Programmed Necrosis, Not Apoptosis, in the Heart

Gloria Kung, Klitos Konstantinidis, Richard N. Kitsis

Abstract: It is well known that apoptosis is an actively mediated cell suicide process. In contrast, necrosis, a morphologically distinct form of cell death, has traditionally been regarded as passive and unregulated. Over the past decade, however, experiments in Caenorhabditis elegans and mammalian cells have revealed that a significant proportion of necrotic death is, in fact, actively mediated by the doomed cell. Although a comprehensive understanding of necrosis is still lacking, some key molecular events have come into focus. Cardiac myocyte apoptosis and necrosis are prominent features of the major cardiac syndromes. Accordingly, the recognition of necrosis as a regulated process mandates a reexamination of cell death in the heart. This review discusses pathways that mediate programmed necrosis, how they intersect with apoptotic pathways, roles of necrosis in heart disease, and new therapeutic opportunities that the regulated nature of necrosis presents. (Circ Res. 2011;108:1017-1036.)

Key Words: cell death ▪ necrosis ▪ apoptosis ▪ myocardial infarction ▪ heart failure

As recently as 30 years ago, cell death was viewed as a passive and unregulated process. Irreversible cellular injury (from physical/chemical/biological insults) was thought to kill solely by overwhelming cellular homeostasis. In this model, the cell was merely the recipient of damage and not a participant in its own demise. Unexplained by this paradigm, however, were the highly reproducible deaths of specific cells during the development of multiple organisms. In fact, these developmental cell deaths (termed “programmed cell death”) had long been recognized but remained poorly understood. Studies in Caenorhabditis elegans showed that a relatively small network of genes (ced-9 ▪ ced-4 ▪ ced-3) regulates the deletion of a specific 131 cells during development. These experiments provided the first evidence that any form of cell death was actively mediated.

Subsequent work demonstrated that these genes had been conserved for more than 600 million years of evolution to humans. The orthologs of ced-9, ced-4, and ced-3 are, respectively, the bcl-2 (B-cell lymphoma 2) family, apaf-1 (apoptotic protease activating factor-1), and the caspase family. Moreover, not only do these genes regulate developmental cell deaths in mammals, they also control the deaths of postnatal cells by a specific process termed apoptosis (discussed below). Taken together, these observations establish that cells often die through active mechanisms that have been highly conserved through evolution.

Research more than the past 2 decades has built on these observations to produce a relatively mature understanding of the pathways that mediate apoptosis. These include an intrinsic pathway, which is conserved back to C elegans, that uses mitochondria and endoplasmic reticulum; and an extrinsic pathway that involves cell surface death receptors. These pathways are critical in the regulation of apoptosis. Apoptosis is characterized by cell shrinkage and fragmentation into membrane enclosed apoptotic bodies that are phagocytosed. Plasma membrane integrity and organelle (eg, mitochondrial) morphology are maintained until late in the process, and inflammation is avoided. Based on morphology, however, it is clear that there are nonapoptotic forms of cell death including necrosis and possibly autophagic cell death. Although the existence of autophagic cell death has not been firmly established, necrosis has been recognized for more than a century. Necrosis is also evolutionarily ancient, dating back to C elegans or possibly earlier. The defining features of necrosis are defective plasma and organelle membranes, cell and organelle swelling, severe ATP depletion, and marked inflammation. Differential features of apoptosis and necrosis are summarized in Table 1.

Based on its morphological and functional characteristics, it is not surprising that necrosis has been considered an “unregulated” or “accidental” form of cell death since Virchow described it. This view has been challenged over the past 10 years, however, by 3 independent lines of investigation. First, screens in C elegans have shown that necrosis is regulated by genes that encode plasma membrane and endoplasmic reticulum (ER) Ca2+ channels, calpains, and cathepsins, the unifying theme being elevated cellular Ca2+ concentrations. Second, biochemical studies of the apoptotic death receptor pathway revealed that this pathway is significantly more complex than initially thought. In addition to...
apoptosis, it can signal survival, necrosis, proliferation, and inflammation. This functional diversity reflects the participation of additional components in the pathway and more complex interactions among them. Third, deletion of \textit{ppif}, encoding cyclophilin D, in the mouse proved the existence of a mitochondrial necrosis pathway.\textsuperscript{9–11} In apoptosis, the central mitochondrial event is permeabilization of the outer mitochondrial membrane (OMM). In contrast, the triggering mitochondrial event in necrosis is opening of an inner mitochondrial membrane (IMM) channel termed the mitochondrial permeability transition pore (MPTP). MPTP is regulated by cyclophilin D, which resides in the mitochondrial matrix.\textsuperscript{12}

Taken together, this body of work strongly supports the concept of regulated or programmed necrosis. The existence of these necrosis pathways, however, does not imply that all necrosis is regulated. In contrast to apoptosis, which by definition is a controlled process, it is possible that some necrotic deaths are regulated, whereas others are not. For example, when a fly hits the windshield, does a regulated death program activate and, if so, does it have sufficient time to kill, or does the fly simply die from unregulated necrosis induced by massive trauma? The answer to this question is not known, nor is the proportion of necrotic deaths that are regulated versus unregulated. These considerations aside, a significant proportion of necrosis appears to be regulated and to play important roles in the pathogenesis of myocardial infarction,\textsuperscript{9,10} heart failure,\textsuperscript{11} stroke,\textsuperscript{12} neurodegenerative diseases,\textsuperscript{14,15} viral infection,\textsuperscript{16} muscular dystrophy,\textsuperscript{17} diabetes,\textsuperscript{18} and pancreatitis.\textsuperscript{19} More work is needed to understand the roles of necrosis in these diseases.

A large number of studies over the past 2 decades have examined the role of cardiac myocyte death in myocardial infarction and heart failure.\textsuperscript{20} The focus was on apoptosis because it was the only form of cell death that was thought to be regulated and, therefore, amenable to experimental manipulation. These studies concluded that apoptosis is an important component in the pathogenesis of myocardial infarction and heart failure. Necrosis, on the other hand, was largely ignored because it was believed to be unregulated. Although cardiac myocyte necrosis is thought to be the major pathologic lesion in acute myocardial infarction, its significance in pathogenesis could not be formally evaluated until recently. Studies, in fact, show that regulated cardiac myocyte necrosis is an important component of myocardial infarction\textsuperscript{9,10} and perhaps heart failure.\textsuperscript{13}

The recognition of necrosis as a regulated entity represents a paradigm shift in biology and medicine. From a fundamental perspective, this notion may provide new insights into how cells live and die while interacting with their environment. But, necrosis is also ubiquitous in disease. Thus, from a translational perspective, the concept of regulated necrosis may shed new light on disease pathogenesis and provide opportunities for therapeutics not thought possible in even the recent past. This review discusses the death receptor and mitochondrial necrosis pathways, how they connect with each other and with apoptosis pathways, and how necrosis may fit into the pathogenesis of the major cardiac syndromes.

### Mechanisms of Necrosis: Death Receptor Pathway

#### Death Receptors

Recent data have shown that programmed necrosis can be stimulated by the same death ligands that activate apoptosis, such as tumor necrosis factor (TNF)\textsubscript{a}, Fas ligand (FasL), and TRAIL (TNF-related apoptosis-inducing ligand). Hence, the
### TABLE 1. Features of Apoptosis and Necrosis

<table>
<thead>
<tr>
<th>Feature</th>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell</td>
<td>Shrinkage</td>
<td>Swelling</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Normal, although swelling possible late in the process</td>
<td>Marked swelling</td>
</tr>
<tr>
<td>Chromatin condensation</td>
<td>Present, classically with margination</td>
<td>Usually not prominent</td>
</tr>
<tr>
<td>Cell fragmentation</td>
<td>Membrane-enclosed apoptotic bodies</td>
<td>Cell rupture</td>
</tr>
<tr>
<td>Membrane blebbing</td>
<td>Present</td>
<td>Not characteristic</td>
</tr>
<tr>
<td>Membrane integrity</td>
<td>Intact in vivo; often lost at late time points (the latter especially in cell culture)</td>
<td>Defective at early stages</td>
</tr>
<tr>
<td>Tissue inflammation</td>
<td>Classically absent, although exceptions</td>
<td>Severe</td>
</tr>
<tr>
<td><strong>Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellular ATP levels</td>
<td>Maintained</td>
<td>Markedly depleted</td>
</tr>
<tr>
<td>Production of ATP</td>
<td>Usually maintained but may decrease</td>
<td>Markedly decreased</td>
</tr>
<tr>
<td>Consumption of ATP</td>
<td>Decreased</td>
<td>Continues</td>
</tr>
<tr>
<td>MPTP opening</td>
<td>May occur late, but not a defining feature</td>
<td>An early defining event in the mitochondrial necrosis pathway</td>
</tr>
<tr>
<td>Loss of ΔΨm</td>
<td>May occur late, but not a defining feature</td>
<td>An early defining event in the mitochondrial necrosis pathway</td>
</tr>
<tr>
<td>Apoptogen release</td>
<td>Present due to Bax/Bak-dependent OMM permeabilization</td>
<td>Not classic, but may be present because of OMM rupture following MPTP opening</td>
</tr>
<tr>
<td>Caspase activation</td>
<td>Cascade of caspase activation critical for cell death</td>
<td>Not classically present but may occur with OMM rupture</td>
</tr>
<tr>
<td>Activation of other proteases</td>
<td>May occur, eg, calpains</td>
<td>Calpains, cathepsins, and other lysosomal proteases sometimes activated and contribute to cell death</td>
</tr>
<tr>
<td><strong>Assays</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphology (light/electron microscopy)</td>
<td>Cell shrinkage and fragmentation, chromatin condensation with margination, plasma membrane blebbing</td>
<td>Cell swelling, organelle swelling, loss of plasma membrane integrity</td>
</tr>
<tr>
<td>PI/trypan blue exclusion in nonpermeabilized cells (microscopy or flow cytometry)</td>
<td>PI/trypan blue excluded until late stages</td>
<td>PI/trypan blue not excluded even at early stages</td>
</tr>
<tr>
<td>MPTP opening (eg, calcein release from matrix)</td>
<td>Not typical</td>
<td>An early event in necrosis</td>
</tr>
<tr>
<td>Loss of ΔΨm (eg, TMRE)</td>
<td>Sometimes present, but usually late</td>
<td>Occurs early</td>
</tr>
<tr>
<td>Caspase activation (eg, Western blots, immunostaining, substrate assays)</td>
<td>Present</td>
<td>Not classically present but may occur with OMM rupture</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Present (reflects effector caspase activation)</td>
<td>Usually TUNEL negative but can occur if caspases are activated by OMM rupture</td>
</tr>
<tr>
<td>Phosphatidylserine externalization (annexin V)</td>
<td>Annexin V–positive with intact plasma membrane integrity early in the process</td>
<td>Annexin V–positive, but usually with loss of plasma membrane integrity</td>
</tr>
<tr>
<td>Extracellular markers (eg, release of HMGB1 [chromatin-associated protein], and cyclophilin A and LDH [cytoplasmic proteins])</td>
<td>Absent, may occur late</td>
<td>Present</td>
</tr>
<tr>
<td><strong>Inhibitors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetic</td>
<td>Bax/Bak knockout</td>
<td>CypD knockout (mitochondrial pathway)</td>
</tr>
<tr>
<td>Drugs</td>
<td>Caspase inhibitors</td>
<td>RIP3 knockout (death receptor pathway)</td>
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Although their morphological definitions are distinct, apoptosis and necrosis are functionally connected at multiple levels. Hence, their differentiation may be difficult in some situations. Timing is an important factor, especially for cells in culture, where what begins as apoptosis can transition to necrotic morphology in the absence of corpse disposal mechanisms.
Receptor-Interacting Protein 1

Receptor interacting protein (RIP) kinases are serine/threonine kinases and constitute a family of seven members that are key regulators of cell survival and death. RIP1 was discovered 15 years ago as an interaction partner of Fas in a yeast two-hybrid screen. RIP1 consists of an N-terminal kinase domain, a RIP homotypic interaction motif (RHIM), and a C-terminal death domain (DD). The DD belongs to an evolutionarily conserved superfamily of death-fold motifs that also includes the caspase recruitment domain (CARD), the death effector domain (DED), and the pyrin domain (PYD). Death-fold motifs exhibit remarkably similar six antiparallel \( \alpha \)-helical structures, despite dissimilar primary amino acid sequences. Primarily homotypic binding of death-fold motifs mediates protein-protein interactions. The DD of RIP1 was shown to be important for binding to the DD of death receptors including TNFR1 (Figure 1), Fas, and TRAILR1 and TRAILR2 (TNF-related apoptosis-inducing ligand receptors 1 and 2). The DD of RIP1 can also bind DD-containing adaptor proteins such as TNF-receptor-associated death domain (TRADD) and Fas associated via death domain (FADD). The serine/threonine kinase activity of RIP1 is necessary for programmed necrosis, but dispensable for both survival and apoptosis.

Necrostatin (Nec)-1 is a potent small molecule inhibitor of programmed necrosis that functions by allosterically inhibiting the kinase activity of RIP1. Nec-1 blocks programmed necrosis without affecting RIP1-mediated survival, and therefore confirms the kinase-independent survival function of RIP1 and its kinase-dependent function in necrosis. Auto-phosphorylation on Ser161 within the activation loop of RIP1 is predicted to activate the kinase; yet, reconstituting RIP1-deficient cells with a phosphomimetic S161E RIP1 mutant cannot induce programmed necrosis by itself, suggesting that other factors are necessary. Nec-1 confers protection in experimental models of ischemic brain injury and myocar-
dial infarction\textsuperscript{29} in vivo, underscoring the importance of the kinase activity of RIP1 for necrosis and its potential as a therapeutic target.

**Receptor-Interacting Protein 3**

RIP3, another member of the RIP serine/threonine kinase family, has recently been implicated as a crucial molecule in the induction of programmed necrosis. RIP3 has a unique C-terminus with a RHIM that facilitates its interaction with RIP1.\textsuperscript{30} Unlike RIP1, RIP3 does not have a DD.\textsuperscript{23} RIP1 and RIP3 share 33\% similarity in amino acid sequence within their kinase domains; however, Nec-1 specifically inhibits the kinase activity of RIP1, but not RIP3.\textsuperscript{16,27}

Recent work has shown that RIP3 is indispensable for TNFα-induced necrotic cell death.\textsuperscript{19} The expression of RIP3 correlates with the ability of cells to undergo necrosis induced by death receptors. Knockdown of RIP3 renders cells resistant to TNFα-induced necrosis. Conversely, reconstituting RIP3-deficient cells with wild type RIP3, but not a kinase-dead RIP3 mutant (K50A), enables cells to undergo necrosis in response to TNFα. Thus, the kinase activity of RIP3 is essential for death receptor–induced necrosis.\textsuperscript{16,19}

RIP3 is phosphorylated on Ser199 during TNFα-induced necrosis. This phosphorylation event is critical for the subsequent cell death, and is most likely mediated by RIP3 autophosphorylation as it is ablated in RIP3 null cells reconstituted with a RIP3 kinase-defective mutant (D160N).\textsuperscript{25} However, RIP3 Ser199 phosphorylation during TNFα-induced necrosis is ablated by Nec-1, indicating that RIP1 kinase activity is also involved directly or indirectly in the phosphorylation of RIP3 Ser199.\textsuperscript{25}

The homotypic interaction between RIP1 and RIP3 through the RHIM is also required for necrosis, and this interaction is detected only with necrosis-inducing stimuli.\textsuperscript{16,19} Nec-1 treatment inhibits both the interaction between RIP1 and RIP3 and RIP3 phosphorylation, suggesting that the kinase activity of RIP1 is required for the interaction of RIP1 and RIP3, and this interaction is required for the subsequent activation of RIP3.\textsuperscript{16,19}

**Distinct TNF Complexes**

TNFα can induce several alternative outcomes: survival, apoptosis, or necrosis (Figure 1).\textsuperscript{21} Hence, the existence of distinct TNFα signaling complexes that lead to the activation of each of these alternative pathways has been investigated intensively.

On ligand binding, activated TNFR1 recruits multiple proteins, including TRADD, RIP1, cellular inhibitors of apoptosis 1 and 2 (cIAP1 and cIAP2), and TNFR-associated factors 2 and 5 (TRAF2 and TRAF5), to form complex I at the plasma membrane.\textsuperscript{8} cIAPs, which bind and inhibit active downstream caspases,\textsuperscript{31} also function as E3 ubiquitin ligases, catalyzing the formation of lysine 63 (K63) polyubiquitin chains onto Lys377 of RIP1.\textsuperscript{32,33} This K63-polyubiquitin chain promotes the recruitment of transforming growth factor-β-activated kinase 1 (TAK1)-binding proteins 2 and 3 (TAB2/3) and TAK1, which activate the IκB kinase (IKK) complex to trigger the activation of NF-κB that transcriptionally upregulates multiple survival genes.\textsuperscript{34} Therefore, RIP1 is thought to be necessary for NF-κB activation, although the kinase activity of RIP1 is dispensable. The necessity of RIP1, however, has recently been challenged.\textsuperscript{35}

A second complex, known as complex II, is formed following endocytosis of complex I and subsequent dissociation from the receptor. TNFα-induced ubiquitination of RIP1 is dynamic and affects the transition from complex I to complex II. Several deubiquitinating enzymes, such as A20\textsuperscript{16} and cylindromatosis (CYLD),\textsuperscript{33} remove the K63-linked polyubiquitin chains from RIP1. This modification is a necessary step in the formation of complex II because knockdown of CYLD can protect cells against TNFα-induced apoptosis and necrosis, the 2 outcomes specified by complex II as is described below.\textsuperscript{37,38}

FADD and procaspase-8 are recruited to complex II, leading to the initiation of apoptosis through forced proximity activation of procaspase-8.\textsuperscript{8,39} Active caspase-8 then proteolytically cleaves RIP1 and RIP3, thereby inactivating their kinase function and precluding the possibility of necrosis. In addition, caspase-8-induced cleavage of RIP1 produces a C-terminal fragment containing the DD, which further drives apoptosis by providing a platform for additional procaspase-8 activation.\textsuperscript{40}

When caspase-8 is deleted or pharmacologically or genetically inhibited, complex II cannot initiate apoptosis, and thus TNFR1 ligation results in programmed necrosis in cell types competent to undergo necrosis.\textsuperscript{26,41} Critical events for necrosis take place in a necrosis-promoting signaling complex, termed the necrosome (Figure 2). In the necrosome, interdependent phosphorylation of RIP1 and RIP3 initiates necrosis as described above. The involvement of FADD or TRADD in programmed necrosis is less clear. FADD-deficient mouse embryonic fibroblasts (MEFs) are resistant to TNFα-induced programmed necrosis.\textsuperscript{40} On the other hand, the absence of FADD sensitizes Jurkat cells to TNFα-induced necrotic death.\textsuperscript{16,26} Moreover, RIP1 phosphorylation still occurs in TNFα-treated FADD-deficient Jurkat cells, which implies that RIP1 and RIP3 can be activated in the absence of FADD. In TRADD-deficient cells, both TNFα-induced apoptosis and necrosis are inhibited.\textsuperscript{42} This observation suggests that TRADD is essential in TNFα-induced cell death in certain experimental settings. However, in some cell types, knockdown of TRADD can stimulate the formation of complex II implying that the assembly and function of complex II does not rely on TRADD in these cell types.\textsuperscript{38} Although a basic understanding of complex II exists, many details await clarification.

**Downstream Execution of TNFR-Induced Necrosis**

Identifying the signaling events that occur downstream of the initiation of programmed necrosis is important in determining how necrosis is executed and for developing potential therapeutic reagents that can target specific events after the initial insult. We discuss several distinct molecular mechanisms that have been described to contribute to the execution of programmed necrosis (Figure 2).
RIP3 has been shown to be required for TNFα-induced reactive oxygen species (ROS) production during necrosis in several cell types, such as L929 murine fibrosarcoma cells, MEFs, and N-type NIH 3T3 cells. Moreover, RIP3 is found to interact with several metabolic enzymes, including glutamate ammonia ligase (GLUL), glutamate dehydrogenase 1 (GLUD1), and glycogen phosphorylase (PYGL). These enzymes are essential for the use of glutamate, glutamine, and glycogen, respectively, as energy substrates for ATP production in oxidative phosphorylation, a major source of ROS. The activation of these enzymes by RIP3 can elevate ROS production leading to TNFα-induced necrosis as a consequence of increased energy metabolism. Indeed, siRNA depletion of PYGL, GLUL, and GLUD1 reduced TNFα-induced ROS accumulation, correlating with a decrease in cell death.

The production of ROS has been shown to be essential for TNFα-induced programmed necrosis in L929 cells and MEFs. Different sources of ROS generation have been reported to be critical including Nox1, an NADPH oxidase at the plasma membrane, and mitochondrial complex I. TNFα-stimulated ROS generation through Nox1 depends on RIP1, whereas TNFα stimulation of mitochondrial ROS requires the RIP1-RIP3 complex. The mechanism by which the RIP kinases affect mitochondrial complex I, however, remains unclear. Treatment with an antioxidant, such as butylated hydroxyanisole, reduces TNFα-induced ROS levels and necrosis in some cell types. However, not all cell lines are protected from programmed necrosis after antioxidant treatment, suggesting that TNFα-induced killing may be mediated by additional mechanisms.

Cellular Energetics, Poly(ADP-Ribose) Polymerase-1 Overactivation, and the Mitochondria

During apoptosis, ATP-consuming processes such as translation, proteasome function, and DNA repair, are minimized through caspase cleavage of specific proteins. On the other hand, ATP consumption persists during necrosis. Continued consumption of ATP during necrosis and impaired ATP production (discussed below) result in markedly decreased ATP levels. Poly(ADP-ribose) polymerase (PARP)-1 is a nuclear protein that is activated during DNA repair and transcriptional regulation. Activated PARP-1 catalyzes the NAD+-dependent synthesis of poly(ADP-ribose) (PAR) onto target proteins resulting in depletion of cellular NAD+. The deficit of NAD+ decreases the rate of glycolysis because NAD+ is an essential cofactor for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Consequently, cells activate other pathways to produce NAD+, leading to excessive ATP consumption. This process has been shown to be involved in the necrotic death of L929 cells in response to TNFα, with PARP-1 overactivation perhaps resulting from ROS-mediated DNA damage. The same process may account for experimental ischemia/reperfusion injury in the brain, which is also accompanied by overactivation of PARP-1, and PARP-1 knockout reduces infarct size. Similarly, PARP-1 absence decreases myocardial infarction size following ischemia/reperfusion. Yet, necrosis induced by PARP-1 overactivation may not be solely attributable to the depletion of ATP. It has been demonstrated that overactivation of PARP-1 can increase mitochondrial complex I production of ROS. Ischemia/reperfusion-induced mitochondrial complex I defects are abrogated in PARP-1-deficient mice. Increased poly(ADP-ribose)ylation of several proteins upstream of mitochondria and translocates to the nucleus in a PARP-1–dependent manner, where in conjunction with an endonuclease, causes DNA damage, possibly resulting in further PARP-1 overactivation. ROS and alkylating agents such as MNNG can also damage DNA and overactivate PARP-1. Overactivation of PARP-1 leads to excessive consumption of NAD+, depleting ATP levels and inducing necrosis. Interestingly, RIP1 has been discovered to function downstream of PARP-1 in programmed necrosis. The precise molecular connections between PARP-1 and RIP1, however, remain unclear.
mitochondrial proteins, including components of the electron transport chain, have been shown in rat liver mitochondria in response to H$_2$O$_2$ or NO, and may also further increase ROS production.$^{58,59}$ In addition, RIP1 appears to mediate some instances of PARP-1-dependent necrosis. For example, MNNG (N-methyl-N'-nitro-N'-nitrosoguanidine), an alkylating agent, activates PARP-1 in MEFs resulting in cell death, which can be rescued by the absence of RIP1.$^{60}$ In addition to the generation of mitochondrial ROS, PARP-1 overactivation can also induce necrosis through mitochondrial release of apoptosis inducing factor (AIF).$^{61-64}$ The mechanism by which PARP-1 induces AIF release from the mitochondria is still controversial. The sequential activation of calpains and Bax was demonstrated to be crucial for the release of AIF.$^{63}$ Other investigators, however, suggest that AIF release is induced by the PAR polymer.$^{57}$ Following mitochondrial release, AIF translocates to the nucleus and, in conjunction with an endonuclease, carries out large-scale DNA cleavage. How AIF induces necrosis is not clear, however, but may involve further PARP-1 activation resulting from AIF-induced DNA damage.

RIP1-dependent signaling can also result in the inhibition of the adenine nucleotide translocase (ANT).$^{65}$ ANT is an integral protein in the IMM that exchanges ATP synthesized in the matrix with cytosolic ADP.$^{66}$ Therefore, a reduction of matrix ADP levels may occur through the inhibition of ANT by RIP1 activation. This, in turn, may lead to the reversal of the F$_1$-Fo ATP synthase and hyperpolarization of the mitochondrial transmembrane potential ($\Delta\psi_m$),$^{67}$ which is observed during the early phases of programmed necrosis.$^{68,69}$ RIP1-dependent inhibition of ANT requires the inclusion of the caspase inhibitor z-VADfmk with TNF$\alpha$ to induce necrosis. However, observations that z-VADfmk itself can interact with ANT raises questions about the general applicability of this mechanism. This will be resolved by testing other necrotic stimuli.

Proteases and Lysosomal Membrane Permeabilization

A proteolytic cascade occurs during apoptosis where the initiator caspases cleave and activate effector caspases. In contrast, a defined proteolytic cascade has not been discovered in the necrotic pathway. Nevertheless, proteases are involved in the execution of necrosis. Calpains are cytoplasmic noncaspase cysteine proteases that are ubiquitously and constitutively expressed in mammalian cells. Under normal conditions, calpastatin is the physiological inhibitor of calpains. However, when cytosolic Ca$^{2+}$ levels increase, calpains are activated.$^{70}$ Activated calpains can cleave the Na$^+$/Ca$^{2+}$ exchangers on the plasma membrane$^{71}$ and mitochondria$^{72}$ leading to Ca$^{2+}$ overload in the cytosol and mitochondrial matrix respectively. The importance of calpain activation in necrotic cell death has been shown in neurons of C elegans,$^{6}$ in dystrophin-deficient muscles of mice,$^{73}$ and in high glucose-induced necrosis in LLC-PK1 cells.$^{74}$ Moreover, in posts ischemic CA1 neurons of primates, activated calpains translocate to the lysosomal membrane$^{75}$ cleaving a form of heat shock protein 70, a chaperone that controls lysosomal membrane integrity, and leading to lysosomal membrane permeabilization (LMP).$^{76}$ Heat shock protein 70 overexpression has been shown to delay LMP and necrosis induced by TNF$\alpha$ or oxidative stress.$^{77,78}$

The disruption of lysosomal membrane integrity allows for the release of proteases that contribute to necrosis.$^{79}$ LMP can be induced by several factors including oxidative stress, lipids, and proteases,$^{80}$ and can lead to cell death through several mechanisms. First, the rupture of lysosomes contributes to the acidification of the cell, which has been shown to be a requirement for necrosis in C elegans.$^{81}$ Second, LMP is associated with the activation of phospholipase A$_2$, which in turn increases the production of ROS.$^{82}$ Third, LMP allows for a massive release of free iron, which through a Fenton-type reaction, produces reactive hydroxyl radicals.$^{83}$ In fact, it has been observed in L929 cells that a sudden increase of free, cytosolic iron is important for TNF$\alpha$-induced necrosis.$^{84}$ Finally, LMP permits the release of proteases, such as cathepsins, into the cytosol allowing for cleavage of various proteins. Ischemic injury in neurons results in the release of cathepsins from lysosomes, and cathepsin inhibitors significantly attenuate neuronal necrosis.$^{85}$ These data suggest an important role for cathepsins in the execution of necrotic cell death.

Phospholipases, Lipoxygenases, and Sphingomyelinases

A key feature of necrosis is the disruption of organelles and plasma membranes. Lipid peroxidation appears to be a mechanism of membrane disruption in necrosis that can be mediated by lipoxygenase.$^{86,87}$ TNF$\alpha$ has been shown in L929 cells to activate phospholipase A$_2$, a family of esterases that produces arachidonic acid from phospholipids.$^{88,89}$ Arachidonic acid is acted on by lipoxygenase, thereby generating ROS and contributing to lipid peroxidation and the disruption of membranes.$^{87}$

Ceramide is a second messenger that elicits pleiotropic effects during necrosis, including activation of nitric oxide synthase, lipid peroxidation, mitochondrial ROS production,$^{80}$ and calpains.$^{91}$ Ceramide can be generated by sphingomyelinase-catalyzed hydrolysis of sphingomyelin, a membrane sphingophospholipid. There are several isoforms of sphingomyelinase that are distinguishable by their subcellular localizations and pH optima. A neutral sphingomyelinate is found at the plasma membrane and an acid sphingomyelinate is localized in the endosomal-lysosomal compartment. A significant increase in ceramide levels has been observed during TNF$\alpha$-induced necrosis in many cell types, including L929 cells, NIH3T3 fibroblasts, and human Jurkat T cells, and the accumulation of ceramide seems to be more pronounced when caspases are inhibited.$^{92-94}$ Cells deficient in acid sphingomyelinate or treated with acid sphingomyelinate inhibitors are more resistant to TNF$\alpha$/z-VADfmk-induced necrosis,$^{94}$ as are cells overexpressing acid ceramidase, which degrades ceramide.$^{89}$ Interestingly, depletion of RIP1 confers protection against ceramide accumulation and necrotic cell death induced by TNF$\alpha$/z-VADfmk in many cell types,$^{94}$ suggesting that RIP1 is essential for the induction of ceramide production in TNF$\alpha$-mediated necrosis.
Mechanisms of Necrosis: Mitochondrial Pathway

Key Mitochondrial Events in Necrosis and Apoptosis

In addition to their role in coupling substrate catabolism with ATP production, mitochondria are intimately involved in regulated forms of cell death. The major mitochondrial event in apoptosis is permeabilization of the OMM. In contrast, the defining mitochondrial event in necrosis is the opening of a channel in the IMM, called the mitochondrial permeability transition pore (MPTP) (Figure 3). Although the biochemical events that mediate OMM permeabilization during apoptosis are poorly understood,95 it is clear that the process is triggered by Bax and Bak, proapoptotic multidomain Bcl-2 proteins. OMM permeabilization allows for the release of mitochondrial apoptogens, such as cytochrome c, into the cytoplasm. These apoptogens promote caspase activation and cell death via the mitochondrial apoptosis pathway. On the other hand, the distinct IMM events that mediate the mitochondrial necrosis pathway is discussed in this section.

Effects of MPTP Opening on Mitochondrial Structure and Function

Although the unpermeabilized OMM does not allow for the passage of apoptogens, such as cytochrome c, it is permeable to ions and small molecules.96 Hence, the cytoplasm is isoelectric with respect to the mitochondrial intermembrane space. In contrast, the IMM in a healthy mitochondrion is impermeable to small molecules and even protons, resulting in an electric and chemical gradient between the intermembrane space and matrix. In particular, the passage of electrons during respiration generates a proton gradient rendering the matrix negative, and this electric potential difference (termed $\Delta \psi_{\text{m}}$) drives the conversion of ADP to ATP. Maintenance of IMM integrity is critical. Conversely, opening of MPTP causes an acute derangement in mitochondrial structure and function that leads to necrotic cell death.

The immediate consequences of MPTP opening include: (1) collapse of $\Delta \psi_{\text{m}}$, leading to cessation of respiration-driven ATP synthesis and reversal of the FoF1-ATP synthase; (2) redistribution of solutes and ions across the IMM; and (3) entry of large amounts of water into the solute-rich matrix to normalize the osmotic gradient. This entry of water results in matrix swelling and expansion of the redundant IMM. Because the OMM lacks the redundancy of the IMM, IMM expansion can lead to OMM rupture and the release of apoptogens, including cytochrome c, into the cytosol. In contrast, the release of apoptogens during apoptosis involves Bax/Bak-dependent OMM permeabilization (not rupture) and usually occurs before loss of $\Delta \psi_{\text{m}}$. Nevertheless, release of apoptogens attributable to OMM rupture following MPTP opening can trigger apoptosome assembly and caspase activation.9 Although severe ATP depletion and loss of plasma membrane integrity are primarily responsible for cell death in necrosis, it is possible that activation of downstream apoptotic signaling also contributes.

The major regulator of MPTP opening is matrix Ca$^{2+}$.97 Multiple additional factors strongly influence pore opening, however, and are thought to work through modulating sensitivity to Ca$^{2+}$.98 Thus, oxidative stress, increased phosphate, and adenine nucleotide (ATP and ADP) depletion augment Ca$^{2+}$ sensitivity to pore opening, whereas acidosis does the opposite.99

Ca$^{2+}$ overload during ischemia is promoted by anaerobic metabolism, lactate production, and intracellular acidosis. H$^+$ is pumped out of the cell by the Na$^+$/H$^+$ exchanger, which in combination with malfunctioning Na$^+$/K$^+$ ATPase (attributable to ATP depletion) results in increased intracellular Na$^+$. Na$^+$ is subsequently exchanged for Ca$^{2+}$ by reverse operation of the sarcolemmal Na$^+$/Ca$^{2+}$ exchanger, resulting in intracellular Ca$^{2+}$ overload. Additional elevations in intracellular Ca$^{2+}$ result from Ca$^{2+}$-induced Ca$^{2+}$ release from the endoplasmic reticulum/sarcoplasmic reticulum (ER/sarcoplasmic...
Components of MPTP: Past and Present

The conceptual basis of MPTP had its origins in experiments performed a half-century ago, in which Ca\(^{2+}\) was noted to trigger marked mitochondrial swelling.\(^{101,102}\) Although various models were considered to explain this observation, the data were most consistent with a nonspecific, high-conductance channel in the IMM. Subsequent experiments demonstrated that this channel could accommodate the passage of molecules <1500 Da.\(^{103}\) Despite considerable effort, the components of MPTP are still not known with certainty. A number of proteins have been hypothesized to comprise and/or regulate this pore including ANT, phosphate carrier (PiC), voltage-dependent anion channel (VDAC), peripheral benzodiazepine receptor, and cyclophilin D. A combination of biochemical and gene knockout studies, however, has ruled out an essential role for many of these proteins as components of the channel, although suggesting a regulatory role for some.

Adenine Nucleotide Translocase

As noted previously, ANT, the most abundant protein in the IMM, functions as an ADP/ATP exchanger. The involvement of ANT in MPTP is supported by correlations between ANT conformation and MPTP opening.\(^{104}\) The ANT ligand carboxyatractyloside stabilizes the cytosolic (“c”) conformational state and stimulates MPTP opening. Conversely, the ANT ligand bongkrekic acid stabilizes the matrix (“m”) conformational state, and inhibits MPTP opening. Ca\(^{2+}\), the major trigger for MPTP opening, stimulates the c conformation of ANT. Although ANT lacks traditional Ca\(^{2+}\) binding motifs, it is possible, but unproven, that Ca\(^{2+}\) binds ANT through carboxyl groups on aspartic acid and glutamic acid residues facing the matrix.\(^{105}\) In addition, the binding of adenine nucleotides to ANT decreases the sensitivity of Ca\(^{2+}\)-induced MPTP opening. Oxidative stress, which potentiates Ca\(^{2+}\)-induced MPTP opening,\(^{104}\) also stimulates disulfide bond formation between matrix-facing cysteine160 and cysteine257 in rat ANT1,\(^{106}\) interfering with the binding of adenine nucleotides to ANT. Although the above data are correlative, they link certain conformational or post-translational states of ANT with MPTP opening, suggesting that ANT is part of MPTP. In contrast, genetic experiments raise significant questions about the necessity of ANT for MPTP function. Initially, there were believed to be 2 mouse ANT genes, ANT1 and ANT2. Mitochondria derived from mice lacking ANT1 and ANT2 still demonstrate MPTP opening in response to Ca\(^{2+}\), suggesting that ANT is not an essential component of MPTP.\(^{107}\) However, the more recent discovery of a third mouse ANT gene, ANT4,\(^{108}\) raises questions concerning redundancy. Whether or not ANT is essential for MPTP opening, it modulates this channel as mitochondria from the ANT1-ANT2 double knockout exhibit reduced Ca\(^{2+}\) sensitivity of MPTP opening.\(^{107}\) In conclusion, ANT is not believed to be a critical component of MPTP, but probably plays a regulatory role.

Voltage-Dependent Anion Channel

VDAC, the most abundant protein in the OMM, functions as a low specificity pore allowing the passage of molecules <5kDa. VDAC was noted to copurify with ANT,\(^{109}\) suggesting that these proteins may interact at contact sites between the OMM and IMM. However, deletion of all 3 mouse VDAC genes (VDAC1, VDAC2, VDAC3) does not affect Ca\(^{2+}\)- and oxidative stress–induced MPTP opening, indicating that VDAC is dispensable for MPTP function.\(^{110,111}\)

Cyclophilin D

Cyclophilin D, encoded by the nuclear gene ppiF, is a peptidylprolyl cis-trans isomerase that resides in the mitochondrial matrix.\(^{112,113}\) Its normal physiological functions are not known with certainty, but some data support a role in Ca\(^{2+}\) efflux.\(^{114}\) Cyclophilin D interacts with ANT and the phosphate carrier (see below).\(^{115}\) The drug cyclosporin A binds to cyclophilin D and inhibits Ca\(^{2+}\)-induced MPTP opening.\(^{116}\) Deletion of ppiF renders mitochondria highly resistant to Ca\(^{2+}\)-induced MPTP opening,\(^{9,10}\) although this will still occur at high Ca\(^{2+}\) concentrations. Conversely, cyclophilin D overexpression induces MPTP opening in the absence of an inciting death stimulus.\(^{9}\) Thus, cyclophilin D plays an important role in promoting MPTP opening. The prolyl isomerase activity of cyclophilin D is important in this function because reconstitution of ppiF null cells with wild type, but not isomerase-deficient, cyclophilin D restores MPTP opening.\(^{8}\) Absence of cyclophilin D does not affect classic mitochondrial apoptotic responses such as cytochrome c release in response to Bax, nor does it protect cells against traditional apoptotic stimuli such as staurosporine. In contrast, the absence of cyclophilin D protects cells in culture and in vivo against necrotic stimuli, whereas overexpression of cyclophilin D does the opposite.\(^{8}\) These results indicate that cyclophilin D is a key regulator of MPTP and necrotic, but not apoptotic, cell death. The fact that MPTP opening can still proceed in the absence of cyclophilin D argues strongly against an essential structural role in the pore.

Phosphate Carrier

PiC is an IMM protein that transports inorganic phosphate (Pi). The ability of the Pi to promote MPTP opening is well known.\(^{1,101}\) More recently, it has been shown that, surprisingly, Pi is also important for desensitization of Ca\(^{2+}\)-induced MPTP opening by cyclosporin A or deficiency of cyclophilin D.\(^{117}\) In fact, PiC binds cyclophilin D in a cyclosporine A-inhibitable manner. In addition, ANT and cyclophilin D interact.\(^{115}\) ANT also binds PiC in a cyclosporine A-independent manner. Thus, it is possible that ANT, PiC, and cyclophilin D form a complex. As previously discussed, ANT and cyclophilin D are not essential for MPTP but play important regulatory roles. The necessity of PiC has yet to be tested in knockout studies. Therefore, one model is that PiC or another yet to be determined protein is an essential MPTP constituent. In this model, the prolyl isomerase activity of cyclophilin D would conformationally regulate PiC, ANT, or
both. The mechanism by which Ca\(^{2+}\) would activate the pore is unknown. Drugs that inhibit cyclophilin D might work through disrupting its interaction with the complex (cyclosporin A) or inhibiting its isomerase activity (sanglifehrin A).\(^{118}\)

**Summary of MPTP Function and Regulation**

There are multiple open questions. Most important, the essential components of the MPTP have not been delineated unambiguously. Genetic studies exclude VDAC and probably ANT as essential components, although ANT may play a regulatory role. Pore opening is not absolutely dependent on cyclophilin D, suggesting that cyclophilin D is not an essential component. However, cyclophilin D is clearly a key regulator. The characteristics of PiC suggest that it may be a core component, but genetic loss of function studies will be needed to test this. Thus, the possibility remains that an essential protein is yet to be identified. Ca\(^{2+}\) is an important trigger for MPTP opening, but it is not clear if its critical targets are PiC, ANT, cardiolipin, or another moiety.

**Explaining the Necrosis Phenotype**
The major manifestations of necrosis are severely decreased cellular ATP levels, defects in membrane integrity, and inflammation.

**ATP Depletion**
As we have already discussed the bioenergetics of necrosis, the key findings are summarized here. Severe decreases in ATP synthesis can result from RIP1-dependent ANT inhibition\(^{106}\) as well as MPTP opening and its consequences.\(^{119}\) Moreover, whatever ATP remains in necrotic cells is squandered by the continued operation of expensive cellular processes such as DNA repair, translation, and others, processes that are halted during apoptosis by caspase cleavage of key proteins.\(^{48—50}\) Thus, cellular ATP depletion during necrosis involves both mitochondrial and peripheral components.

**Membrane Dysfunction**
Necrosis also involves defects in the integrity of plasma membranes (resulting in loss of cellular homeostasis and cell swelling), ER membranes (resulting in increased intracellular Ca\(^{2+}\)), and lysosomal membranes (resulting in the release of proteases such as cathepsins). This aspect of the necrotic phenotype is very important but not well understood. Aspects of the molecular mechanism have been reviewed above. In addition to these factors of general interest, mechanical stress can contribute to plasma membrane leakiness in striated muscle cells. The sarcolemma of actively contracting cells is susceptible to damage. For example, transient leakiness of the cardiac sarcolemma can be induced by exercise and \(\beta\)-adrenergic stimulation in rats.\(^{120}\) Membrane stress attributable to ischemic contractures during infarction may contribute to plasma membrane defects.

Moreover, skeletal and cardiac myocytes in patients experiencing a variety of muscular dystrophy syndromes exhibit increased sarcosomal fragility.\(^{121}\) Plasma membrane damage in these situations is counteracted by ongoing repair mechanisms involving dysferlin\(^{122,123}\) and mitsugumin 53.\(^{124—127}\) Sarcosomal abnormalities in animal models of muscular dystrophy appear to activate necrosis signaling as evidenced by increased cellular Ca\(^{2+}\) and swollen mitochondria.\(^{128}\) Although deletion of ppif does not correct the genetically determined plasma membrane dysfunction, it improves mitochondrial abnormalities and ameliorates muscle degeneration.\(^{129,130}\) These studies underscore that mechanical stress in muscle cells may be an important factor in plasma membrane dysfunction leading to necrosis.

The muscular dystrophy work also unmasks a bidirectional relationship between necrosis signaling and plasma membrane defects. Traditionally, we think of necrosis pathways as inducing plasma membrane dysfunction. However, the baseline plasma membrane dysfunction in muscular dystrophy appears to activate necrosis pathways, which play an important role in pathogenesis. Thus, it would appear that activation can proceed bidirectionally. This model predicts that key membrane-associated proteins as well as components of the endogenous membrane repair systems might be targets of proteases activated during necrosis in heart failure unrelated to the various dystrophic syndromes. In fact, dystrophin cleavage has been observed in patients with idiopathic heart failure.\(^{129}\)

**Inflammation**
As described previously, apoptosis has traditionally been considered a death process that avoids inflammation, whereas necrosis is highly inflammatory. Although this dichotomy is accurate as a first approximation, recent observations suggest increased complexity regarding the occurrence of inflammation in apoptosis and necrosis. As the details of the postdeath clean-up operation, including recruitment of inflammatory cells and phagocytosis, are beyond the scope of this review, we summarize several key points. The reader is referred to an excellent recent essay for an in-depth consideration of this area.\(^{130}\)

The essential principle is that a combination of factors determines whether dying cells undergo silent (no inflammation) removal versus noisy (marked inflammation) removal. These include the active release of soluble “find me” signals to attract phagocytes. For example, “find me” signals in apoptosis include lysophosphatidylcholine and sphingosine-1-phosphate, which attract macrophages while altering their release of cytokines to avoid inflammation.\(^{131}\) “Eat me” signals to induce phagocytosis include phosphatidylserine\(^{132}\) displayed at the surface of apoptotic cells. “Eat me” signals bind to specific serum proteins and interact with receptors on phagocytes triggering engulfment. Silent removal may also be the fate of cells undergoing necrosis that have not yet lost plasma membrane integrity when these cells exhibit phosphatidylserine at the cell surface\(^{133}\) thereby facilitating phagocytosis.\(^{134}\) In contrast, necrotic cells that have undergone plasma membrane permeabilization release a variety of proteins and nucleic acids factors that are not only markers of membrane dysfunction, but also mediate the inflammatory response. For example, the release of the histone-associated protein HMGB1 (high-mobility group protein B1) stimulates inflammation through multiple mechanisms involving Toll-like receptors and Receptor for Advanced Glycation End-products.
Connections Between Cell Death Pathways
Peripheral Signaling Pathways That Can Regulate Apoptosis and Necrosis

Multiple signaling pathways affect both apoptosis and necrosis and often exert concordant effects.

Akt and Pim-1
The serine/threonine kinase Akt inhibits apoptosis through its phosphorylation of specific targets, which alters the function and/or subcellular localization of these proteins (eg, FoxO [forkhead box O], Bad [Bcl-2 associated agonist of cell death], Bax, glycogen synthase kinase [GSK3β]). Akt can also translocate to the mitochondrial matrix and inhibit MPTP opening, but its targets in this situation are unknown. Similarly, Pim-1, itself a serine/threonine kinase and downstream effector of Akt, antagonizes apoptosis through substrates that are distinct from, and overlap with, those of Akt. Pim-1 also translocates to the mitochondria following ischemia/reperfusion and inhibits MPTP opening through an unknown mechanism.

Protein Kinase C-ε
Protein Kinase (PK)C-ε inhibits MPTP opening, an effect requiring its kinase activity. In this case, there are some hints as to mechanism: PKC- ε interacts with ANT1, VDAC1, and hexokinase II, and recombinant PKC- ε can phosphorylate VDAC1. Under hypoxic conditions, PKC- ε can also associate with and phosphorylate cytochrome oxidase (mitochondrial complex IV). The mechanistic relationships between these phosphorylation events and MPTP opening, however, are unclear.

Glycogen Synthase Kinase 3β
GSK3β promotes apoptosis (eg, by phosphorylating Bax and facilitating its mitochondrial translocation). GSK3β is also an important point of convergence for multiple signals that regulate MPTP opening in cardiac myocytes. Specifically, GSK3β inactivation by phosphorylation (pharmacological agents) or knockdown with RNAi decreases the sensitivity of MPTP opening. Although the underlying molecular mechanism has not yet been delineated in cardiac myocytes, an association has been identified in cancer cells. GSK3β interacts with cyclophilin D, which becomes phosphorylated, but it is not known whether phosphorylation of cyclophilin D is responsible for the increased sensitivity of MPTP opening.

Connections Between Death Receptor and Mitochondrial Necrosis Pathways

The death receptor and mitochondrial necrosis pathways are functionally interconnected. Necrosis induced by the TNF death receptor pathway (TNFα/SMAC-mimetics/z-VADfmk) is substantially rescued in MEFs lacking cyclophilin D. RIP1 is necessary for MNNG-induced loss of Δψm, suggesting a connection between RIP1 and MPTP opening. Administration of nec-1 reduces infarct size in wild type mice subjected to ischemia/reperfusion injury, but does not reduce infarct size further in mice already cardioprotected because of the absence of cyclophilin D. These observations suggest that RIP1 and cyclophilin D reside in the same genetic pathway, although the molecular connections await elucidation.

The death receptor and mitochondrial necrosis pathways are connected through several potential mechanisms. One possible connection is ROS. As previously described, RIP3 binds and activates catabolic enzymes that generate ROS, and ROS increases the sensitivity of MPTP opening. A second connection may be provided by other substrates of RIP3 which have yet to be identified. Some may be components of the mitochondrial death machinery or regulate these components indirectly. A third possibility is RIP1, which can translocate to the mitochondria in response to TNFα. Although possible effects of RIP1 on ANT have been discussed previously, its localization at the mitochondria may provide an opportunity for additional regulation.

Connections Between Apoptosis and Necrosis: Death Receptor Pathway

The death receptor apoptosis and necrosis pathways are intimately connected through multiple shared constituents (ligands, receptors etc; Figure 1). They differ with respect to some of the complexes that are formed (eg, necrosome; Figure 2). The decision to die through either death program is driven, in part, by K63-deubiquitination of RIP1 and the transition from complex I to complex II. The choice to undergo apoptosis rather than necrosis, however, is determined by whether caspases are activated, and RIP1 is cleaved. Thus, in the death receptor pathway, necrosis is a default outcome when apoptosis is inhibited.

The death receptor apoptosis and necrosis pathways are also linked by Bmf (Bcl-2-modifying factor), a BH3-only member of the Bcl-2 family. In healthy cells, this protein is sequestered on the myosin V-actin motor complex, but is released to translocate to mitochondria and induce apoptosis in response to stimuli such as anois. Bmf appeared in an siRNA screen for mediators of death receptor–induced necrosis and was confirmed to be necessary for this form of cell death, although the mechanism is not known.

Connections Between Apoptosis and Necrosis: Mitochondrial Pathway

In contrast to the death receptor pathway, it appears that either apoptosis or necrosis can be a primary outcome in the mitochondrial pathway. Therefore, a mechanism may exist to coordinate these processes in this pathway, but its molecular nature is not understood.

Connections Between Outer and Inner Mitochondrial Membranes

As apoptotic OMM and necrotic IMM events are separated by only microns, one would expect cross-talk between these processes. We discuss examples of signaling between Bcl-2 proteins on the OMM and ANT on the IMM; how necrotic mitochondrial events can activate downstream apoptosis signaling; and how caspase cleavage during apoptosis could produce a necrotic phenotype.

During apoptosis induced by growth factor withdrawal or etoposide, ADP/ATP exchange by ANT decreases. Antiapoptotic Bcl-2 and Bcl-Xl interact with ANT and stimulate ADP/ATP exchange. These findings were interpreted a de-
Expression of a noncleavable mutant of NDUFS1 (on the wild type background) maintains consistent with MPTP opening), decreased ATP levels, and ROS production, loss of ∆ψ\text{m}, mitochondrial swelling, which are all features of necrosis. The swelling of the mitochondrion leads to OMM rupture and results in the release of cyt c, which occurs independently of Bax/Bak. The transfer of Ca\textsuperscript{2+} from the ER to the mitochondria is postulated to be the stimulus for MPTP opening. Adapted from Kitsis and Molkentin.\textsuperscript{14} with permission from the National Academy of Sciences (illustration credit: Cosmocyte/Ben Smith).

NADH dehydrogenase (ubiquinone) Fe-S protein 1 (NDUFS1 or p75), a component of mitochondrial complex I, is a caspase-3 substrate that gets cleaved during staurosporine-induced apoptosis. NDUFS1 faces the mitochondrial intermembrane space, and caspase-3 presumably gains access during apoptosis through the already permeabilized OMM. Cleavage of NDUFS1 disrupts electron transport leading to ROS production, loss of ∆ψ\text{m}, mitochondrial swelling (consistent with MPTP opening), decreased ATP levels, and defective plasma membrane function, all features of necrosis. Expression of a noncleavable mutant of NDUFS1 (on the wild type background) maintains ∆ψ\text{m}, ATP levels, and mitochondrial morphology, limits ROS production, and delays plasma membrane leakiness.\textsuperscript{146} The fact that these effects occur early in this model support their relevance to cell killing. However, the overall importance of these events to the mechanisms by which apoptosis brings about cell death is not clear.

Proteins That Activate Both Apoptotic and Necrotic Signaling

Bak resides constitutively in the ER membrane and OMM, and Bax trafficks to these locations in response to some death stimuli.\textsuperscript{95} In addition to their role in promoting OMM permeabilization and apoptogen release, Bax and Bak also exert effects on ER Ca\textsuperscript{2+} handling and cell death. Thus far, the details of this process have been studied only in MEFs.\textsuperscript{147,148} An important mechanism for ER-mediated cell killing is the transfer of a bolus of Ca\textsuperscript{2+} from ER to mitochondria, either through the cytoplasm or via direct connections between the 2 organelles. The delivery of Ca\textsuperscript{2+} to the mitochondria may trigger necrosis through MPTP opening and possibly apoptosis through unknown mechanisms. Antia apoptotic Bcl-2 inhibits ER-mediated cell death by interacting with the type 1 inositol-1,4,5-triphosphate receptor (IP\textsubscript{R}-1) to induce a Ca\textsuperscript{2+} leak.\textsuperscript{149} The resulting decrease in baseline ER luminal Ca\textsuperscript{2+} concentration limits the magnitude of the Ca\textsuperscript{2+} bolus elicited by death stimuli relevant to this pathway (eg, oxidative stress, lipids), and cell death is blunted. In contrast, in the presence of proapoptotic Bax, Bcl-2 dissociates from IP\textsubscript{R}-1 abrogating the Ca\textsuperscript{2+} leak. The baseline ER luminal Ca\textsuperscript{2+} concentration increases as does the bolus of released Ca\textsuperscript{2+} and amount of cell death elicited by a death stimulus.

Standard BH3-only proteins induce apoptosis, and this requires the BH3 domain, which mediates interactions with Bax or Bak. In contrast, BH3-only-"like" proteins possess an atypical BH3 domain,\textsuperscript{12} which is not required for killing.\textsuperscript{150} Cell death induced by BH3-only-like proteins can exhibit features of apoptosis or necrosis. Nix/BINip3L, a BH3-only-like protein, is induced in cardiac myocytes by hypertrophic stimuli.\textsuperscript{151} Nix/BINip3L overexpression elicits heart failure primarily resulting from cell death,\textsuperscript{151} whereas cardiac-specific deletion of Nix ameliorates cardiomyopathy induced by hemodynamic overload.\textsuperscript{152,153} Nix/BINip3L is normally found at the OMM and ER membrane.\textsuperscript{154} Targeting Nix/BINip3L to mitochondria versus ER shows that it is capable of inducing more than one type of cell death (Figure 4).\textsuperscript{155} Mitochondrially-targeted Nix/BINip3L causes Bax/Bak-dependent OMM permeabilization, apoptogen release, caspase activation, and apoptosis. In contrast, ER-targeted Nix/BINip3L triggers MPTP opening, swollen mitochondria, and cytochrome c release independent of Bax/Bak and presumably attributable to OMM rupture. Ca\textsuperscript{2+} was not
examined in this study. But, an earlier report showed that Nix/BINip3L null cells have reduced levels of ER Ca\(^{2+}\) and cell death, and Ca\(^{2+}\) repletion partially restores killing.\(^{154}\) Thus, apoptotic or necrotic cell death is induced depending on whether Nix/BINip3L is mitochondrially or ER-localized.

The ability of Nix/BINip3L to induce a mixed death phenotype in a population of cells is interesting and raises some mechanistic questions. Standard BH3-only proteins (eg, BimEL, PUMA) targeted to the ER require Bax or Bak specifically at the ER to kill.\(^{156}\) Thus, it is curious that ER-targeted Nix/BINip3L can induce cell death in the absence of Bax and Bak. In contrast, mitochondrially-targeted Nix/BINip3L still requires Bax or Bak to affect cytochrome c release. The reasons for this difference are not clear, but may be related to fundamental differences between BH3-only and BH3-only-like proteins. These studies highlight that various proapoptotic Bcl-2 proteins can activate central events in apoptosis signaling at the mitochondria as well as critical ER BH3-only-like proteins. These studies highlight that various proapoptotic Bcl-2 proteins can activate central events in apoptosis signaling at the mitochondria as well as critical ER BH3-only-like proteins. However, the specificities of these BH3-only proteins are not understood. Do signals suppress one death process to allow the other to proceed? Or do both processes proceed with the effects of one dominant over the other? Additional work will be needed to sort this out.

### Necrosis in Heart Disease

Numerous studies have demonstrated that cell death is an important component in the pathogenesis of myocardial infarction and heart failure.\(^{20}\) Although a variety of cell types may be involved, this discussion focuses on cardiac myocytes. The magnitude and kinetics of cell death during myocardial infarction and heart failure differ greatly. Myocardial infarction is characterized by a large burst of cardiac myocyte death that takes place in the 24 hours following the onset of ischemia.\(^{157}\) In contrast, failing hearts exhibit ongoing cardiac myocyte death over months to years at levels that are low but still 100-fold higher than those seen in nonfailing hearts.\(^{158}\) The recognition that not only apoptosis, but also necrosis, may be actively mediated has renewed interest in the role of regulated cell death in heart disease. Specifically, which cell death processes operate during myocardial infarction and heart failure and, based on this, which antideath strategies may be therapeutically useful (Table 2).

#### Recognizing Cell Death Programs In Vivo

Apoptosis and necrosis can be identified in cell culture systems using established morphological and biochemical markers (Table 1). For apoptosis, these include chromatin condensation, cell shrinkage and fragmentation, cytochrome c release, caspase activation, cleavage of caspase substrates, cell surface exposure of phosphatidylserine ( Annexin V binding), and DNA cleavage (eg, TUNEL). Necrosis indicators include cell and organelle swelling, MPTP opening, loss of Δψ\(_{\text{m}}\), decreased cellular ATP levels, loss of membrane integrity, and release of endogenous proteins such as creatinine kinase, lactate dehydrogenase, troponin, HMGB1, and cyclophilin A.\(^{159}\) Even in cultured cells, however, the separation between apoptosis and necrosis is not as clean as might be expected. For example, cytochrome c release can result from both apoptotic OMM permeabilization and necrotic OMM rupture, such that the resulting caspase activation, cleavage of caspase substrates, and TUNEL may not be specific for either form of cell death. Conversely, loss of Δψ\(_{\text{m}}\), a primary feature of necrosis, can occur late in apoptosis. Timing can also be an important factor. When the disposal of corpses is slow (or nonexistent as in cell culture), cells in late phases of apoptosis can lose membrane integrity and transition toward a necrotic phenotype. For this reason, it is important to analyze cell death early in the process and, optimally, at more than a single time point.

In addition to these generic ambiguities, the in vivo setting presents additional challenges in differentiating between apoptosis and necrosis. First, the sensitivities and optimal time windows of markers vary, confounding direct comparison of apoptosis versus necrosis. Second and most important, there is currently a paucity of necrosis markers for in vivo studies. Although general markers (eg, the release of “cardiac enzymes” into the circulation and histological evidence of myocardial inflammation) exist, other more specific indicators (eg, release of HMGB1) have been problematic in the in vivo setting. There has been some success, however, in documenting myocardial necrosis by the in vivo administration of antimirosin antibodies or Evans blue (becomes bound to albumin in vivo) to assess plasma membrane integrity. Because of these issues, electron microscopy is sometimes used. This allows apoptosis and necrosis to be assessed using a single technique, although sensitivities of detection of apoptotic and necrotic cells differ.

#### Myocardial Infarction

Only limited information exists concerning the frequencies of cardiac myocyte necrosis and apoptosis in myocardial infarction. Necrosis has traditionally been considered the means by which cardiac myocytes die during myocardial infarction. These observations predate the recognition of apoptosis as an entity and necrosis as a regulated process. The occurrence of necrosis during myocardial infarction has been delineated only in permanent occlusion models. An electron microscopy study in 1959 showed that necrosis takes place within one hour of the onset of ischemia.\(^{160}\) In a 1996 study,\(^{157}\) the frequencies of apoptosis and necrosis were evaluated using TUNEL to assess DNA fragmentation and a myosin antibody administered in vivo to assess plasma membrane integrity. Apoptosis was detectable following 2 hours of ischemia (the earliest time point examined), became maximal at 4.5 hours (≈6 000 000 myocytes), and fell off sometime between 6 and 24 hours. In contrast, necrosis was not present at significant levels until 6 hours (≈1 000 000 myocytes) and persisted at these levels until 24 hours. This study concluded that apoptosis is the predominant form of cell death during myocardial infarction, but little was known about the specificities of apoptosis and necrosis markers at that time. Accordingly, it is possible that TUNEL overestimated the frequency of apoptosis at the expense of necrosis as discussed above. In conclusion, the relative frequencies of apoptosis and necrosis in myocardial infarction remain unclear.
Which necrosis pathways are involved in myocardial infarction? Although only primitive knowledge exists at present, the answer appears to be that both the death receptor and mitochondrial pathways mediate cardiac myocyte necrosis during ischemia/reperfusion. A role for the death receptor necrosis pathway is not surprising in light of the involvement of the death receptor apoptosis pathway in myocardial ischemia/reperfusion. For example, infarct size is reduced markedly in mice with Fas loss of function mutations. Moreover, although the effect of these mutations has been attributed to decreases in apoptosis, it is not clear, from today’s perspective, whether changes in necrosis, apoptosis, or both are responsible.

Genetic manipulation of the TNF signaling axis has provided additional insights into the role of the death receptor necrosis pathway in myocardial infarction. Simultaneous deletion of both TNFR1 and TNFR2 exacerbates infarct size following permanent coronary occlusion. Conversely, overexpression of TNFα (at low levels) or TRAF2 on a wild type background ameliorates myocardial damage. In the case of TRAF2, this is associated with NF-κB activation. These observations suggest that the survival arm of the death receptor pathway can limit myocardial infarction during ischemia/reperfusion.

The role of the mitochondrial pathway in cardiac myocyte apoptosis is well established. Overexpression of Bcl-2 and loss of function mutations in Bax, Bak, and procaspase-9 reduce infarct size in response to ischemia/reperfusion. The mitochondrial necrosis pathway also plays a central role in cardiac myocyte death during ischemia/reperfusion.

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Some, but not all, studies are discussed in text. CK, creatine kinase; I/R, ischemia/reperfusion; ppi-, peptidylprolyl isomerase f; LAD, left anterior descending artery; LDH, lactate dehydrogenase; LVDP, left ventricular developed pressure; PCI, percutaneous coronary intervention; STEMI, ST-segment elevation myocardial infarction; TAC, transaortic constriction.
myocardial infarction. Deletion of cyclophilin D markedly decreases infarct size during ischemia/reperfusion in vivo, supporting the idea that MPTP opening is involved in pathogenesis. Similarly, cyclosporine A and sanglifehrin A, which bind and inhibit cyclophilin D (discussed above), protect isolated cardiac myocytes from reoxygenation injury, and decrease infarction in isolated, perfused hearts and the myocardium in vivo during ischemia/reperfusion.

These exciting findings have been translated into a pilot study in humans. Patients with ST-segment elevation acute myocardial infarction received either cyclosporine A (n = 30) or saline (n = 28) before undergoing percutaneous coronary intervention (angioplasty and stenting). Serum creatine kinase (days 1 to 3; \( P < 0.04 \)) and infarct size (day 5, measured by MRI in 27 patients; \( P < 0.04 \)) were decreased in cyclosporine A-treated patients. It is curious, however, that troponin I, a sensitive and specific marker for myocardial necrosis, was not significantly affected. Follow-up MRI 6 months later showed cyclosporine A-treated patients exhibited persistent reduction in infarct size \( (P < 0.04) \) and a nonsignificant trend toward improved left ventricular function. Cardiac mass was similar in treated and control groups, of potential significance in light of the possibility that cyclosporine A might blunt hypertrophy through inhibition of calcineurin. Although this study is too small to provide definitive conclusions, the data suggest that cyclosporine A may reduce infarct size in humans. These results need to be confirmed in a larger number of patients.

**Heart Failure**

In comparison with the massive, short-lived burst of cell death during myocardial infarction, the absolute magnitude of cell death (TUNEL) in failing human hearts is quite low (0.08 to 0.25% of cardiac myocytes), but is of orders of magnitude higher than in control hearts (0.001 to 0.01%). Moreover, cardiac myocytes continue to die over the course of advanced heart failure, suggesting that low levels of cell death could lead to significant cumulative cardiac myocyte loss. Multiple studies have demonstrated that cardiac myocyte apoptosis is a critical component in the pathogenesis of heart failure. Transgenic experiments demonstrate that very low levels of cardiac myocyte apoptosis (0.023%) are sufficient to cause a lethal cardiomyopathy. Conversely, genetic inhibition of cardiac myocyte apoptosis ameliorates heart failure induced by a variety of stimuli.

The frequency of necrosis in heart failure models has not been studied intensively. Because abnormalities in Ca\(^{2+}\) handling are a component of heart failure and also a trigger of MPTP opening, transgenic mice were created with inducible, cardiac-specific overexpression of the \( \beta 2a \) subunit of the L-type Ca\(^{2+}\) channel. These mice exhibit Ca\(^{2+}\) overload, spontaneous myocardial necrosis, and heart failure. Interestingly, this phenotype is rescued by the deletion of cyclophilin D (but not Bcl-2 overexpression), implicating necrosis (but not apoptosis) as a causal component in pathogenesis. This conclusion was also tested in a more clinically relevant model of heart failure, doxorubicin-induced cardiomyopathy. The absence of cyclophilin D confers significant protection against heart failure in this model. Taken together, these data raise the possibility of a role for cardiac myocyte necrosis in heart failure.

To test this concept further, mice lacking cyclophilin D have been subjected to hemodynamic overload induced by transverse aortic constriction. Unexpectedly, the absence of cyclophilin D was associated with more – not less – heart failure compared with wild type animals. Even more striking, mice lacking cyclophilin D transitioned into heart failure in response to swimming, a classic stimulus for physiological hypertrophy. These phenotypes resulted from the absence of cyclophilin D in cardiac myocytes as the generalized knockouts were rescued by cardiac myocyte-specific transgenic replacement of cyclophilin D. Notably, \( p p i f^{-/-} \) and wild type mice do not exhibit baseline differences in cardiac function. Rather, differences in heart failure susceptibility are evident only under stress. Because the increased propensity of cyclophilin D–deficient mice for stress-induced heart failure appears to run counter to the benefit that its absence confers on ischemia/reperfusion, other actions of cyclophilin D were sought. NMR spectrometry under basal conditions demonstrated a shift from fatty acid oxidation to glycolysis in cyclophilin D–deficient hearts. In addition, microarray analysis demonstrated increases in pyruvate dehydrogenase and \( \alpha \)-ketoglutarate dehydrogenase transcripts. Although corresponding protein levels were unchanged, the activity of each of these enzymes was increased. Pyruvate dehydrogenase and \( \alpha \)-ketoglutarate dehydrogenase are mitochondrial matrix proteins that are activated by Ca\(^{2+}\). Mitochondria lacking cyclophilin D or treated with cyclosporine A exhibit increased levels of matrix Ca\(^{2+}\) with decreased Ca\(^{2+}\) efflux. These data suggest a novel physiological function for cyclophilin D in promoting Ca\(^{2+}\) efflux, possibly through MPTP. The relationship between decreased Ca\(^{2+}\) efflux and the stress-induced decompensation exhibited by cyclophilin D–deficient mice as well as the explanation for why cyclophilin D deletion is protective in some heart failure models but not others will require further investigation.

**Potential Therapeutic Opportunities**

Programmed necrosis of cardiac myocytes is critical in the pathogenesis of myocardial infarction resulting from ischemia/reperfusion and may also play a role in heart failure. Accordingly, there is a strong rationale for seeking pharmacological approaches to inhibit necrosis in heart disease. We consider the merits of various strategies to inhibit necrosis and discuss some currently available drugs.

A key issue for any anti–cell death strategy is whether to target proximal versus distal pathways. An advantage of distal inhibition is that it may avoid the redundancy resulting from multiple proximal inputs. This advantage comes at a price, however, in that distal blockade may not rescue key organelles. For example, caspase inhibitors often fail to preserve mitochondrial function in apoptotic cells. In the case of necrosis, the organelles that need protection are the mitochondria, which sustain considerably more damage than in apoptosis, and the various cell membranes (plasma, ER, and lysosomal). Thus, we suspect that inhibition of proximal pathways will be needed to preserve mitochondrial and

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**Kung et al** Programmed Necrosis 1031
membrane function, both of which are critical for cell viability.

Several types of small molecules inhibit proximal events: (1) nec-1,\textsuperscript{17} which antagonizes the kinase activity of RIP1, blocks the death receptor necrosis pathway; (2) PARP inhibitors\textsuperscript{17}; and (3) cyclosporine A\textsuperscript{169,170} (and related compounds), which binds cyclophilin D and inhibits its interaction or function, thereby decreasing the likelihood of MPTP opening. Nec-1 appears to reduce infarct size markedly,\textsuperscript{143} although these data come from a single study with a small number of animals (Table 2). In contrast, substantial data support the notion that cyclosporine A can reduce infarct size in various animal models and perhaps humans. The reported overlap between the effects of nec-1 and cyclophilin D absence on infarct size reduction suggest that there would be little benefit to combining nec-1 with cyclosporine A. However, the effects of combinations of these drugs have been studied in relatively few animals and at only a single time point. There are likely to be some actions of nec-1 that do not overlap with those of cyclosporine A. For these reasons, it will be important to re-evaluate necrosis inhibition by nec-1 and cyclosporine A, alone and in combination.

Even if proximal inhibition proves most critical, the addition of distal inhibitors may be advantageous. Examples of compounds that may work through distal inhibition include calpain antagonists\textsuperscript{178} and polymers that seal plasma membranes.\textsuperscript{179}

Concluding Remarks

The recognition that a substantial proportion of necrotic death is regulated impacts on multiple areas of science and medicine. From a fundamental perspective, it raises questions about physiological roles of necrosis, molecular connections between necrosis and other death processes, and evolutionary relationships among various forms of cell death. Necrosis is also tremendously important in the pathogenesis of multiple diseases. From the perspective of heart disease, cardiac myocyte necrosis plays a critical role in myocardial infarction and may also be important in heart failure. The fact that cardiac myocyte necrosis is regulated opens up the possibility of novel pharmacological approaches to inhibiting this form of cell death and limiting cardiac damage and dysfunction.

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Disclosures

None.

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Dysferlin-mediated membrane repair protects the heart in deficient muscular dystrophy.


Programmed Necrosis, Not Apoptosis, in the Heart
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