-specific markers in tissue sections (57), even with prolonged (<12 h) postmortem delays in processing (65) (Fig. 3). However, three important caveats limit its specificity. It detects necrotic cells, which have extensive double-strand breaks and long 3’-prime overhangs (9), live cells with active transcription (36), and active DNA repair (78). This recognized lack of specificity of TUNEL regarding the true detection of apoptosis warrants the use of alternative approaches, such as active caspase 3 detection. Furthermore, nonspecific staining should be controlled by optimization using negative controls, based on omission of the enzyme terminal deoxynucleotidyl transferase and positive controls (DNase 1-treated cells), particularly when targeted for lung sections (Fig. 3) (38, 50, 79). On the other hand, apoptosis-specific single nucleotide 3’ overhangs can be labeled in tissue sections by in situ ligation of a labeled 200-bp PCR product (9). This assay works well in lung sections, with a high specificity for apoptosis but somewhat lower sensitivity than TUNEL. Single-stranded DNA antibodies detect breaks revealed in tissue sections subjected to heat denaturation and have strengths similar to that shown with situ-labeled DNA ligation (17, 73).

Early in apoptosis, cells maintain their DNA supranucleosomal order of packaging. Later, degradation of DNA leads to condensation and oligomerization, initially into 50- to 300-kbp fragments and eventually into multimers of ~200 bp (which provides the classical DNA ladder in ethidium bromide-stained gels). In this stage, the DNA forms globules of ~30 nm in

![Fig. 3. Transferase-mediated dUTP nick end labeling (TUNEL) in alveolar tissue of a normal mouse lung. A: positive control for TUNEL with DNase 1-treated histological section, showing widespread nuclear positivity. B: negative control in which the lung section was processed as in A but with the enzyme terminal deoxynucleotidyl transferase. C: example of TUNEL-positive (green signal), prosurfactant protein C (SpC)-positive type II cell (red signal) (long arrow), in a mouse lung section. Viable SpC-positive cells lack TUNEL positivity (arrowhead), whereas apoptotic non-type II cells can also be detected in the section (short arrow).](http://ajplung.physiology.org/content/294/4/L605/F3)

---

Invited Review

L605

AJP-Lung Cell Mol Physiol • VOL 294 • APRIL 2008 • www.ajplung.org

Downloaded from http://ajplung.physiology.org/ by 10.220.33.3 on April 19, 2017
diameter (12). DNA laddering detection requires at least $5 \times 10^5$ DNA cell equivalents (60). An attractive alternative is the use of linker-based amplification of 200-bp oligomers, which can detect <2% apoptotic cells in a sample (45). This apoptotic-specific assay offers the advantage of only needing small samples of lung and potential quantification using the density of one of the oligomer bands, normalized by an internal control apoptotic DNA (32) (Fig. 4). Furthermore, oligomers can be quantified with an ELISA with histone antibodies (60). On a single cell level, the Comet assay

![Images of cellular structures with arrows indicating DNA ladders and other relevant patterns.](http://ajplung.physiology.org/)
allows the measurement of total DNA and DNA with strand breaks in a given cell (15).

**Cell proteolysis.** Degradation of cell structures is carried out by a series of cysteine proteases, caspases, particularly caspase 3, and other enzymes, such as proteosomal proteases and calpain. Active caspase 3 is probably involved in most alveolar apoptotic processes (see for example, Ref. 81). Antibodies directed to the cleavage site of the 18-kDa (active) form of caspase 3 have been instrumental in the documentation of apoptosis at the tissue and cell culture levels (70) (Fig. 4). An important consideration in the optimization of immunohistochemical detection of tissue markers is the need for antigen retrieval with heat denaturation in the presence of specific buffers, such as citrate-based solutions or the Borg Decloaker. Although, active caspase 3 immunolocalization provides solid support for apoptosis, the specific retrieval procedures for active caspase 3 immunohistochemistry limit the concomitant immune detection of specific markers of lung cells (55, 57). The localization assays can be further complemented with activity assays performed in tissue lysates (that often measure active caspase 7 as well) (55). Antibodies against specific epitopes on protein targets of caspase 3 provide additional support of apoptosis in the lung, such as lamin (2), poly ADP(ribose) polymerase, and cytokeratin 18 (54).

**WHAT IS NEEDED TO DOCUMENT APOPTOSIS IN THE LUNG**

Several of the methods discussed previously are better suited for qualitative analyses, such as those based on electron microscopy. Importantly, most of the assays lend themselves to quantification. However, it is not appropriate to compare multiples of changes among the different assays. These are assays based on different platforms, with particular characteristics concerning the dynamic range and specificity, providing a snapshot of a dynamic process within a heterogeneous tissue (i.e., lung).

Precise quantification of apoptotic endpoints among different cells and compartments within the lung tissue can only be provided by FACS or labeling on tissue sections. FACS combines the advantage of a multistep quantitative approach for a refined delineation of apoptosis in specific (i.e., lymphoid) cells (39, 81). Quantification of apoptotic cells (e.g., using TUNEL or active caspase 3 immunohistochemistry) requires the application of rules of stereology. In a 5-μm section, a positive “cell” in fact represents a positive cellular profile or outline. The statistical chance that a positive cellular profile is positive “cell” in fact represents a positive cellular profile or the application of rules of stereology. In a 5-μm section, a positive “cell” in fact represents a positive cellular profile or outline. The statistical chance that a positive cellular profile is positive “cell” in fact represents a positive cellular profile or

**INCREASED APOPTOSIS VS. DECREASED CLEARANCE**

The most obvious, and common, interpretation of detecting apoptotic cells in the lung is that it represents increased cell death and demands an assessment of why and how this may be occurring. However, apoptotic cells are also rapidly and efficiently cleared from most tissues so that the net observation at any one point in time (e.g., a tissue section) is a combination of apoptosis induction and apoptotic cell removal (effector cytokinosis). Because most cells in the body undergo some endogenous rate of turnover, defective clearance could result in the appearance of increased numbers of apoptotic cells in a tissue even in the absence of enhanced cell death processes. The overall efficiency of these clearance processes (see below) have led us to suggest that finding apoptotic cells should automatically generate questions of clearance defects or overload as well as those of increased cell death, or more likely all acting together.

One of the first demonstrations of how efficient and capacious the apoptotic cell clearance processes could be was in a study by Scott et al. (67). They induced apoptosis of the thymocytes in mice with dexamethasone. Examination of the thymus in wild-type mice yielded scanty apoptotic cells at any adjacent planes are recorded as a single positive cellular event, i.e., one cell positive for a particular marker. This can be accomplished easily with confocal immunofluorescence. Bright-field signals require a thick section (~20–25 μm in thickness) and imaging through tissue planes obtained along a z-plane (through the thickness of a section). This can be accomplished with a lens with a high numerical aperture (e.g., ×60 oil) (31). The results can be normalized by total 4',6-diamidino-2-phenylindole-positive nuclei (in case of single labeling) or cells expressing a specific cell marker (i.e., prosurfactant C).

In summary, no single method can accurately describe and document apoptosis in the lung. A complementary approach based on at least two or more assays probing into different aspects of apoptosis will therefore provide the most compelling evidence of lung cell apoptosis. TUNEL is one of the most used assays and offers the advantage of colocalization imaging with cell-specific markers. Given its important caveats, it is recommended that assessment of active caspase 3 expression (immunohistochemical detection and quantification) or activity assays using lung lysates be used to support the findings obtained with TUNEL. In situ approaches have the advantage of preservation of valuable tissue samples, the evaluation of the identity of a given apoptotic cell, and analysis of localized events, particularly aimed at late in the apoptotic process. FACS of single cells prepared by lung digestion offer the potential for dissection of early and late events in specific lung cells. However, these procedures may induce apoptosis themselves or cause loss of surface markers due to the enzymatic digestion.

---

**Fig. 4.** A and B: active caspase-3 expression (arrows) detected by immunohistochemistry in a rat lung treated with a combination of an anti-VEGF receptor 1 (MF-1) and receptor 2 (DC101) antibodies, which causes alveolar cell apoptosis and progressive alveolar enlargement. Negative control consisted of rabbit serum in place of anti-caspase 3 antibody, which showed complete lack of reaction with the lung tissue. Note that there is a marked reduction in caspase 3-positive cells in rat IgG-treated rats (B). C–E: colocalization of TUNEL (arrows) (green signal) and endothelial cell (CD34, red; C or SpC (red; D) of rat lungs treated with DC101 plus MF-1 (as shown in A). Note the marked lack of TUNEL positivity in IgG-treated rat lungs in E (as in B). F and G: colocalization of TUNEL (green, arrow) and smooth muscle cell α-actin (red) in rats exposed to chronic hypoxia (F) or room air (controls; G). In F, note an apoptotic endothelial cell (arrowhead) and a positive cell outside the vascular smooth muscle cell ring (arrow). H: evidence of apoptosis in rat lungs treated with the VEGF receptor blocker SU5416 for 3, 7, and 21 days vs. vehicle control (reproduced with permission from Ref. 33). I and J: single-stranded DNA immunohistochemistry of a rat lung treated with SU5416 for 3 wk (arrows; I) compared with vehicle control (arrows; J) (reproduced with permission from Ref. 73).
time during the subsequent clearance process. On the other hand, dexamethasone caused large numbers of apoptotic thymocytes in mice lacking in Mer, a receptor tyrosine kinase that is involved in recognition and ingestion of apoptotic cells. The rates and extent of thymocyte apoptosis were not different between the different mice. On reflection, we can also infer the tremendous capacity of the clearance processes from the huge ongoing turnover, i.e., removal, of circulating blood cells (for example, >10^{11} neutrophils per day in humans). Resolution of inflammation is another situation in which apoptosis of the inflammatory cells usually leads to their extremely efficient removal. An example from the lung is the detection of remarkably low numbers of apoptotic cells in lavage analysis of patients with community-acquired pneumonia (10) or in our own studies of apoptosis during resolution of experimental inflammation in mice (30). Even in these circumstances, one must also question whether the apoptotic cell that is detected has already been ingested but not yet digested, for example by a macrophage. By contrast, in some inflammatory lung diseases, larger numbers of apoptotic cells are detectable and have been shown to be associated with local defects in clearance [e.g., cystic fibrosis (77) and COPD (28, 49)].

However, we should note that most of the evidence for very high capacity clearance in vivo has come from deletion of hematopoietic cells. Does this also apply to pulmonary epithelial (or for that matter, endothelial) cells? Apoptosis of cells in an epithelium has been suggested to be accompanied by active extrusion from the monolayer (58); whether this process occurs before or after or whether it accompanies the apoptotic process is so far unclear. This would suggest removal of apoptotic epithelial cells in the alveolar lumen (or via the mucociliary escalator in the lung or into the intestinal lumen in the gut). On the other hand, epithelial cells can function as highly efficient phagocytes for apoptotic cells. An example here is in the postlactation involution of the mammary gland. Forced weaning of mice (also not uncommon in humans) leads to complete removal of the mammary alveolar epithelium in 4 days, a process of apoptosis and ingestion of the apoptotic cells by remaining viable epithelial cells (46). Some of the epithelial cells are extruded from the monolayer and ingested secondarily by the phagocytic epithelial cells, and some appear to be ingested in situ in the epithelium without apparent extrusion. Can these types of clearance mechanisms occur in the lung? How much clearance of apoptotic epithelial cells results from extrusion? How much is carried out by macrophages, and how much by epithelial cells that have become activated (as in the mammary gland) to become more efficient phagocytes? How much clearance of apoptotic cells in the airway lumen (including of inflammatory cells) is carried out by the epithelium? These questions and others still need to be answered. Similar issues and questions could be raised for apoptosis in the alveolar endothelium.

What then is the capacity for cell removal in different compartments of the lung? The emphasis above has been placed on local ingestion by phagocytes of varying types, which we feel is the usual mode of apoptotic cell clearance. However, as noted, clearance via the airways may represent a mode for removal in the lung, and the relative proportions of cells that are removed from the alveolar or airways lumen by each mechanism are very difficult to determine. An additional point here is that pulmonary dendritic cells are also likely candidates for phagocytosis of apoptotic cells, with potential consequences for either suppression or activation of local adaptive (auto)immune responses.

### EFFECTS OF RECOGNITION AND UPTAKE OF APOPTOTIC CELLS IN THE LUNG

Although an immediate implication of finding apoptotic cells in the lung is the effect of losing functional cells from the tissue, the actual process of apoptotic cell recognition also has important secondary consequences. Recognition and uptake of apoptotic cells are themselves noninflammatory and in fact have been shown to lead to an anti-inflammatory and anti-immunogenic environment. These effects are mediated in part by inhibitory intracellular signaling pathways (5) and in part by generation of anti-inflammatory mediators such as transforming growth factor (TGF)-β, IL-10, PGE2, PGI2, and the like (14, 19). This is usually contrasted with the proinflammatory effects induced by necrotic cells. However, it should be noted that most of the contrasts involve artificial experiments using necrotic cell debris induced by excessive heat or freezing and thawing, conditions not usually seen in real life. The stimuli on apoptotic or necrotic cells that induce these contrasting effects are not fully understood. Recognition of exposed phosphatidylinerine appears to drive much of the anti-inflammatory and anti-immunogenic effects (14, 19, 29), whereas release of intracellular constituents during necrosis, including from mitochondria, lysosomes, and nucleus, likely all contribute to the subsequent enhanced inflammation. Nevertheless, cell disruption also exposes phosphatidylinerine, and, on the other side, apoptosis can lead to exposure of proinflammatory stimuli such as DNA (53) and calreticulin (23). In each case, therefore, a balance between potential suppressors and inducers of inflammation and immunity might be expected, with varying local effects depending on cell type, mode of PCD induction, local environment, and efficacy of clearance. In general, the rapid removal of cells undergoing PCD is thought to limit the generation and/or release of proinflammatory stimuli, and the anti-inflammatory effects are suggested to dominate. Abnormalities of this balance may then be sought in conditions of overexuberant or prolonged inflammation or in examples of autoimmunity.

These anti-inflammatory consequences of apoptotic cell recognition (which by the way can occur from cell contact alone and do not require actual uptake of the apoptotic cell) are thought to play a major role in normal resolution of inflammation, including that in the lung (27, 30, 64). The concept leads inevitably to the question of defects in the process in chronic inflammatory conditions, for example, in cystic fibrosis, asthma or COPD.

Another intriguing consequence of apoptotic cell recognition may be to help initiate repair. Normal tissue homeostasis presumably involves efficient replacement after death of individual cells. Generation of growth factors in response to apoptotic cell recognition could provide a local stimulus for this replacement. Thus the demonstration that apoptotic cells can stimulate the production of VEGF (25) or hepatocyte growth factor (48), growth factors for endothelial and epithelial cells, respectively, is in keeping with this suggestion. Although direct demonstration of this effect in vivo is thus far lacking, one might even extend the concept to ask whether recognition...
of apoptotic cells might lead to production of chemotactic factors for tissue precursor cells that could also mediate repair of the injured lung by replacement of the apoptotic cells.

However, repair can also represent fibrotic changes in the tissue (as in classical wound healing). Thus the well-documented ability of apoptotic cells to stimulate the production of active TGF-β from responding cells (3, 14) provides yet another consequence of recognition, although here representing a double-edged sword. Apoptotic cells and the phosphatidyserine exposed on their surface have, therefore, to be considered as a candidate for the induction of the profibrogenic TGF-β in the various lung diseases in which local or generalized fibrosis occurs.

CONCLUSIONS AND IMPLICATIONS

Finding apoptotic cells in lungs under various pathophysiological states is important. However, we suggest that it is now incumbent to further consider in each circumstance the identity of the cells undergoing PCD, the mechanisms of induction and execution, and tissue. On the other hand, despite these various implications, we should note that definitive demonstration of the effects of apoptosis, or its recognition, in lung diseases is extremely difficult. Inhibition of executioner caspases has been used, for example, to block development of emphysematous changes in experimental models of emphysema (e.g., Ref. 33). However, the administration of such agents may not be feasible in longer term pathogenic processes. Furthermore, blocking caspase effects likely allows damaged cells, unable to undergo normal apoptosis and removal, to undergo later nonapoptotic forms of death and cell destruction (necrotic processes), which have their own complex biological effects in the lung. Because the biological response to apoptotic cells is a highly redundant process, involving numerous recognition ligands, receptors, and bridge molecules, it is also not easy to simply manipulate the consequences of apoptosis at this recognition step. Nevertheless, difficult does not mean impossible, and we suggest that there is much to learn from addressing the questions raised, both for understanding pathogenesis and for potential new therapeutic approaches to lung diseases.

ACKNOWLEDGMENTS

The authors thank Shehzin Mozammel and Rheda Girgis for the caspase 3 images and Ann Hoa Le Thi for the TUNEL images.

GRANTS

This work was supported by National Institutes of Health Grants HL-68864, HL-81151, GM-61031, and HL-66554.

REFERENCES


