ANNALS OF SURGERY
Vol. 231, No. 3, 424–431
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Cell Death in Human Lung Transplantation: Apoptosis Induction in Human Lungs During Ischemia and After Transplantation

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Objective
To examine the presence and extent of apoptosis as well as the affected cell types in human lung tissue before, during, and after transplantation.

Summary Background Data
Apoptosis has been described in various human and animal models of ischemia-reperfusion injury, including heart, liver, and kidney, but not in lungs. Therefore, the presence of apoptosis and its role in human lungs after transplantation is not clear.

Methods
Lung tissue biopsies were obtained from 20 consecutive human lungs for transplantation after cold ischemic preservation (1–5 hours), after warm ischemia time (during implantation), and 30, 60, and 120 minutes after graft reperfusion. To detect and quantify apoptosis, fluorescent in situ end labeling of DNA fragments (TUNEL assay) was used. Electron microscopy was performed to verify the morphologic changes consistent with apoptosis and to identify the cell types, which were lost by apoptosis.

Results
Almost no evidence of apoptosis was found in specimens after immediate cold and warm ischemic periods. Significant increases in the numbers of cells undergoing apoptosis were observed after graft reperfusion in a time-dependent manner. The mean fraction of apoptotic cells at 30, 60, and 120 minutes after graft reperfusion were 16.6%, 22.1%, and 34.9% of total cells, respectively. Most of the apoptotic cells appeared to be alveolar type II pneumocytes, as confirmed by electron microscopy.

Conclusions
Programmed cell death (apoptosis) appears to be a significant type of cell loss in human lungs after transplantation, and this may contribute to ischemia-reperfusion injury during the early phase of graft reperfusion. This cell loss might be responsible for severe organ dysfunction, which is seen in 20% of patients after lung transplantation. Therefore, this work is of importance to surgeons for the future development of interventions to prevent cell death in transplantation.

Since the first successful human lung transplantation, reported by Cooper in Toronto in 1983,[1] this procedure has become standard treatment for end-stage lung diseases such as cystic fibrosis, emphysema, primary pulmonary hypertension, idiopathic pulmonary fibrosis, and others.[2] As with other forms of organ transplantation, there remains a significant shortage of available donor organs. Human lung transplantation is further limited, however, by the fact that only approximately 20% of lungs from potential donors are suitable for transplantation due to factors such as trauma, pulmonary edema, and aspiration.[3,4] After lung transplantation, severe life-threatening graft dysfunction occurs in up to 30% of patients, related to ischemia/reperfusion (I/R) injury.[5] This injury is due primarily to mechanisms that increase pulmonary vascular permeability and cause alveolar epithelial cell damage.[6] Currently, there is no histologic

Supported by a grant from the National Sanitarium Association of Canada.
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Accepted for publication June 17, 1999.

I/R injury scoring system available to quantify the degree of injury during preservation and reperfusion in transplanted organs. Edema, inflammation, neutrophil migration, intraparenchymal hemorrhage, and necrosis have all been accepted as histologic markers of injury after organ transplantation.[7,8]

When sufficient injury is inflicted on a cell, there are two major types of cell death that can occur: necrosis and apoptosis. Necrosis is a form of irreversible cell death accompanied by the loss of cell membrane integrity and ion pump damage. This results in cell swelling, lysis, and release of intracellular enzymes and lysozymes, followed by neutrophil migration, inflammation, and edema.[9] In clinical and experimental work, it has been found that the degree of necrosis in transplanted organs does not necessarily parallel the posttransplant physiologic organ function.[10,11] Another form of cell death, apoptosis, an area of intense clinical and scientific investigation, is thought to be a process of programmed cell death. As observed by electron microscopy, apoptotic cell death is morphologically characterized by overall cellular condensation, shrinkage, and plasma membrane blebbing.[9] Nuclear changes are characterized by chromatin margination and nuclear condensation followed by segmentation and DNA fragmentation. Finally, the apoptotic cell is broken up into smaller membrane-bound apoptotic bodies that are usually phagocytosed by macrophages.[9] An important morphologic distinction from necrosis lies in the fact that apoptotic cell death lacks inflammation.[12]

There are strong indications that apoptosis plays an important regulatory role in various disease processes such as cancer,[13,14] autoimmune diseases,[15,16] and others.[17–21] Increasing evidence suggests that alterations in the genetic control of cell survival and elimination seem to be important regulators in these disease states. Of particular importance in organ transplantation, apoptosis has recently been reported in several animal models to occur after I/R injury to the kidney, retina, brain, heart, liver, and adrenal gland.[22–27]

Many laboratory studies have shown a relation between organ injury, including I/R injury, and apoptosis induction, whereas it is unknown whether a more significant injury triggers necrosis and a milder injury initiates apoptotic cell death.[28–32] It has been reported that apoptosis appears in rabbit cardiomyocytes after ischemia followed by reperfusion, but it is not detectable after ischemia alone.[23] These findings are of particular interest to transplantation medicine and surgery.

At present, little is known about the relation between I/R injury and the induction of apoptosis after human organ transplantation. Isolated reports of a descriptive nature are available regarding apoptosis in human liver,[33] kidney, pancreas,[34] and heart transplantation,[35] whereas apoptosis after human lung transplantation has not been previously described. Given that lung preservation differs from all other organs in that lungs stored inflated with oxygen undergo aerobic, ischemic, hypothermic preservation[36,37] (in contrast to other organs, which undergo anaerobic, ischemic, hypothermic preservation), the nature of the injury at the cellular and subcellular level could indeed be different. Consequently, the aims of this study were to examine apoptosis after human lung transplantation and to investigate the relation between I/R injury and the induction of apoptosis. Further, we explored the potential of apoptosis as a marker for severity of I/R injury and its relation to organ function after human lung transplantation. We also report the time course of the induction of apoptosis after graft reperfusion, including quantification of apoptosis and the cell types affected in human lung allografts before, during, and after transplantation.

PATIENTS AND METHODS

Lung tissue was obtained from 20 consecutive patients undergoing lung transplantation. Consent for biopsy to be taken from the transplanted lung as part of our study of I/R injury was obtained. The study was reviewed and approved by the Ethics Review Committee of The Toronto General Hospital. The indications for transplant were emphysema in six patients (30%), cystic fibrosis in five (25%), idiopathic pulmonary fibrosis in four (20%), bronchiectasis in two (10%), and primary pulmonary hypertension, lymphangioleiomyomatosis, and bronchiolitis obliterans in one patient each (5% each). Samples from lungs were taken by stapled resection of a small wedge of lung at the end of cold ischemia time at 4°C, which ranged from 1 to 5 hours, after warm ischemia (i.e., during graft implantation; samples were taken at 60 minutes if the implantation time exceeded this period of time) and after graft reperfusion (at 30, 60, and 120 minutes). If the donor lung was volume-reduced to fit into the recipient, larger tissue specimens were available. Six patients (30%) underwent transplantation with cardiopulmonary bypass support.

This study was undertaken during the period in which our lung transplant program switched from Euro-Collins solution to low-potassium dextran glucose solution for clinical lung preservation. Euro-Collins was used in seven patients (35%), low-potassium dextran glucose solution (Perfadex; Biophausia, Uppsala, Sweden) in the other 13 (65%). Prostaglandin E_1 (500 μg/L) was used as an adjunct to the lung preservation technique in all patients.[38] All donors received 2 g methylprednisolone before organ donation.

In 19 patients, bilateral lung transplantation was performed; in the remaining patient, who had idiopathic pulmonary fibrosis, a single lung was transplanted, as described by Cooper and Patterson,[39] with the modification of telescoped bronchial anastomoses and a completely everting, endothelium-to-endothelium, atrial anastomosis, which is our current standard technique.

Lung Specimens

Lung biopsies, which were obtained after cold ischemic preservation, warm ischemic implantation time, and at 30,

60, and 120 minutes after graft reperfusion, were snap-frozen in liquid nitrogen and stored at −70°C. For in situ apoptosis detection, tissue segments were thawed and fixed in 10% buffered formalin. We have found this technique to provide reliable results comparable to direct formalin fixation (data not shown). Specimens were then embedded in paraffin blocks and cut in 4-μm sections to avoid overlapping of cells on microscopic evaluation.

In Situ TUNEL Assay

For in situ apoptosis detection, the ApopTag Kit (Oncor, Gaithersburg, MD) was used according to the manufacturer's instructions. This has been shown to be the most sensitive method for quantitative apoptosis detection available at present.[40]

The method used is based on the biochemical property of terminal deoxynucleotidyl transferase (TdT), which catalyzes a template-independent addition of deoxyribonucleotide triphosphate to the 3′-OH ends of double- or single-stranded DNA.[40] After completing the protocol, slides were photographed at low magnification (×100) on a color slide film using a fluorescent microscope with a 520-nm filter (for propidium iodide staining) and a 590-nm filter (for fluorescence staining). To localize apoptotic cells, randomly selected specimens of lung tissue taken at 120 minutes after reperfusion were photographed under the fluorescent light microscope at high magnification (×400).

Apoptotic cells were counted from four randomly chosen fields per slide. Therefore, propidium iodide nuclear staining (red) represents the total number of cells per field; these were counted first, followed by apoptotic cell counts (yellow to bright green) in the same fields. Results for apoptotic cells are given as a percentage of total propidium iodide-stained cells. Two individuals masked to the identity of the specific specimens performed the counting.

Electron Microscopy

Lung biopsy samples similar to those obtained for light microscopy were studied ultrastructurally. Small pieces of fresh tissue were immersed in universal fixative (1% glutaryl-aldehyde, 4% paraformaldehyde, pH 7.4) immediately after biopsy, postfixed in 2% osmium tetroxide, dehydrated in graded acetons, and embedded in an Epon-Araldite mixture (Fisher Scientific Corp., Toronto, Ontario, Canada). Selected blocks were thin-sectioned, mounted on copper grids, and contrasted with uranyl acetate and lead citrate. The grids were examined in a Philips 201 electron microscope (N.V. Philips, Gloeilampenfarbrieken, Eindhoven, The Netherlands).

Statistical Analysis

Statistical analyses were performed using SigmaStat version 3.0 (Jandel Scientific, San Rafael, CA), comparing the percentage of apoptotic cells in tissue samples taken at the different time points using a one-way analysis of variance. Post hoc analysis was performed using the Student-Newman-Keul test. Differences were considered significant at $P < .05$. For evaluation of a correlation between clinical outcome and the amount of apoptotic cells 60 and 120 minutes after reperfusion, the Pearson product moment test was used.

Table 1. DONOR PROFILE

Number of donors	20
Gender	
Female	10
Male	10
Mean age (years)	37 (range 11–61)
Cause of death	
Intracerebral hemorrhage	8
Head injury	8
Cerebrovascular accident	2
Anoxia	1
Suicide	1
Cardiopulmonary resucitation	3
Positive smoking history	9
Mean length of intubation before retrieval (days)	1.6 (range 1–3.5)
Preretrieval mean Po2* (mmHg)	448.7 (range 301–560)
Preretrieval mean Pco2* (mmHg)	37.8 (range 31–46)
Preservation solution	
Euro-Collins solution	7
Low-potassium dextran glucose solution	13

* Fio2 = 1.0, positive end-expiratory pressure = 5 cm.

Table 2. RECIPIENT PROFILE

Number of recipients	20
Gender	
Female	10
Male	10
Mean age (years)	40 (range 15–60)
Reason for lung transplantation	
Emphysema	6
Cystic fibrosis	5
Idiopathic pulmonary fibrosis	4
Bronchiectasis	2
Lymphangioleiomyomatosis	1
Primary pulmonary hypertension	1
Bronchiolitis obliterans	1
Type of transplant	
Single lung	1
Bilateral sequential (double) lung	19
Mean immediate postoperative Po2* (mmHg)	365.2 (range 201–508)
Mean postoperative Po2* in intensive care unit (mmHg)	343.8 (range 68–540)
Use of cardiopulmonary bypass	6

* Fio2 = 1.0, positive end-expiratory pressure = 5 cm

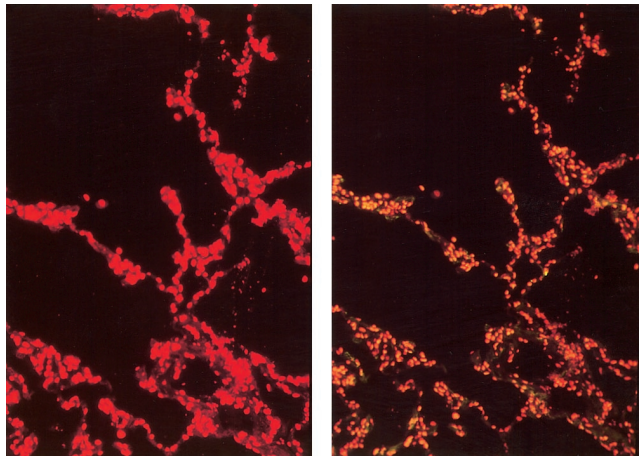

Figure 1. TUNEL immunofluorescence staining showed absence of apoptotic cells in lung tissue after cold ischemic preservation. Lung biopsy taken 3 hours after cold preservation period. (Right) There is no reactivity for apoptosis as shown in Figure 2. Red propidium iodide nuclear staining of the same microscopy slide area (left) shows total number of cells. (Magnification ×100)

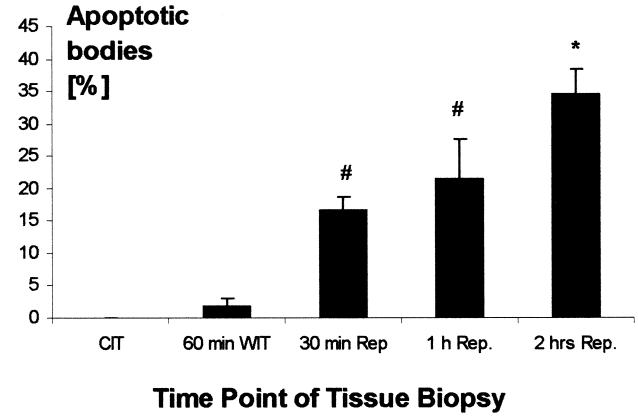

Figure 3. Time course of the induction of apoptosis in human lungs before, during, and after transplantation. CIT, cold ischemic time (preservation time); WIT, warm ischemic time (implantation time); Rep, after organ reperfusion. Data are presented as mean ± SD from different samples. $P < .001$ as determined by one-way analysis of variance. $^*P < .05$ versus all other groups. $\#P < .05$ versus cold ischemic time and 60 minutes warm ischemic time, as determined by post hoc analysis using the Student-Newman-Keul test.

RESULTS

Patient Profile

Donor and recipient patient profiles are summarized in Tables 1 and 2. The surgical (30-day) death rate was 10% (2/20). The mean total ischemic time was 267 minutes (range 178–430) and the mean warm ischemia time was 61 minutes (range 45–83).

In Situ TUNEL Staining

Results of apoptosis staining showed minimal to no apoptosis in specimens obtained after cold and warm ischemia, independent of the length of ischemic time. At short (<2 hours), intermediate (2–4 hours), or long (4–5 hours) cold ischemic intervals, little if any appreciable apoptosis was detected (Fig. 1). After a subsequent period of warm ischemia (the implantation period), lung tissue samples continued to demonstrate only minimal numbers of apoptotic cells. After graft reperfusion, however, lung tissue samples started to show a significant ($P < .001$) increase in the percentage of cells undergoing apoptosis. These increased numbers of apoptotic cells were evident as early as 30 minutes after reperfusion of the transplanted lung, when the mean percentage of ap-

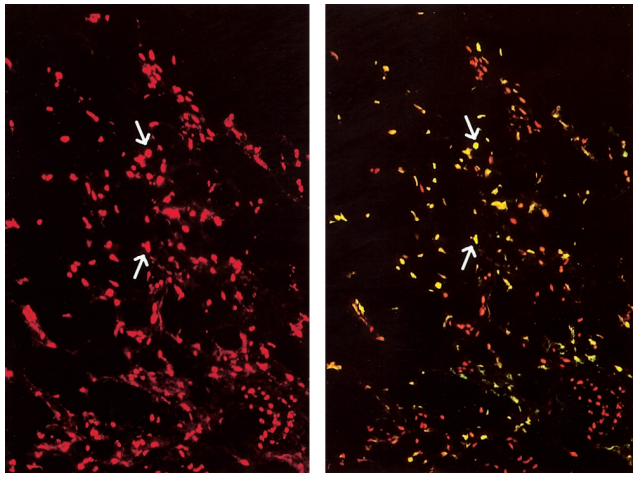

Figure 2. Presence of apoptotic cells in lung tissue 120 minutes after reperfusion. Representative picture of the TUNEL immunofluorescence staining (bright yellowish) of apoptotic cells in lung tissue taken 120 minutes after graft reperfusion after transplantation. The propidium iodide staining (red) can be used to identify nuclei (total number of cells) in the same area of tissue section in comparison to immunofluorescent stained apoptotic cells (arrows). (Magnification ×100)

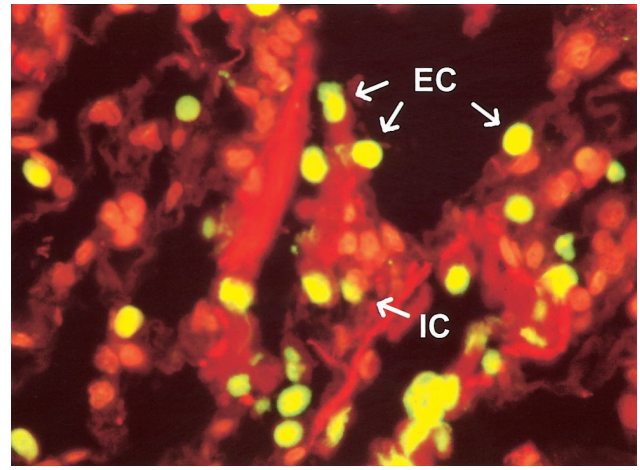

Figure 4. Representative view at higher magnification of lung biopsy taken 2 hours after graft reperfusion. Specimen was stained using the TUNEL technique. Most of the bright-yellow apoptotic cells were identified as epithelial lining cells (EC). A few cells were interstitial cells (IC). (Magnification ×400)

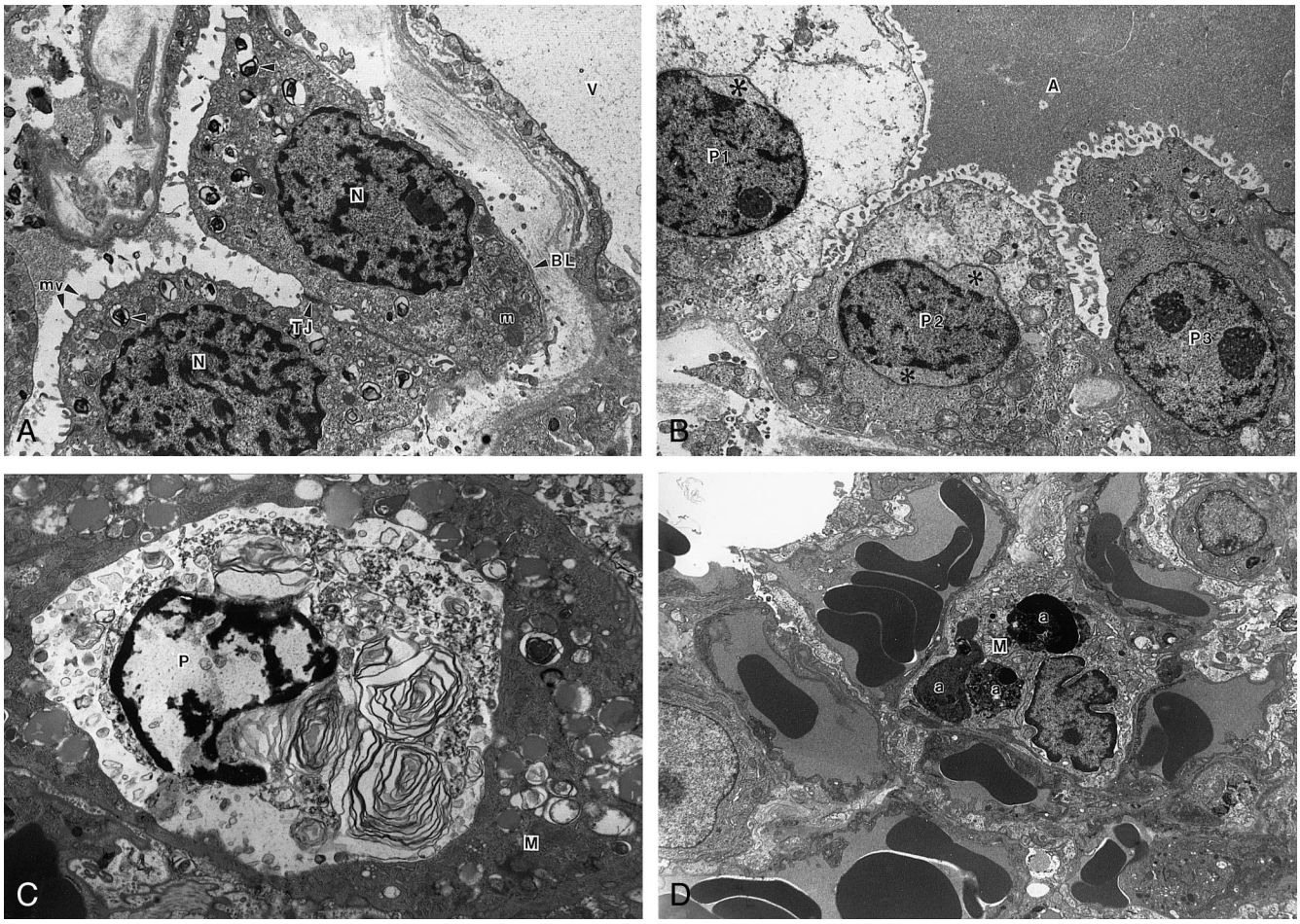

Figure 5. (A) Electron micrograph of lung after cold ischemic preservation. Portions of type II pneumocytes and a blood vessel (V) are shown. The nucleus (N) and cytoplasm of the pneumocytes are within normal limits. Lamellar bodies (arrowheads), mitochondria (m), tight junction (TJ), microvilli (mv), and basal lamina (BL) profiling these epithelial cells are shown. Note intact endothelium lining the blood vessel. (Magnification ×12,175) (B) Electron micrograph of lung after cold and warm ischemia and reperfusion for 120 minutes. Alveolar space and portions of three type II pneumocytes are shown. The type II pneumocyte (P1) shows extensive cytoplasmic swelling and vacuolation. There is blebbing of the nuclear membrane with nuclear shrinkage, leaving a clear empty space (asterisk). The type II pneumocyte in the middle (P2) shows less cytoplasmic vacuolation but more extensive nuclear shrinkage and nuclear membrane blebbing (asterisks). The third type II pneumocyte (P3) shows a relatively normal nucleus and cytoplasmic organelles including mitochondria, rough endoplasmic reticulum, and lamellar bodies. The alveolar space contains edema fluid (A). (Magnification ×7,800) (C) Electron micrograph of lung after cold and warm ischemia and reperfusion for 120 minutes. Apoptotic type II pneumocyte (P) phagocytosed by an alveolar macrophage (M). Note the condensation of nuclear chromatin and cytoplasmic degenerative changes in this pneumocyte. (Magnification ×11,920) (D) Electron micrograph of lung after cold and warm ischemia and reperfusion for 120 minutes. An interstitial macrophage (M) with nuclear fragments of several apoptotic bodies (a). These electron-dense bodies are membrane-bound with pleomorphic shape and variable size. Several blood vessels show intact endothelial lining. Note the absence of an inflammatory cell infiltrate. (Magnification ×6,330)

optotic cells was 16.7 ± 2.2%. These numbers continued to increase at 60 minutes as well as 120 minutes after graft reperfusion (Fig. 2), with mean apoptotic cell percentages of 21.7 ± 6.2% and 33.6 ± 4.0%, respectively (Fig. 3). Photomicrographs taken at high magnification (×400) demonstrated that apoptotic cells were mainly localized to the alveolar epithelial lining, but a few apoptotic cells were also seen in the interstitium (Fig. 4).

Electron Microscopy

Ultrastructural evaluation of lung biopsy samples after cold ischemic preservation showed well-preserved lung architecture (Fig. 5). Type II pneumocytes showed no evidence of apoptosis, and the vascular spaces showed intact endothelial lining cells. Minimal or no edema fluid was seen within the alveolar spaces. However, ultrastructural exam-

ination after 120 minutes of reperfusion showed lung tissue with extensive nuclear and cytoplasmic changes and also increased phagocytic activity of apoptotic cells. Clearing of the cytoplasmic matrix accompanied by shrinkage of the nucleus was seen. Phagocytosed apoptotic cells and remnants in an alveolar and interstitial location were observed at 120 minutes after perfusion, when edematous fluid also appeared in the alveolar spaces. Of note was the absence of inflammatory cells in the lungs, even though vigorous apoptotic activity was occurring.

Clinical Observations

The degree of apoptosis did not significantly correlate with short-term clinical outcome measures such as Po_2 after reperfusion, first Po_2 in the intensive care unit, 30-day death rate, and ventilation time after surgery. Further, differences in the total ischemia time of the lungs, preservation solution, or ventilation time of the donors before organ retrieval did not significantly correlate with the degree of apoptosis at any of the time periods.

DISCUSSION

Apoptosis has been studied in various organs and disease entities such as cancer,[41] I/R injury,[33,42] and organ rejection.[43] This specific form of cell death has yet to be described in the setting of human lung preservation and transplantation. Also, not only the phenomenon of apoptosis itself, but also its induction during the time course of retrieval, preservation, and transplantation, as well as its dependence on total ischemia time have not been previously described. This is highly relevant to further considerations regarding the extension of ischemic times for organ preservation in our attempts to increase the number and quality of available donor organs.

Our findings of apoptosis in human lung transplantation raise several important points. First, cold ischemic preservation of the lungs before transplantation does not seem to induce apoptosis. This was true for short (1–2 hours), intermediate (2–4 hours), and long (4–6 hours) periods of cold ischemia. This is consistent with previous studies in liver and intestinal I/R injury in animal models.[28,42] This phenomenon may result from decreased cellular metabolism secondary to the hypothermic (4°C) preservation. Second, we found that apoptosis remained at low levels after cold ischemia and during the interval of warm ischemia necessary for graft implantation. This is partly consistent with previous studies of warm ischemia.[26,44] Third, we found a significant increase in apoptosis after reperfusion of the transplanted lung. This increase was noted as early as 30 minutes after reperfusion, and a trend toward increased levels of apoptosis over time was noted during the 120 minutes after reperfusion. This is consistent with research in human liver tissue, which describes a similar rise in apoptosis after liver reperfusion.[33] Our results further confirm the finding described in intestinal I/R injury that apoptosis increases significantly only after reperfusion and is virtually undetectable during cold ischemic preservation.[44]

Graft reperfusion is a process associated with an acute increase in the production of oxygen free radicals and a significant accumulation of intracellular calcium. Both of these phenomena are potent inducers of apoptosis.[41] This is in keeping with the theory that oxidative stress related to an abundance of reactive oxygen species and metabolites may induce apoptosis by direct DNA damage, oxidation of lipid membranes, or upregulation of regulatory "apoptosis genes."[45] This hypothesis would explain the acute increases noted in apoptotic cell numbers (mostly type II pneumocytes) in the human lung samples in our study after reperfusion.

The fact that we did not find a significant correlation between apoptosis and clinical physiologic function of the transplanted lungs should not viewed as conclusive, given that the sample size was small and the variation of disease severity and etiology in our patients contributed to significant heterogeneity, which may affect the ultimate outcome related to the implanted lung. Therefore, animal studies under rigorously controlled conditions are required to answer questions about the relation between the amount of apoptosis observed and physiologic function after lung transplantation. These studies are ongoing in our laboratory.

Severe oxidative injury, such as prolonged ischemia, has been known to cause cell death by necrosis. Recently, it has been shown that I/R injury can induce apoptosis as well.[46,47] The finding that apoptosis is induced in the human lung transplantation setting, with such significantly elevated levels after graft reperfusion, is intriguing. Clearly, further investigation is required to define the implications of apoptosis in this setting. This may indeed represent a marker of cell injury resulting from ischemic preservation and the oxidative stress of reperfusion, heralding a poorer posttransplant prognosis. Alternatively, apoptosis may represent a host-protective form of selective cell death, inevitable, to a certain degree, after the stresses imposed by the transplantation process. In a sense, "controlled" cellular senescence may be favored over the "destructive" and inflammatory cellular death seen in necrosis, which results in the local and systemic release of injurious and noxious intracellular components and proinflammatory cytokines such as tumor necrosis factor alpha. Further, because apoptosis is a genetically controlled, programmed cell death, it might indeed be possible to modify this type of cell death in transplanted organs by interventions such as gene therapy. Necrotic cell death may be more difficult to influence.

Apoptosis could also be viewed as an adaptive mechanism that aids the body in its response to a significant injury. This view of the process hypothesizes that a high index of apoptosis in a transplanted organ indicates that organ's ability to eliminate injured cells and avoid the more destructive process of necrosis. This theory views apoptotic cell death not negatively as a loss of organ tissue and function,

but positively as an important part of the process of tissue healing from reperfusion injury. This is similar to the field of fetal organogenesis, where it has been demonstrated that apoptosis is an important aspect of the remodeling of an effective pulmonary alveolar–capillary interface in fetal lungs during middle to late gestation.[48]

Future clinical and experimental investigations are needed to evaluate the role of apoptosis as a marker of the extent of reperfusion injury and the resultant condition of the organ after preservation and transplantation. It also remains to be determined when the process of apoptosis peaks, and its rate of recovery. Details regarding the specific cell types affected and their relative proportions also need to be ascertained. Further, the relation between the early index of apoptosis and the development of chronic obliterative bronchiolitis, which occurs in approximately 50% of patients after lung transplantation, needs to be studied.

In summary, we have found that in human lung transplantation, up to 34% of pulmonary cells undergo apoptotic cell death after reperfusion. Cold, aerobic, ischemic preservation up to 5 hours did not appear to induce apoptosis; nor did a period of warm ischemia before implantation. Clearly, further work is required to determine the implication of this magnitude of apoptotic cell death on ultimate organ function. Patients obviously did well in the short term, but the ability of the organ to recover from this type of injury may indeed have significant implications regarding long-term lung function after transplantation and the development of bronchiolitis obliterans.

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