# Apoptosis in Lung Transplantation

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*Graft* 2001; 4; 481

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Apoptosis or *programmed cell death* is currently an area of intense clinical and experimental interest. This process plays a significant role in tissue development and homeostasis. The number of diseases in which it is becoming evident that apoptosis plays a role as a causal or protective factor is rapidly increasing. The induction of cellular apoptosis presents an exciting potential therapeutic approach in the treatment of diseases such as cancer. In clinical and experimental transplantation, it appears that apoptosis aids in the removal of cells that are damaged during the overall process of transplantation because of acute injury or immunologic processes. In the transplanted lung, a significant amount of graft cells undergo apoptosis in the very early phase following graft reperfusion. This active and silent genetic shutdown of damaged cells relates to the length of ischemic preservation. The underlying molecular mechanisms of apoptosis induction in transplanted lungs remain to be discerned. In this review, we provide an overview of apoptosis with respect to its potential regulation in the setting of lung transplantation, particularly with respect to ischemia-reperfusion injury.

## Introduction

Lung transplantation has become a widely accepted standard treatment modality for end stage lung diseases, such as cystic fibrosis (CF), emphysema, primary pulmonary hypertension, idiopathic pulmonary fibrosis, and others. Since the initial success in Toronto in 1983, approximately 14,000 lung transplantations have been performed worldwide, as reported by the Registry of the International Society for Heart and Lung Transplantation in 2000.[2]

## Ischemia Reperfusion Injury

In the very early phase after lung transplantation, severe life-threatening graft dysfunction related to ischemia-reperfusion (IR) injury occurs in up to 20% to 30% of patients, which significantly contributes to the overall 1st year mortality of approximately 25% of patients posttransplant.[3] Clinically, IR injury manifests as pulmonary edema and acute respiratory distress syndrome, characterized by significant pulmonary infiltrates and severe oxygenation difficulties.[4] Consequently, patients are subjected to prolonged mechanical ventilatory support, with or without inhaled nitric oxide (NO), ventilator-associated infections, longer stays in the intensive care unit, and increased early postoperative mortality.[5]

### *Pathophysiology of IR Injury*

Endothelial dysfunction is an important early phenomenon in virtually all forms of ischemia-reperfusion, including organ transplantation.[6] The dysfunction appears to be triggered within minutes of the endothelial generation of a large burst of superoxide radicals.[7] However, the initial dysfunction may be amplified by neutrophil-generated factors, including oxygen-derived free radicals, proinflammatory cytokines, and lipid peroxidation.[8] Moreover, activated neutrophils migrate across the endothelium into the pericapillary space,[9] where they cause tissue destruction by releasing oxygen free radicals, proteolytic enzymes (cathepsin G, elastase, collagenase), and peroxidase.[10] Sequestration of activated neutrophils is an important step in the development of graft failure, or even multiple organ failure following transplantation.[11] The pathophysiology of IR injury in the lung following transplantation also involves platelet activation and tissue inflammation, followed by alveolar-capillary barrier leakage, ultimately causing interstitial and alveolar edema and

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cell injury.[12] Lipid peroxidation is the most damaging effect of free radicals in IR injury.[13]

Cell death following exposure to different stimuli, for example, heat stress, is primarily due to free radical reactions with DNA.[14] The release of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-2 (IL-2), as well as the up-regulation of their receptors (such as IL-2R),[15] are involved in the complex system that mediates IR injury following pulmonary graft preservation and transplantation.

Currently, there is no reliable, reproducible histological IR injury scoring system available to measure accurately the degree of tissue or cellular injury during preservation and reperfusion in transplanted lungs. Edema, pulmonary parenchymal and airway inflammation,[16] neutrophil migration, intraparenchymal hemorrhage, and cell death (apoptosis and necrosis) have all been utilized as histological markers of injury following organ transplantation.[17] In general, however, such examinations of the pathological correlates of IR injury have not been particularly helpful, as they are nonspecific, insensitive, and nonpredictive.

## Cell Death

Recently, increasing interest has been demonstrated in the induction of cell death in transplanted organs during ischemia and after transplantation. A basic premise of transplantation rests upon the theoretical belief that cells can endure a degree of injury and still maintain the ability to recover. Ultimately, however, if a sufficient degree of injury is inflicted upon a cell, the cell may die. There are two major ways in which this might occur—namely, *necrosis* and *apoptosis*.

### *Necrosis*

Necrosis is a form of irreversible cell death accompanied by the loss of plasma membrane integrity and ion pump damage, leading to the loss of sodium and calcium ion balance, followed by acidosis, osmotic shock, clumping of chromatin, and nuclear disruption.[18] Sodium ions move into the cell, drawing with them a volume of water to maintain osmotic equilibrium with the surrounding interstitial space, while potassium ions escape from the cell into the interstitium.[19] These changes are accompanied by activation of mitochondrial phospholipase, a precipitant loss of oxidative phosphorylation, and a drop in adenosine tri-phosphate (ATP) production, which leads to failure of synthetic and homeostatic capability.[18] Calcium overload leads to mitochondrial membrane dysfunction and irreversible damage. Secondary autolysis, accompanied by lysosomal swelling, dilatation and vesiculation of the endoplasmic reticulum, and release of intracellular enzymes and proteins, and the loss of cellular compartmentalization occur. In vivo, necrosis of cells induces neutrophil migration, a significant tissue inflammatory response, and edema.[20]

### *Apoptosis*

An alternate form of cell death is apoptosis or *programmed cell death*. Physiologically, apoptosis is a genetically controlled mechanism used to eliminate aging, genetically damaged, or "sick" cells. Thus, as a counterpart to mitosis, apoptosis is an important regulator in development and maintenance of tissue homeostasis in multicellular organisms.[21] Glucksmann originally described the death of cells during normal development of vertebrates and invertebrates in 1951.[22] In 1965, Kerr described different types of liver cell death after portal vein branch ligation.[23] In 1972, Kerr and Searle proposed the name "apoptosis" for this phenomenon (from the Greek, [illegible], which translates as "falling off") and suggested it might play a role opposite to that of mitosis.

As observed by electron microscopy, apoptotic cell death is morphologically characterized by overall cellular condensation, shrinkage, and plasma membrane blebbing.[20] Nuclear changes are characterized by chromatin margination and nuclear condensation followed by segmentation. During this time, DNA fragmentation into 180 to 200 base pair (bp) fragments occurs. Finally, the apoptotic cell is broken into smaller membrane-bound apoptotic bodies that are usually phagocytozed by macrophages.[24] Using both light microscopy and electron microscopy, an important morphologic distinction from necrosis lies in the fact that apoptotic cell death lacks inflammation.[24] Daemen and colleagues, however, have recently described a possible association between the occurrence of apoptosis

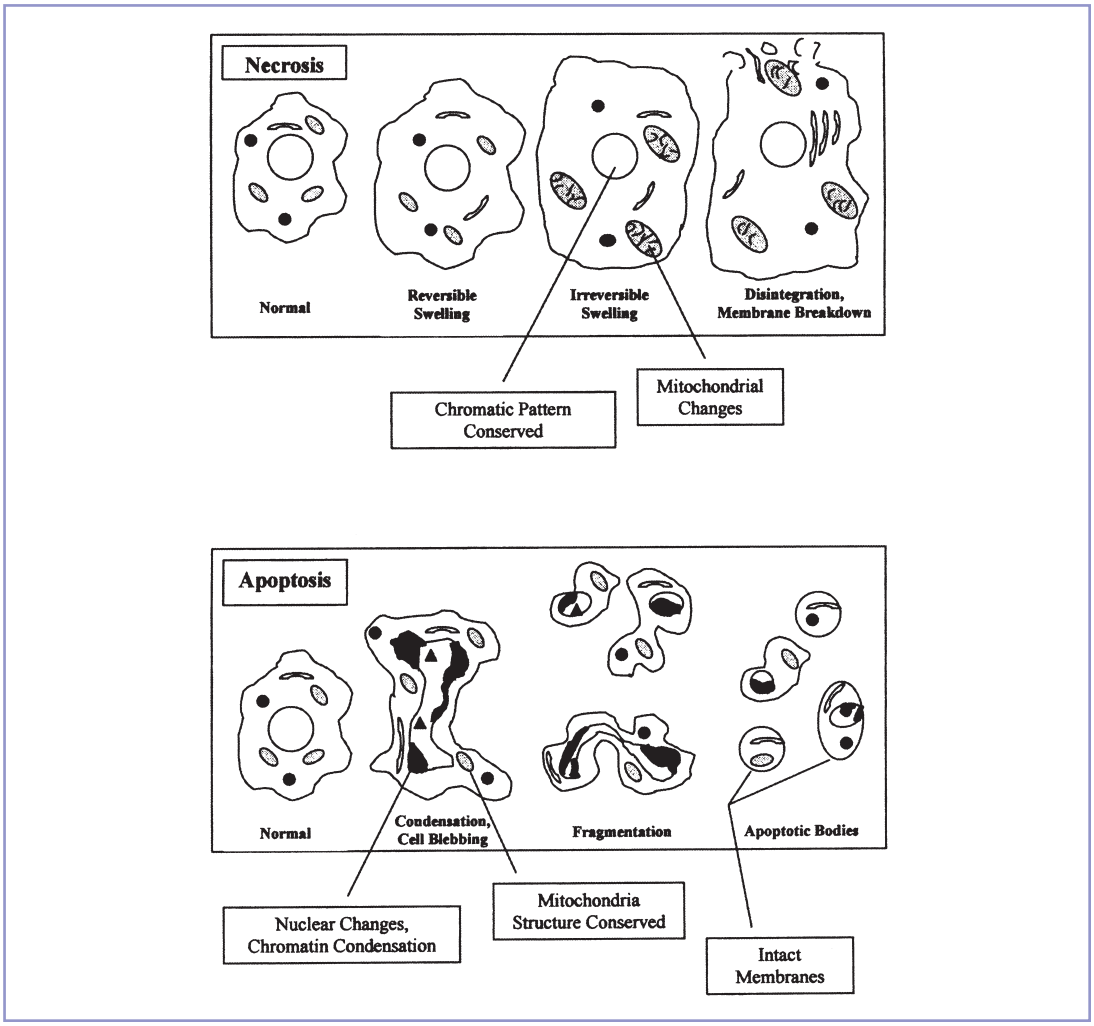


Figure 1. Morphological differences between apoptosis and necrosis.

and neutrophil influx and sequestration in reperfused kidneys.[25] The mechanism for this phenomenon, which suggests that apoptosis may indeed lead to secondary inflammation, is not clear. However, hypothetical saturation of the apoptotic cell removal systems (i.e., the macrophage system) by an overwhelming number of apoptotic cells, might subsequently lead to *secondary* necrosis of apoptotic cells and thus cause inflammation. Furthermore, the extent of cellular necrosis in these organs was not reported. The characteristic morphological features of the two different modes of cell death—necrosis and apoptosis—are depicted in Figure 1.

## Role of Apoptosis in Disease

There are strong indications to believe that the dysregulation of apoptosis plays an important regulatory role in various disease processes, such as cancer, autoimmune diseases, and other disease states.[26] Increasing evidence suggests that alterations in the genetic control of cell survival and elimination are important regulators in these disease states. Of particular importance in organ transplantation, apoptosis has recently been reported in several clinical and experimental studies following IR injury to the kidney, retina, brain, heart, liver, and adrenal gland.[27-32]

Many laboratory studies have demonstrated a relationship between organ injury (including IR injury) and apoptosis induction. However, it is unknown whether a more significant injury triggers necrosis and a milder injury initiates apoptotic cell death. It has been reported that apoptosis appears in rabbit cardiomyocytes after ischemia followed by reperfusion, yet it is not detectable following ischemia alone.[28] These findings are of particular interest to transplantation medicine and surgery.

Presently, very little is known about the relationship between IR injury and the induction of apoptosis after human organ transplantation. Isolated reports of a descriptive nature are available regarding apoptosis in human liver, kidney, pancreas, and heart transplantation.[33-35]

## Apoptosis in Human Lung Transplantation

In a clinical study designed to determine if apoptosis is present in human lung tissue before, during, and after transplantation, we collected tissue biopsies from 20 consecutive human lungs for transplantation following cold ischemic preservation (1 to 5 h), after warm ischemia time (during implantation), and 30, 60, and 120 min following graft reperfusion.[36] Apoptosis was detected and quantified by fluorescent in situ end labeling of DNA fragments (TUNEL). Almost no positive staining was detected in specimens following both cold and warm ischemic periods. Significant increases in the numbers of cells undergoing apoptosis were observed following graft reperfusion in a time-dependent manner. The mean fraction of apoptotic pulmonary cells at 30, 60, and 120 min after graft reperfusion were 16.6%, 22.1 %, and 34.9% of total graft cells, respectively. As confirmed by electron microscopy, the majority of apoptotic cells were alveolar type-II pneumocytes.[36] We concluded that apoptosis appears to be a significant type of cell death following human lung transplantation, which may contribute to lung dysfunction during the early phase of graft reperfusion. This knowledge is of importance for potential future development of interventions to prevent lung dysfunction after transplantation, perhaps by using antiapoptotic strategies to limit programmed cell death.

## Cell Death in Experimental Lung Transplantation

Following organ transplantation, the mode of cell death appears to be related to the ischemic time and the process of reperfusion. Recently, we developed a triple-staining technique to quantify apoptosis and necrosis in the same specimens of lung tissue,[37] a technique that has been validated by other groups working on cell culture models.[38] We used this method (which quantifies the degree of apoptosis versus necrosis) to determine the time course of each type of cell death during lung preservation, transplantation, and reperfusion. We determined the impact of the specific types of cell death on posttransplant pulmonary function.[37] We noted that apoptosis is the predominant type of cell death in transplanted lungs following moderate periods of cold storage (6 and 12 h), with 28% of cells being apoptotic after reperfusion for both of these times. The amount of dead cells in these lungs after hypothermic storage (prior to reperfusion) was less than 2%. On the other hand, necrosis was the predominant type of cell death after long-term storage prior to reperfusion (18 and 24 h). In these lungs, necrotic cell death was already evident after cold storage in approximately 10% and 24% of graft cells, respectively. The percentages of necrotic cells in these lungs after transplantation were approximately 21% and 29%, respectively, whereas the amount of apoptotic cells remained low, at levels of less than 2%. Table 1 illustrates the amounts of apoptosis and necrosis in lungs at various states of the lung transplantation process.

Furthermore, the amount of necrotic cells in the lungs after transplantation correlated with the degree of organ dysfunction 2 h after reperfusion.

## Mechanisms and Signaling Pathways

Apoptosis in organ transplantation is a relatively young field of investigation, and until now there has been little in the literature that focuses on the examination of underlying signaling pathways of apoptosis induction in transplanted lungs. Thus, at present one can only extrapolate from what investigators have reported in apoptosis-related research on topics other than lung transplantation. Vaux and Strasser have recommended dividing the

Table 1 | **Cell Death in Lungs after Different Periods of Ischemic Preservation and after Same Ischemic Periods Followed by Transplantation and 2 h of Graft Reperfusion**

| | Apoptosis | Necrosis |
|---|---|---|
| Short cold ischemia (20 min) | 0 | 0 |
| Warm ischemia (60 min) | 0 | 0 |
| Reperfusion after cold ischemia (20 min) | 0 | 0 |
| Intermediate cold ischemia (6 and 12 h) | 0 | 0 |
| Reperfusion after intermediate cold ischemia (6 and 12 h) | +++ | 0 |
| Extended cold ischemia | 0 | ++/+++ |
| Reperfusion after extended cold ischemia (18 and 24 h) | 0 | +++ |

process of physiologic cell death (apoptosis) into 4 different phases for improved understanding:[39]

1. apoptotic stimulus;
2. signal detection and transduction;
3. effector phase/caspase activation; and
4. postmortem phase/cellular degradation.

The nematode *Caenorhabditis elegans* has provided an excellent model system in which the stages of physiological cell death can be observed. During development, *C. elegans* has 1,070 cells of which 131 must die. Mutants in which cell death is abnormal have provided invaluable insight into the "death" genes that implement it.

At present, it is not clear what stimulus induces apoptosis in transplanted lungs. It is hypothesized that certain presuppositions have to be fulfilled for a cell to initiate its suicidal genetic program and undergo apoptosis. First, there must be a specific stimulus that initiates cell death. In organ transplantation, this could be an endogenous trigger such as cellular or DNA damage during ischemia or from preservation. It could also be an exogenous stimulus, such as proinflammatory cytokines, that initiates apoptosis through specific cell surface death receptors. For instance, TNF-α, a very potent apoptosis inducer, is up-regulated within 30 min following lung transplantation.[40] Second, even though the cell is injured, it must still be able to initiate the genetic program to undergo apoptosis, which is an energy-requiring process. Therefore, the cell must contain at least some ATP resources. This could possibly explain why transplanted lungs in our experimental study showed high amounts of apoptotic cells after short ischemia and of necrosis after long-term preservation. In lungs after short ischemic times, almost no dead cells were seen. However, it is still unknown how many cells were injured (but did not die) from ischemia before transplantation. Furthermore, it is unknown how many of these injured cells recovered after transplantation and how many underwent any type of cell death. Following lung transplantation, approximately 30% of graft cells were obviously damaged but not damaged enough to start the apoptotic program.[36,37] In lungs after long ischemic times, 10% to 30% of graft cells were already necrotic before the lung was transplanted, suggesting that the stimulus was too strong (lethal) for the cells to undergo apoptosis. These cells died randomly by necrosis, which was worse after transplantation and reperfusion of these lungs.[37] We hypothesize that the high amounts of cellular necrosis in lung grafts after long-term preservation cause tissue inflammation. This inflammation is due to the release of intracellular proteins into the surrounding tissue, which further increases cellular necrosis. These novel observations indicate that the mode of cell death that occurs in transplanted lungs is dependent on the prior ischemic injury, plus the insult due to graft reperfusion following lung transplantation.

### *Cell Death Receptors*

Various characterized apoptotic signaling pathways involve the binding of proteins, such as TNF-α, Fas-L, and TNF-related apoptosis-inducing ligand (TRAIL/Apo-3L), to their cell surface receptors TNFR1, Fas, and death receptor (DR) series.[41] TNF-receptors (TNFR) bind 2 related TNF molecules: TNF-α and TNF-β. TNF-α is one of the principal mediators of inflammation in lung transplantation, and interaction between TNF and TNFRs produces a range of effects, which are depend-

ent on the nature of the target cell. The respective role of the two TNFR chains in triggering cell death, however, is disputed. Some studies suggest that TNFRp55 is the principal mediator,[42] whereas others contend that p70 may be equally effective.[43] Because several of the commonly used target cells constitutively express one or both TNFRs, it has been difficult to assess the effect of each receptor chain independently. The possibility of direct or indirect cooperation between the two polypeptides has not been excluded in the literature.

Fas (Apo-1/CD95) antigen is a 43-kD cell surface glycoprotein that is constitutively expressed in a variety of normal tissues and tumor cell lines while being inducible in others.[44] Administration of anti-Fas mAb in vitro[44] and in vivo[45] triggers apoptosis of Fas-expressing cells, suggesting that Fas may play a critical role in the regulation of cell death in a broad range of tissues. Mutations that inactivate Fas have been shown to be associated with the lymphoproliferative disorder of lpr/lpr mice, and Fas has recently been implicated in cytotoxic T lymphocyte-mediated target cell death.[46]

Although Fas and TNFRs are currently grouped together as the principal cell death receptors, it is unclear whether they trigger the death signal along the same or distinct pathways, whether the potency of each receptor in any given cell is comparable, and whether their function is subject to similar or distinct regulatory circuits. Some studies indicate that different biochemical pathways are activated by Fas and TNFR-1,[47] whereas others demonstrate a cooperation of both receptors.[48] However, besides the caspase 9–involving intracellular apoptosis activation due to drugs, toxins, and radiation, as mentioned above, there are 2 major pathways that play a role in initiating and controlling apoptosis through these receptors and their death domains, which involves caspase 8 activation. After binding of peptides such as TNF-α and Fas-ligand (Fas-L), their receptors oligomerize and recruit the adapter proteins FADD (Fas-associated death domain) and TRADD (TNF-receptor associate death domain) to form death-inducing signaling complexes (DISC).[49] This activates procaspase 8, which further activates the initiator caspase 8 (Figure 2). Caspase 8 activates downstream the effector caspases (e.g., caspase 3).[50] The TNF-α/TNFR-1 complex can also elicit an anti-apoptotic response by recruiting TRAF-2 (TNF receptor associated factor 2), which results in NF-κB-mediated up-regulation of antiapoptotic genes through activation of the stress-activated protein kinase (SAPK)/jun kinase (JNK).[51]

If the TNF pathway indeed regulates apoptosis in transplanted lungs in the very early stage following graft reperfusion, then the transfection of lungs with anti-inflammatory cytokines may reduce the inflammatory tissue reaction, which is worse the more cells undergo necrosis from the release of intracellular proteins by necrotic cells.[20] In fact, Itano and colleagues have recently shown that adenoviral-mediated transfection of rat lungs with IL-10 leads to a significant reduction in IR injury and improved graft function.[52] This is consistent with what we have found in a similar study. In our study, we observed a decrease of TNF-α and IFN-γ levels in lung tissue, as well as in recipient plasma 2 h after transplantation of lungs that were transtracheally hIL-10-transfected using adenovirus Ad5 in the living donor 24 h prior to graft retrieval. Interestingly, in this study, we observed an increase in apoptotic cells in transplanted grafts after 24 h of cold preservation plus 2 h of reperfusion, and a significant decrease of necrotic cells. We concluded that the increased early expression of the anti-inflammatory protein IL-10 either preserved the cells' ability to undergo apoptosis even after prolonged ischemia or reduced the tremendous inflammatory reaction in lungs after such a long preservation time, which, as mentioned above, usually leads to cellular necrosis rather than apoptosis.

## Conclusion

Several laboratory studies have shown a relationship between organ injury, including IR injury, and the induction of apoptosis.[53-55] Cell death, especially apoptosis, is a tightly regulated process that involves numerous initiators as well as specific transduction and signaling pathways.

We and others have shown that there are two types of cell death that can occur in transplanted lungs: apoptosis and necrosis. Their occurrence is mainly dependent on the length of ischemia before transplantation. Apoptosis appears not to be initiated during cold ischemic preservation at 4°C. That might be explained by the fact that apoptosis is an energy-requiring process, which is suppressed by

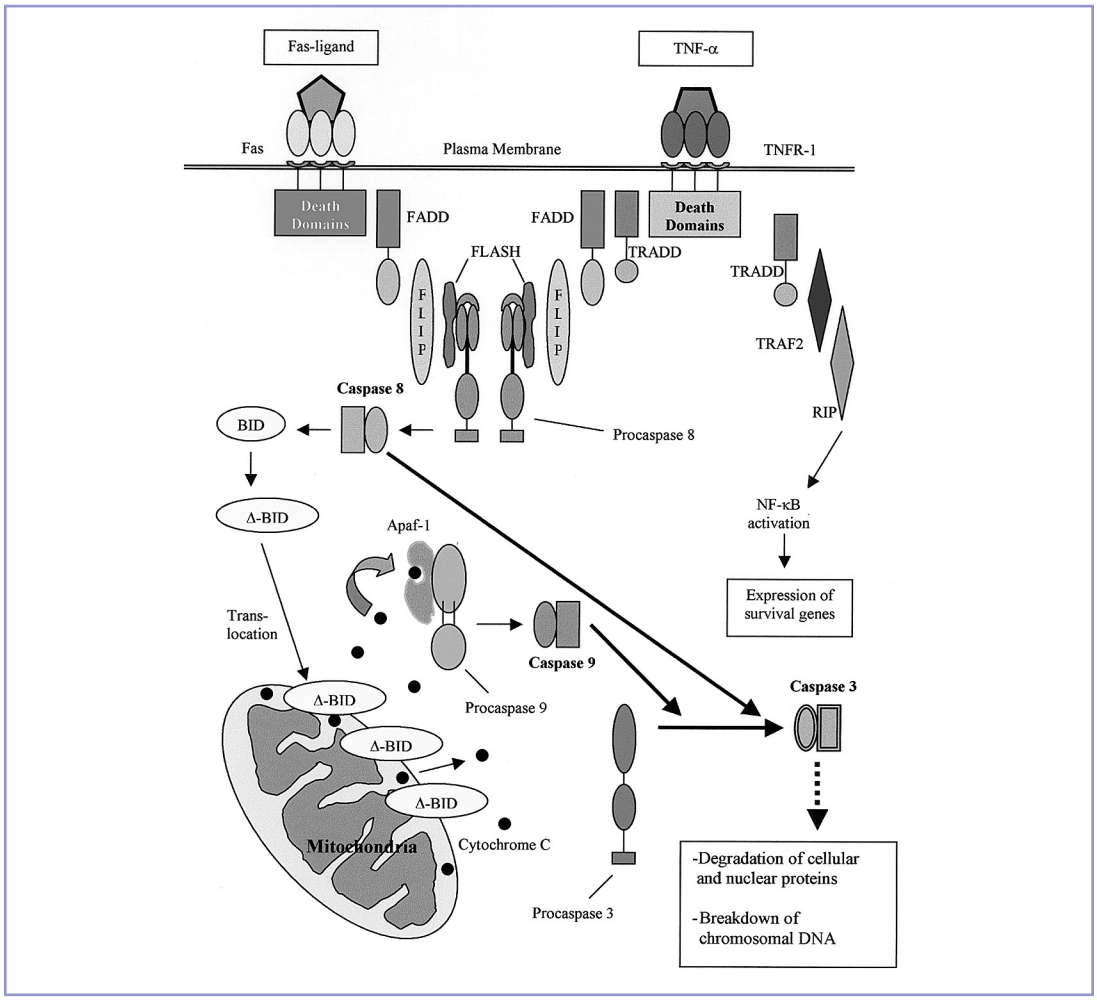


Figure 2. The two major pathways that mainly use the initiator caspase 8 to trigger effector caspases, which are initiated by the tumor necrosis factor receptor-1 (TNFR-1) or Fas.

hypothermia. However, a mild cellular injury during this phase of the lung transplantation process cannot be excluded. During warm ischemia, apoptosis levels in lung grafts following short periods of preservation remain at low levels but increase after reperfusion within the first 2 h to approximately 30%.[36] After long-term preservation, lungs show already high levels of necrotic cell death, which increase after reperfusion, suggesting a cellular injury due to ischemia that is beyond the ischemic limit that graft cells can survive.[37] From our experimental study, we would set the cutoff point of tolerable ischemic time for lung grafts at approximately 15 h. This is consistent with our observation of satisfactory lung function following 12 h hypothermic preservation in our clinical lung transplant program. Yet, this fact also indicates the limitation of current techniques for lung preservation, which must be further modified to make prolonged lung preservation feasible. We recently described decreased cell death and improved function in rat lungs that were stored in a raffinose-modified low potassium dextran glucose preservation solution for 24 h.[56,57] These and other studies suggest cell death to be an important and unique marker in lung preservation– and transplantation–related research. The most important clinical endpoint in the early phase following lung transplantation is the graft's

ability to oxygenate blood measured by blood gas analysis (arterial $Pa0_2$ levels). We have shown a correlation between posttransplant lung function and the predominant type of cell death.[37] This further supports the importance of cell death in clinical and experimental lung transplantation, because it opens new avenues to improve the outcome of lung transplantation by developing strategies to prevent or modify cell death. Useful approaches might be direct caspase inhibition, transfection of anti-inflammatory genes, or modifications of current preservation strategies by cell-stabilizing agents, such as the trisaccharide raffinose.

From the limited evidence on cell death in the lung, we believe that cell death is more a result of incidences occurring at earlier time points in the lung transplantation process rather than a mechanism itself. Obviously, pulmonary graft cells become increasingly injured the longer the lung is preserved before transplantation. Thus, the "real" insult that causes lung dysfunction and the injurious trigger that later will lead to the initiation of cell death after transplantation occurs during ischemia. Consequently, the most useful approaches to improving the outcome following lung transplantation will be directed toward events before transplantation, possibly even when the graft is still in the donor individual.

Another very important question is how the transplanted lung recovers from such a high cellular loss following transplantation. Although a mechanistic relationship between IR injury, or early graft dysfunction, and chronic graft dysfunction has yet to be elucidated, abnormal healing processes and the development of a pro-fibrotic environment after posttransplant IR injury may be a possible link to obliterative bronchiolitis (OB), which is the histological manifestation of end-stage (and irreversible) chronic graft failure in transplanted lungs. Recently, Crocetti and colleagues reported on the relationship between early and late pulmonary graft dysfunction, comparing the degree of postreperfusion pulmonary edema in x-rays obtained from human lung transplant recipients and the occurrence of OB.[58] The role of apoptosis in chronic lung dysfunction after transplantation is now being investigated by a small number of research groups. However, the limited evidence that has been achieved thus far suggests that apoptosis is the predominant mode of cell death during active OB processes in the transplanted lung.[59] Once again, however, the important question is whether apoptosis is a destructive mechanism of cell elimination initiated by immunologic processes or whether it is the response of the host to eliminate rejected graft cells to prevent cellular necrosis and damage of other nonrejected cells. This is unclear and should be investigated in future studies.

The finding that apoptosis is induced in the lung transplantation setting, with significantly elevated levels after graft reperfusion, is an intriguing observation. Clearly, further investigation is required to define the implications of apoptosis in lung transplantation. This type of cell death may indeed represent a marker of cell injury resulting from ischemic preservation, and the oxidative stress of reperfusion, heralding a poorer posttransplantation prognosis. Alternatively, apoptosis may represent a host-protective form of selective cell death, inevitable, to a certain degree, following 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 pro-inflammatory cytokines such as TNF-$\alpha$. The idea of apoptosis being a protective mechanism in transplanted lungs following reperfusion is further supported by the observation that apoptosis can be induced with high levels of nitric oxide and that high levels of nitric oxide are clinically used to improve graft function in lungs with severe IR injury. This concept, however, has yet to be proven, but an ongoing randomized clinical trial at our institution is focusing on the role of nitric oxide in transplanted lung IR injury. Furthermore, because apoptosis is a genetically controlled, programmed cell death, it might indeed be possible to modify this type of cell death in transplanted organs with interventions, for example, gene therapy. Necrotic cell death may be more difficult to influence directly, but it's likely that this can be done indirectly by improvements in lung preservation.

Apoptosis could also be viewed as an adaptive mechanism, which 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 is indicative of 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 mid to late gestation.[60]

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