Review

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Programmed cell death with a necrotic-like phenotype

Abstract: Programmed cell death is the process by which an individual cell in a multicellular organism commits cellular 'suicide' to provide a long-term benefit to the organism. Thus, programmed cell death is important for physiological processes such as development, cellular homeostasis, and immunity. Importantly, in this process, the cell is not eliminated in response to random events but in response to an intricate and genetically defined set of internal cellular molecular events or 'program'. Although the apoptotic process is generally very well understood, programmed cell death that occurs with a necrotic-like phenotype has been much less studied, and it is only within the past few years that the necrotic program has begun to be elucidated. Originally, programmed necrosis was somewhat dismissed as a nonphysiological phenomenon that occurs *in vitro*. Recent *in vivo* studies, however, suggest that regulated necrosis is an authentic classification of cell death that is important in mammalian development and other physiological processes, and programmed necrosis is now considered a significant therapeutic target in major pathological processes as well. Although the RIP1-RIP3-dependent necrosome complex is recognized as being essential for the execution of many instances of programmed necrosis, other downstream and related necrotic molecules and pathways are now being characterized. One of the current challenges is understanding how and under what conditions these pathways are linked together.

Keywords: caspase-independent cell death; necroptosis; programmed cell death; programmed necrosis.

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Introduction

Programmed cell death is a cellular death process in which an individual cell (or cells) within a multicellular organism is eliminated in response to an inherent and genetically defined set of internal molecular events. The elimination of individual cells under physiological circumstances by this process is necessary to the overall well-being of the organism. Meanwhile, nonprogrammed cell death, or what could be termed 'classical necrosis', is distinguished from programmed cell death by its nonphysiological or pathological cause coupled with its lack of requirement for specific internal cellular machinery. Programmed cell death, therefore, plays extensive roles in many physiological processes in a developing organism, allowing tissues and organs to be shaped and maintained. In an adult organism, programmed cell death continues to play roles in tissue remodeling and organ and tissue homeostasis; it also contributes to the protection of the health of the organism through immunity, tumor suppression, etc. Programmed cell death also plays a significant role in pathological situations that invoke cellular stresses, such as ischemia-reperfusion injury, oxidative stress, pathogen infection, or DNA-damaging agents.

Although programmed cell death by definition requires that cell death occur as a result of an internal cellular 'program', or a genetically encoded sequence of events, the 'program' – the internal switch for initiating the events as well as the set of events themselves – may vary tremendously depending on the type of cell and stimulus. Programmed cell death has traditionally been divided into three categories based on the type of 'program', with apoptosis designated as type I, autophagic cell death as type II, and programmed or regulated necrosis classified as type III (1, 2). Although the result of programmed cell death is the death of the cell regardless of the way in which the cell commits cellular suicide, the classifications of programmed cell death are important for at least two reasons. First, the identification of the cell death program being executed is important if one seeks to intervene therapeutically to inhibit or augment the cell death processes (i.e.,

one may develop an inhibitor of cell death only when one knows the cause of cell death and appropriate pathway to inhibit). Second, there are important downstream consequences that occur as the direct result of the way in which a cell dies – biochemical consequences affect the remaining nondying cells of an organism. For instance, apoptosis is thought to occur primarily without triggering inflammation, which further sets it apart from necrosis, which is highly proinflammatory (3, 4).

The three traditional classifications of programmed cell death were originally based mainly on morphological criteria (1, 2). For instance, apoptosis was characterized by Kerr et al. (5) as involving cellular shrinkage, nuclear fragmentation with condensed chromatin, and membrane blebbing with the pinching off of membrane-bounded bodies containing organelles and other cellular contents, whereas autophagic cell death was originally classified based on the appearance of the double-membrane autophagosome structure during the death process. Programmed necrosis was originally classified as programmed death not having the characteristics of the first two classifications and morphologically very similar to the nonprogrammed classical necrosis, characterized by cellular swelling, plasma membrane rupture, and the swelling and dysfunction of organelles, such as the mitochondria (3, 4), even though in the case of programmed or regulated necrosis, specific molecular machinery is required for these events to occur. Therefore, although morphological distinctions continue to be important for classification purposes (1), more modern classifications are based largely on biochemical events, which are more specific and allow one to distinguish mechanisms even when morphological events are similar (6). Moreover, it is important to realize that although we make such classifications, an individual cell may die with the characteristics of one or more categories, and there also perhaps exist modes of cell death that do not fit well into one of these categories (6). Nevertheless, it is useful to classify death into three broad traditional categories to compare the similarities and differences in ways in which cells die, with the implicit understanding that these classifications are somewhat oversimplified and that the underlying specific mechanisms of cell death are probably more important.

Apoptosis

Apoptosis is largely and primarily executed by the activation of cysteine proteases of the caspase family. Apoptotic caspases are designated as one of two kinds: first, those caspases that are the upstream initiators of the apoptotic signal; second, those effector caspases that act downstream and that execute most of the apoptotic program by cleaving the majority of the downstream substrates (7). Initiator caspases, such as caspases 8 and 9, contain long prodomains and are activated primarily by dimerization, whereas effector caspases have short prodomains and are activated by the cleavage of the catalytic domain (7, 8). The downstream substrates of caspases are numerous (9–11) but include nuclear and cytoplasmic structural proteins (such as lamins, tubulin, and actin), DNA repair enzymes [such as poly(ADP-ribose) polymerases, or PARPs], proteins involved in regulation of the cell cycle (such as Rb, p21, and p27), and other enzymes (such as iCAD/DFF45) that inhibits the caspase-activated deoxyribonuclease CAD (12).

There are two major pathways that lead to the activation of caspases and apoptosis: intrinsic and extrinsic (6). Apoptosis through the intrinsic pathway is triggered by many stressors, such as DNA damage or cytokine withdrawal, that activate a number of signaling networks that converge on the permeabilization of the outer membrane of the mitochondria (also called MOMP) (13, 14). This permeabilization leads to the release of proapoptotic factors from the mitochondria, including cytochrome C, SMAC/Diablo, endonuclease G, and HTRA2 (15). Once released, cytochrome C is free to bind with APAF1 and dATP to form a complex that activates caspase 9 called the apoptosome (16). Caspase 9 activation then results in the activation of the executioner caspases 3, 6, and 7 (17). Whereas the apoptosis-inducing factor (AIF) and endonuclease G mediate caspase-independent functions in large-scale DNA fragmentation, SMAC/Diablo and HTRA2 inhibit the members of the inhibitor of apoptosis (IAP) family, thus preventing their inhibition of caspases (15). Mitochondria permeabilization and the activation of the extrinsic pathway are regulated by proapoptotic and antiapoptotic members of the BCL-2 family. Conformational changes of one of two proapoptotic members, BAK or BAX, are thought to be essential for mitochondrial permeabilization and result in the homo-oligomerization of these proteins on the outer mitochondrial membrane and resulting pore-forming ability (18, 19). The proapoptotic members of the BCL-2 family are antagonized by the binding of the antiapoptotic BCL-2 family members, including BCL-2, BCL-xL, BCL-W, MCL-1, and A1 (20). It is not entirely clear as to how the other proapoptotic members of the family (the BH3-only members BID, BIM, PUMA, BMF, BAD, BIK, HRK, and NOXA) trigger apoptosis, but it is believed to be either through the direct binding of some of them to BAX or BAK to activate them and/or by neutralizing the antiapoptotic

family members and allowing BAK or BAX to oligomerize on their own (18–20).

The extrinsic pathway is activated primarily by the transmembrane death receptors of the TNF superfamily (21), such as Fas (22), TNFR1 (23), DR4, and DR5 (24). The activation of the extrinsic pathway by such death receptors relies on death domain-mediated recruitment of the death domain protein FADD and its recruitment of caspases 8 and 10 into a death-inducing complex (21, 22). This process is antagonized by the cellular inhibitor of apoptosis proteins (cIAPs), which are proteins recruited to the complex having an E3 ubiquitin ligase function that prevents caspase activation (25, 26), and by c-FLIP, which is a molecule with a high homology to caspase 8 that lacks catalytic activity (27, 28). Once activated, caspase 8 may then cleave the executioner caspases, resulting in a caspase cascade. Inside some cells, the level of caspase 8 activation is not sufficient by itself to induce death, and the cleavage of the BH3-only BCL-2 family member BID by caspase 8 amplifies the signal through the activation of the intrinsic pathway (29).

Autophagic cell death

Autophagic cell death, which has also been classified as type II programmed cell death, involves the process of autophagy. Autophagy, which comes from Greek words meaning 'to self eat', is an evolutionarily conserved process by which a cell recycles its basic components ranging from individual molecules to organelles (30, 31). It does this through the process of lysosomal degradation of cellular components, thereby promoting survival during nutrient deprivation and other stresses by providing substrates for the cellular energy production machinery as well as structural and functional components for cell processes (32–34). Autophagy also protects cells from cellular injury by recycling damaged or aggregated proteins and organelles (35, 36). Targeting of the cellular components to the lysosome for this process requires a system of autophagic machinery that is carried out by a system of more than 30 autophagy-related (ATG) genes, many of which were discovered or characterized in yeast (32, 34). The process requires the formation of an autophagosome, which is a double-membrane structure that is capable of engulfing large organelles, macromolecules, and cytoplasm (34, 37), much of which are specifically targeted cellular components and which, like the proteosome system, are targeted for lysosomal degradation by ubiquitination events (38).

The term 'autophagic cell death' is quite confusing because it originally referred to cell death in the presence of autophagic features, namely, the double-membrane structure of the autophagosome (31, 39). This original definition does not provide much information about the death process because autophagy is triggered by many cellular stresses and can therefore occur when a cell is dying of apoptosis or necrosis (31, 36, 40). A more current definition of autophagic cell death is death that requires autophagy to proceed and does not therefore occur in the absence of the required ATG proteins of the autophagic machinery (31). An additional requirement of the stricter definition of autophagic cell death is that the death of the cell is actually mediated by the autophagic machinery, as opposed to being merely required for cell death (i.e., the cell requires autophagy to die but does so through an apoptotic or necrotic mechanism) (31). Under this strictest requirement, autophagic cell death is exceedingly rare and somewhat difficult to substantiate, given that many ATG proteins have both autophagic and nonautophagic functions, some of which can have effects on apoptosis and necrosis (41, 42). Perhaps one of the more convincing examples of physiological autophagic cell death is developmental cell death in *Drosophila* salivary glands where their degradation is inhibited in *atg* mutants, whereas the induction of autophagy induces premature cell death by a caspase-independent mechanism (43). Although it is unclear why the autophagic process kills cells under some special situations but not under other conditions, the autophagy process itself as a whole is generally well characterized.

Programmed necrosis

Programmed necrosis, which has also been referred to as type III programmed cell death, is perhaps the least mechanistically characterized of the three types of cell death. As mentioned earlier, the term 'necrosis' was originally used to refer to a cell death morphology that included cell and organelle swelling and dysfunction and plasma membrane rupture. Because this morphological changes occur in classical as well as programmed necrosis, the terms 'programmed necrosis', 'programmed necrotic cell death', or 'regulated necrosis' are used to distinguish the process carried out by a specific program of genetically encoded cellular apparatus from classical necrosis, which is a random but primarily injury-initiated process that occurs passively after cell damage. Thus, the main difference between the two processes other than their initiating

factors is that specific gene products are required for programmed or regulated necrosis but not for classical necrosis. Although there is much that we do not know about the mechanisms of programmed necrosis, studies within the past 3 years have substantially increased our understanding of the process, and we shall spend the remainder of the review discussing what is known of the mechanisms of programmed necrotic cell death.

Historically, the major models for studying programmed necrosis have involved the stimulation of the death receptor subtypes of the TNF receptor superfamily. Although Fas and TNFα ligands stimulate apoptosis in many cell types and under some conditions, in other situations, they kill in a nonapoptotic manner with a necrotic morphology (44–48). This death receptor-specific necrotic death was eventually designated 'necroptosis' (49) (see Figure 1). In some cell types, such as the murine fibrosarcoma cell line, L929, necrotic cell death is the default mode of cell death induced by TNFα treatment. The phenomenon of programmed necrosis was observed as early as 1972 in L929 cells treated with lymphotoxin (50), and these cells, although somewhat unusual, remain a useful model. In other cell types, such as U937 cells, apoptotic cell death is the accompanying default cell pathway in cells treated with TNFα, and caspase inhibitors such the pancaspase inhibitor zVAD are used to switch the mode of death to programmed necrosis (49). Cells that are deficient in NF-κB activity, such as p65/RelA, TRAF2, and TRAF5,

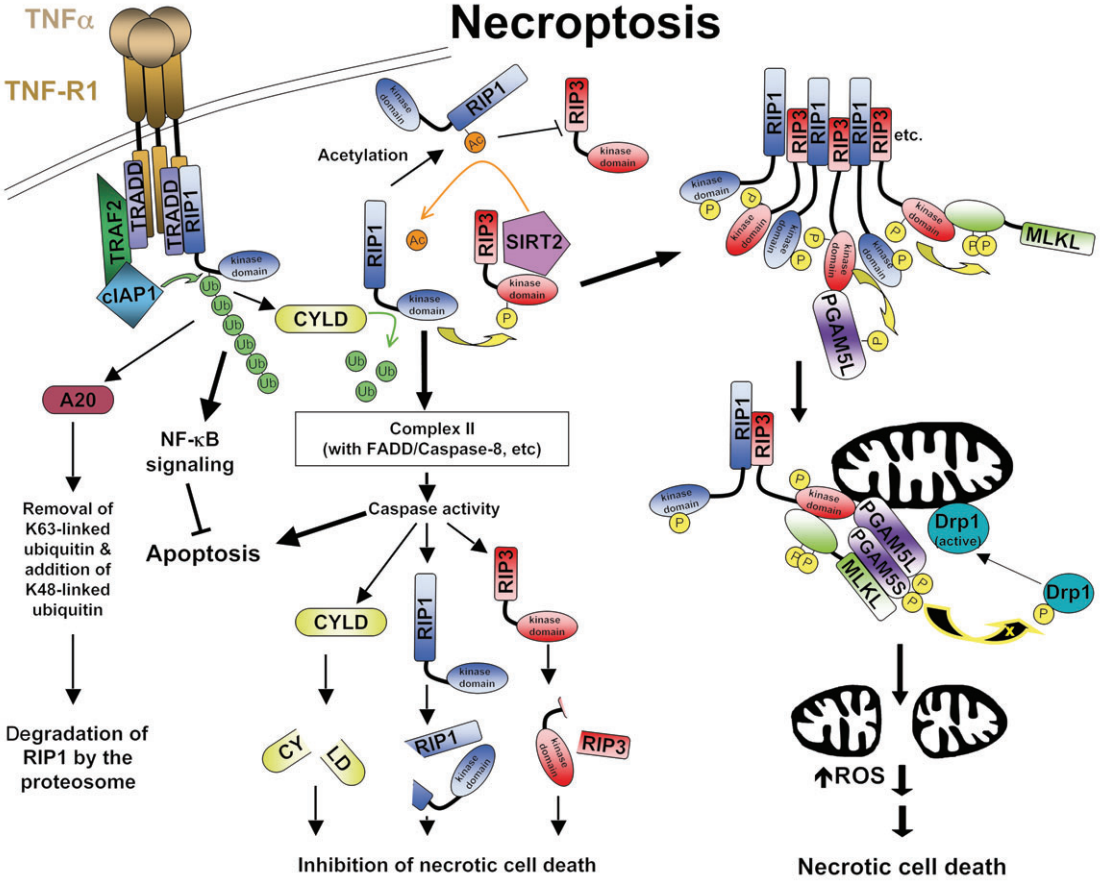

Figure 1 Necroptosis, a specific example of programmed necrosis.
Necroptosis occurs downstream of death receptors, such as TNF receptor 1 (shown). K63-ubiquitination of RIP1 prevents its interaction with the apoptotic and necrotic machinery and contributes positively to NF-κB signaling. Upon deubiquitination by CYLD, the RIP1 protein may interact with the proapoptotic machinery that is involved in caspase 8 activation (in which case, necrotic cell death is inhibited by the cleavage of various players in the necrotic pathway). Under certain conditions, such as caspase inhibition, RIP1 may interact with RIP3 and the necrosomal complex. Phosphorylation events, as well as the recruitment of MLKL and PGAM5L to the necrosomal complex, are important in the activation of downstream signaling events, including, among others, the recruitment of PGAM5S and the dephosphorylation of Drp1, which then acts to induce mitochondrial fission. The remainder of the events in the necrotic pathway are not clearly understood, but mitochondrial fission is known to contribute, and may make the mitochondrion more susceptible to undergo the permeability transition and produce ROS, which can initiate downstream damage.

are also susceptible to necrotic cell death by TNFα ligand in the presence of zVAD, whereas in other cell types, the addition of SMAC mimetics, transcriptional or protein synthesis inhibitors, in combination with caspase inhibitor, is necessary to observe programmed necrosis (51–53).

The necrotic cell death machinery

RIP1

The serine-threonine kinase RIP1 was the first genetic element identified as being essential for the cellular necrotic program machinery downstream of the Fas and TNFR1 signaling complexes (46, 52). Although it has a central role in most programmed necrotic processes, this appears to not be its primary role in death receptor-mediated functions, and RIP1 plays other important roles in many death receptor signaling pathways, including the TNFα pathway, where it is essential for the efficient activation of NF-κB and the ERK, JNK, and p38 MAP kinases (54). With the exception of ERK activation, these signaling pathways do not require its kinase activity (55) but are activated primarily by its intermediate domain, which is recruited to the death receptor complexes by its death domain. The pronecrotic activity of RIP1, however, does require its serine-threonine kinase activity, and RIP1 is autophosphorylated on serine 161 (56), and the kinase then becomes active in response to pronecrotic, but not proapoptotic, stimulation of TNFR1 (57). Under some circumstances, such as SMAC mimetic treatment or extensive DNA damage, RIP1 kinase activity is believed to contribute to autocrine TNFα production in a mechanism that may involve NF-κB and/or JNK activation, creating a feed-forward loop (58, 59). The compound necrostatin 1 was identified in a small-molecule screen as an inhibitor of programmed necrotic cell death and was later shown to be an inhibitor of the kinase activity of RIP1 (49, 56).

RIP3

A major breakthrough in the mechanism of programmed necrosis occurred when it was determined that RIP3 interacts with RIP1 under necrotic cell death conditions and is an essential downstream partner for RIP1 in programmed necrosis (53, 57, 60).

RIP3 had long been designated an RIP kinase family member based on kinase domain similarity, but RIP3 lacked the death domain usually involved in the protein recruitment to death receptors. However, RIP1 interacts with RIP3 through a specific region at the C-terminal end of its intermediate domain known as a homotypic interaction motif (RHIM) (61). The resulting interaction with the C-terminal RHIM of RIP3 may result in the formation of a filamentous structure similar to β-amyloids, and therefore, necrosis can be somewhat inhibited by amyloid dyes (62). The RIP1-RIP3 complex, with its associated proteins, is commonly referred to as the 'necrosome', and its stable formation depends on the kinase activity of RIP1; thus, the compound necrostatin 1 inhibits necrosome formation (53, 57). Although the kinase activity of RIP3 is not required for the formation of the necrosome complex, it is required for the downstream signaling events in necrotic cell death (53, 57, 60) and may possibly contribute to RIP1 phophorylation (57).

With the identification of RIP3 as an important part of the necrosome complex, the pathways downstream of RIP3 are now being identified. Among the substrates of RIP3 that have been identified is the mixed lineage kinase domain-like protein (MLKL), which will be discussed in the following paragraphs. Using liquid chromatography-tandem mass spectrometry, Zhang et al. (60) identified several metabolic enzymes as potential RIP3 interactors, including glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL), glutamate dehydrogenase 1 (GLUD1), as well as fructose-1,6-bisphosphatase 2 (FBP2), fumarate hydratase (FH), glycosyltransferase 25 domain containing 1 (GLT25D1/COLGALT1), and isocitrate dehydrogenase 1 (IDH1). PYGL, GLUL, and GLUD1 were verified in their interaction with RIP3 in overexpression systems (60). The prevalence of the association of RIP3 with metabolic enzymes may suggest that RIP3 may regulate energy production pathways associated with glycolysis and the mitochondria. The potential association of the mitochondria with proteins, including GLUD1, may underscore the important nature of the necrosomal relationship with the mitochondrion. The upregulation of metabolic pathways may possibly lead to the generation of reactive oxygen species (ROS) and impact mitochondrial function (63).

MLKL and other downstream events

Recently, two independent laboratories identified MLKL as the protein that interacts with RIP3 during programmed necrotic cell death (64, 65). This interaction is dependent on the kinase activity of RIP3 (64, 65) and also its phosphorylation at serine 227 (65). RIP3 phosphorylates MLKL at both threonine 357 and serine 358, and these phosphorylation events are required for cell death

(65). The drug necrosulfonamide was identified as an inhibitor of necrotic cell death through a mechanism that prevents MLKL binding to other proteins downstream the RIP1-RIP3 complex. (62). Interestingly, MLKL is predicted to be an inactive pseudokinase; however, this has yet to be formally tested. It may therefore act as a scaffolding protein for downstream events. Two splice variants of the PGAM5 mitochondrial phosphoglycerate mutase, which uses an alternative catalytic activity to function as a Ser/Thr phosphatase (66), are recruited to the necrosome, and the phosphorylation of the short variant requires the presence of MLKL (67). Both variants are required for efficient necrosis and are possibly phosphorylated by RIP1 or RIP3 (67). The active PGAM5 variants then recruit and activate the protein Drp1, which may involve the dephosphorylation of Drp1 by the phosphatase activity of PGAM5 (67). Drp1 is GTPase that can activate mitochondrial fission and fragmentation (68), and its inhibition can protect from necrosis (67). Thus, the main downstream target of necrotic cell death pathway may be mitochondrial fragmentation, which is supported by other studies that show that the opposite process, mitochondrial fusion, protects from necrotic cell death (69).

Regulation of necrosome components

The ubiquitination of RIP1 regulates the signaling within the TNFR1 complex and controls access of RIP1 to the necrosomal complex. The RING finger ligases TRAF2 and cIAP1/2 and the LUBAC protein complex formed by HOIL-1, HOIP, and SHARPIN cooperate to catalyze the ubiquitination of complex components, including RIP1 (70–72). The initial noncanonical K63 ubiquitination of RIP1 not only contributes to the activation of NF-κB (73, 74) but also prevents RIP1 from interacting with the apoptotic and necrotic complexes (75–77). At least two deubiquitinases are therefore thought to be important for the regulation of cell death signaling. CYLD, which is a tumor-suppressor gene, removes the K63-linked ubiquitin chains and thereby represses NF-κB activation (78). Therefore, by K63-linked ubiquitin removal (allowing RIP1 interaction with the necrosome) and by the repression of NF-κB activation (preventing the expression of NF-κB-dependent antioxidant proteins), CYLD potentially contributes in two ways to the ability of the cell to initiate necrotic cell death. A20 also removes K63-linked chains, but it also has a second domain that adds K48-linked chains, which results in the degradation of RIP1 by the proteosome (79), and therefore, A20 contributes to the suppression of both apoptotic and programmed necrotic cell death by the TNFα pathway (80–82) but may sensitize to necrotic cell death by direct oxidative stress (81).

The RIP1-RIP3 complex formation is also apparently negatively regulated by the acetylation of RIP1 on lysine 530, which is near its RHIM domain, and thus, the deacetylation of RIP1 must be carried out by the RIP3-binding deacetylase SIRT2 for necrosis to proceed (83).

As the stability of the RIP1-RIP3 complex is crucial to programmed necrotic cell death, important regulation of RIP1 and its associated proteins occurs when complexed with FADD, cFLIP, and caspase 8. Caspase inhibitors prevent apoptosis; however, these inhibitors potentiate necrotic cell death (45), due to their inhibition of caspase 8 (and other caspases)-dependent cleavage of RIP1 (84), RIP3 (85), and the CYLD deubiquitinase (86).

The regulation of RIP by the apoptotic machinery underlies the main evidence that the necrotic cell death pathway is physiologically and pathologically relevant in development. The developmental defects and lethality of some gene deletions, including FADD, caspase 8, cFLIP-FADD double knockout (but not cFLIP knockout alone), XIAP-cIAP1 double knockout, and cIAP1-cIAP2 double knockout are rescued completely or to some degree by RIP1/RIP3 deficiency (87–94). This suggests that the apoptotic machinery is necessary to prevent RIP1/RIP3-mediated necrosis during development and also suggests that the necrotic machinery can be active *in vivo*.

Other molecules and pathways involved in programmed necrosis

Although the RIP1-dependent necrosome plays a significant and important role in the majority of most programmed necrotic events, there are now several publications that have indicated that it may not be completely required in some specific situations (57, 60, 95, 96). For instance, RIP1 is not required for mutant cytomegalovirus infection-mediated necrosis (95). Instead, necrosis proceeds by RIP3 binding to the DAI protein *via* an RHIM-dependent interaction (97), and the viral M45-encoded inhibitor of RIP activation (viral) typically binds RIP3 and disrupts this interaction, preventing necrosis (95). There are other molecules that also have proven functions within programmed necrotic pathways. In many cases, these molecules act in concert with (mostly downstream of) the RIP1-RIP3-mediated pathway. The mitochondrion may play multiple roles in these pathways, providing a major connection between the molecular necrotic machinery, whereas the lysosome may also play several roles in necrotic death.

It has long been known that programmed necrosis induced by TNFα occurs together with, and requires the production of, ROS (4, 45, 51, 52, 76, 98–101). Mitochondrially derived ROS, primarily initiated by electrons from mitochondrial electron transport chain complexes, are thought to be of particular importance (96, 98, 100–103). Lysosomal ROS may also contribute to programmed necrotic events (96). The production of ROS in necrotic cell death may also sometimes be mediated by NADPH oxidases, dependent or independent of RIP1.

Among other molecules that are involved in programmed necrotic pathways are cyclophilin D (104–107) and lysosomal proteases, such as cathepsins (108, 109). Additionally, PARPs (110, 111), AIF (112), and perhaps nonlysosomal proteases, such as calpains (113), constitute a second necrotic pathway that has been designated with the term 'parthanatos' (Figure 2) (114). The parthanatic pathway often has strong interdependent interactions with the RIP1-RIP3 pathway but is also thought to execute independently under some conditions. The activation of the stress-activated map kinase JNK, which often occurs in response to ROS intermediates, and upstream or downstream of the mitochondria may also have important roles in necrotic signaling (115).

The mitochondrial permeability transition pore channel and cyclophilin D

Spanning both the inner and outer mitochondrial membranes, the mitochondrial permeability transition pore channel (MPTP) is a channel made up of the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT), cyclophilin D (CypD), and perhaps several other proteins, including the mitochondrial phosphate carrier (PIC/SLC25A3) (116, 117). CypD, which is a prolyl isomerase, regulates the opening of the channel upon oxidative stress or calcium, resulting in an influx of ions and a loss in mitochondrial membrane potential, preventing oxidative phosphorylation and ATP production. Mitochondrial membrane rupture may also occur as a result of water influx. MPTP opening and loss of membrane potential can trigger ROS release, which can then contribute to necrosis. An overexpression of CypD causes sensitivity to ROS, whereas CypD knockout cells are substantially resistant to necrosis triggered by oxidative stress (104–107) CypD knockout mice are also resistant to ischemia-induced necrosis *in vivo* (104, 106, 107). Recent data suggest that MPTP opening can occur due to the accumulation of p53 within the mitochondrial matrix and its physical interactions with CypD (118), connecting oxidative damage as sensed by p53 with a cellular response.

Lysosomal involvement

It is not surprising that lysosomal proteases are sometimes activated during programmed necrosis and can contribute to the execution of cell death. Because organelles are significantly damaged during necrotic cell death, either in response to direct oxidative damage of membrane lipids or as a downstream consequence of other events resulting in swelling and rupture, lysosomal permeabilization or rupture also occur, thus releasing proteolytic enzymes, and a decrease in intracellular pH by lysosomal rupture could also contribute to direct toxicity to the cell (119). Although many lysosomal enzymes are active only at the low lysosomal pH, several lysosomal enzymes, including the cathepsin proteases, remain significantly active at cytosolic pH and thereby contribute to necrotic cell death execution (120). Lysosomal function is required for necrotic cell death in *Caenorhabditis elegans* (121), and cathepsin release from the lysosome is important for the programmed necrotic process (109). Lysosomal proteases may also be involved in programmed necrotic processes mammalian cells under certain circumstances (108, 122).

In addition to contributing proteases, the lysosome is also a potential source of ROS because it often has high iron content from the recycled proteins (123). The lack of H_2O_2-detoxifying enzymes makes it more likely that iron will convert H_2O_2 by the Fenton reaction into the highly reactive hydroxyl radical (63, 124). The iron chelator desferrioxamine inhibits the lysosomal permeability that precedes cell death in programmed necrosis initiated by exogenous ROS, indicating that the lysosome seems to be particularly important for necrosis in this context (96).

The parthanatic pathway: PARP and AIF

PARPs are a family of enzymes that transfer the ADP-ribose moiety of NAD^+ to an amino acid, thus placing poly(ADP-ribose) (PAR) polymers on various proteins (125). Initially characterized as enzymes involved in DNA damage detection and repair, PARPs, especially PARP1 and PARP2, are now known to play significant structural, regulatory, and organizational roles within nuclear chromatin (125, 126). Additionally, PARP1 may be involved in metabolic regulation (126). PARP1 is activated in response to DNA damage and can have multiple effects on necrotic cell death: first, its prolonged activity causes NAD^+ depletion, which is

replenished by ATP consumption, thereby depleting cellular ATP (127–129). Second, because NAD^+ is an important cofactor for glycolysis (130), there is further reduction in ATP production because glycolysis is slowed (131). Third, the depletion of NAD^+ promotes MPTP opening, leading to mitochondrial membrane depolarization (131, 132). This may be due to the reduction in the activity of SIRT3, an NAD^+-dependent enzyme that maintains CypD in a deacetylated and less active state (133, 134). Lastly, PARP facilitates the release of AIF from the mitochondria (135). Upon the translocation of AIF from the mitochondria to the nucleus, AIF causes cell death distinguished by chromatin condensation and high-molecular-weight DNA fragmentation (136). The PAR polymer produced by PARP is itself a cytotoxic signal to the cell (137), and the mitochondrial release of AIF requires AIF's high-affinity PAR-binding site binding directly to the PAR polymer (138). In addition to DNA damage-mediated necrotic death, PARP1 has also been reported to contribute to necrotic cell death initiated by TNFα (110) and TRAIL (139). PARP1 contributes to almost all of the PARP activity in a cell; however, PARP2 has also been shown to contribute to TNF-induced necrotic death (140).

The alkylating agent *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) requires PARP1 to induce necrotic cell death (111). Some reports have indicated that JNK activation downstream of RIP1 and TRAF2 is also required, and therefore, RIP1 may be downstream of PARP1 in some cases (111). The release of AIF from the mitochondria initiated by PARP1 has been proposed to depend on calpain and Bax (113), although calpain is apparently not required under all circumstances (141). BCL-2 may therefore regulate PARP1-dependent necrosis by preventing Bax activation or by the direct binding to PARP1 (142). Under conditions of apoptosis, PARPs are cleaved by caspases, thus inhibiting the necrotic pathway. In contrast, the inhibition of PARP promotes apoptosis over necrosis (143, 144).

The importance of PARP1 in pathological necrotic cell death *in vivo* has been shown in knockout mouse models of PARP1, where tissues from these mice are resistant to various types of ischemic injury (145–147), and in conditions where there is an acute systemic inflammatory response, such as hemorrhagic or septic shock (148–150) – situations where necrotic death mechanisms play a significant role in pathological damage.

ROS and JNK

As mentioned before, many of the early observations of programmed necrotic cell death induced by TNFα included the generation, and also the requirement for the production, of ROS (4, 45, 51, 52, 98–101). ROS are known to induce both apoptosis and necrosis, and the cell death mechanism can be dependent upon the level of ROS exposure (151–153). Prolonged activation of the stress-activated map kinase JNK is also an important factor in cell death in both apoptotic and necrotic settings (51, 99, 154–157). Because ROS and JNK activation are often coincident and are also causatively related, we will discuss them briefly together here because their relationship in programmed necrotic death has been discussed in more detail in a recent review (158).

An important aspect of ROS involvement in necrosis is that ROS can contribute as signaling messengers and can also inflict direct cellular damage; in some cases, damage produces an amplification of ROS. In many cases, ROS generation lies downstream of JNK and the mitochondria, whereas in other cases, ROS contributes to JNK activation – perhaps through the activation of the upstream MAP3K ASK1 (158). JNK and ROS signaling generally contribute to necrotic cell death through signaling pathways that impinge on the mitochondria. The mitochondrion, which is the main source of cellular energy, can also be a main source of cellular ROS during necrosis. ROS generated by mitochondrial electron transport chain complexes, especially complex I, is important during programmed necrotic death. JNK, but not p38, has been shown to activate the mitochondrial production of superoxide on complex I-dependent manner (159). Rotenone, an inhibitor of complex I, has been shown to inhibit TNF necroptosis (96, 103, 160). ROS can directly result in oxidative damage to mitochondrial proteins, which can then amplify ROS through a variety of mechanisms (161), including oxidative reactions with iron-sulfur proteins (162).

NADPH oxidases from the Nox family are another potential source of ROS during programmed necrosis (163, 164), and TNFα can directly activate NADPH oxidases in various cell types (165), which can be required for cell death in some cases (166, 167). The potential mechanisms of direct NOX1 activation by death receptors have been proposed to be mediated by RIP1/TRADD-dependent interactions with NOXO1 and Rac1 in the TNF receptor signaling complex (167) or by a receptor/TRADD-mediated interaction with riboflavin kinase (RFK), which is important for p22phox and NOX1 recruitment as well as FAD loading of NOX1 (168, 169). The superoxide produced by NADPH oxidases may potentially lead to downstream mitochondrially generated ROS (158), and under some conditions, NOX1 activation has also proposed to be downstream of mitochondrial

or other ROS (170), such as during serum withdrawal-induced necrotic cell death (171). The overexpression of NOX1 with its regulatory proteins, NOXO and NOXA1, can trigger TNFR1-dependent activation of JNK and cell death (172), indicating that ROS can upregulate receptor-mediated activation and potentially further receptor-activated NADPH oxidases.

JNK kinase activity is thought to contribute its pronecrotic role through actions involving the mitochondria. JNK has been shown to translocate to the mitochondria under some circumstances and regulate MPTP opening (173). JNK may regulate this opening directly or it may do so indirectly through regulation of BCL-2 family members. Many BCL-2 family members are targets of JNK phosphorylation, including BCL-2 itself (174). For instance, JNK phosphorylates BMF (175) and BAX (176), which are required for TNFα-induced necrosis (140) and MNNG-mediated AIF release (113), respectively. JNK also phosphorylates 14-3-3 proteins that anchor BAX in the cytoplasm, causing BAX release (177). Although BAX and BAK are thought of as apoptotic proteins, deletion of BAX and BAK reduces necrotic injury during myocardial infarction in mice similar to cyclophilin D deletion, whereas the triple knockout mice that lack BAX/BAK/CypD are not further rescued (69). ROS- and JNK-dependent signaling events can result in loss of mitochondrial membrane potential *via* the opening of the MPTP, leading to a loss in ATP production and other significant events including an influx of solute and mitochondrial rupture.

Although JNK and ROS are involved in a majority of programmed necrotic cases, they may not be required under every circumstance. ROS scavengers do not seem to prevent cell death in FADD-deficient Jurkat T cells (49). Neither does JNK appear to be required for necrotic cell death in Jurkat cells (178) or in the HT-29 cell model. However, inhibition of JNK protects from ischemic brain injury in rats and mice (179, 180), whereas JNK-1$^{-/-}$ or JNK-2$^{-/-}$ mice are protected from cardiac ischemia-reperfusion injury (181). Similar studies have shown protection from antioxidants on ischemia-reperfusion injury, indicating that ROS has a significant role in necrotic damage.

Potential implications in the treatment of disease

Until several years ago, due to the lack of strong biochemical markers for programmed necrotic death coupled with the postnatal lethality of RIP1 knockout mice (182), scientists had very limited tools to address the physiological and pathological relevance of programmed necrosis. However, the development of pharmacological inhibitors combined with experiments in RIPK3-deficient animal models has resulted in the generation of a large amount of recent data that have implicated programmed necrotic cell death in a variety of pathological conditions. For instance, necrostatin 1 was originally identified as a compound that inhibits ischemic brain injury (49) and has since been found to not only limit damage in various types of ischemia-reperfusion brain injury (49, 183, 184) but also protects against traumatic brain injury (185), myocardial infarction (186–188), Huntington disease (189), TNF-induced systemic inflammatory response syndrome, and ischemic kidney injury (190). Because necrostatin 1 is not completely specific for RIP1 (59, 191–193) and because the kinase activity of RIP1 is not only important for necrotic cell death but also for RIP1-dependent ERK activation (55, 76), these data do not necessarily prove that programmed necrosis is involved in each case. However, these data are consistent with the notion that it is involved in various pathological conditions. Undoubtedly, protective effects will have to be verified in RIP3-deficient mice, which have been shown to be resistant to pathological states in mouse models of TNFα-mediated shock (194), retinal detachment (195), retinal degeneration (196), and ethanol-induced liver injury (197) as well as the development of advanced atherosclerosis (198). RIP3-deficient cells have allowed researchers to also implicate that programmed necrotic cell death is important in the elimination of cells infected with virus and bacteria (57, 95, 97, 199) and some pathogens have developed machinery to inhibit it (95, 97).

Clearly, there is much to be done in determining the biological situations where programmed necrosis has a role. However, when one considers that just a few years ago, programmed necrosis was not well accepted as a genuine physiological or pathological phenomenon, much progress has clearly been made in showing that this process is clearly important and a worthy therapeutic target for drug development. Undoubtedly, further progress will be made in the next few years and the use of inhibitors of programmed necrotic cell death pathways will have a large potential impact in the treatment of disease.

Outlook

Although the RIP1-RIP3-dependent necrosome complex is recognized as being essential for the execution of many instances of programmed necrosis, other downstream and

related necrotic molecules and pathways are now being characterized, and with recent evidence that these pathways are physiologically and pathologically relevant, the future of the necrotic cell death field looks bright. One of the current challenges is understanding how and under what conditions the various pathways are linked together. There are many questions remaining. For instance, is there a 'core downstream component' of the necrotic machinery that is shared by all necrotic signaling pathways or are we actually dealing with several distinct pathways with similar outcomes? If this is the case, how central or connected is the RIP1/RIP3 necrosome with the central necrotic machinery? Why are ROS and JNK necessary for some necrotic death events, but not in others? What are the central connections between the mitochondria and ROS, as well as other ROS-generating mechanisms? Further and a more complete characterization of the necrotic machinery may provide additional means of therapeutic intervention in disease states where necrotic cell death substantially contributes to the pathology of the disease.

Highlights

- Programmed cell death is an 'active', genetically encoded, and executed form of altruistic cellular 'suicide'.
- Programmed cell death is usually classified into three categories, with apoptosis designated as type I and autophagic cell death as type II. Programmed or regulated necrosis, which is less characterized than the others, is classified as type III.

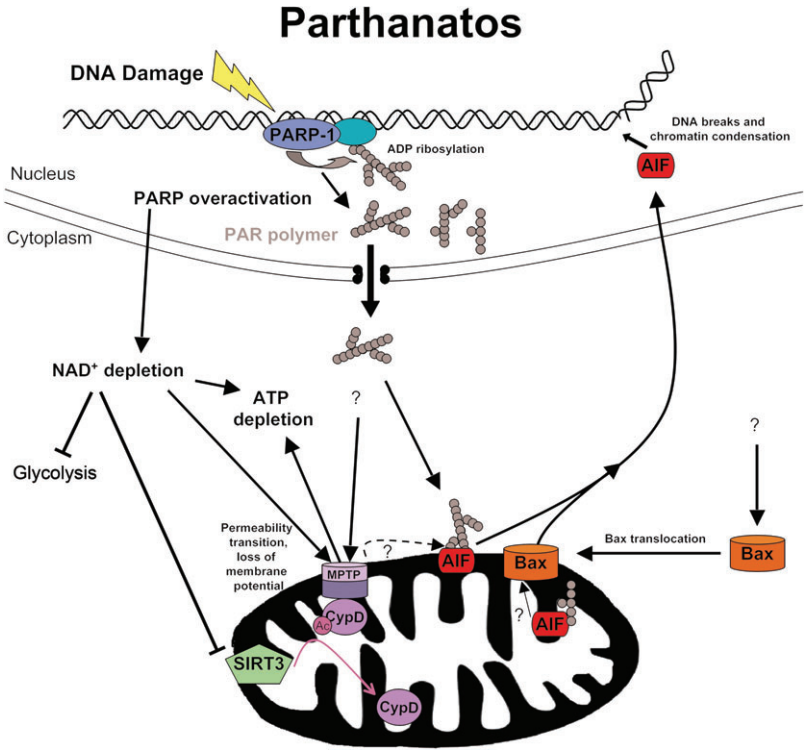

Figure 2 Parthanatos, a specific example of programmed necrosis.
Parthanatos is a PARP-dependent process. Upon DNA damage, PARP is activated. When an overactivation of this enzyme occurs, several events are triggered. First, NAD^+ becomes heavily used because this is a substrate of PARP. Replenishment of NAD^+ requires ATP, and thus, cellular ATP stores are taxed. NAD^+ depletion also inhibits glycolysis and prevents the formation of pyruvate and other downstream substrates for mitochondrial respiration pathways. While the mitochondrion maintains its own internal pools of NAD^+, eventually, the high ratio of NADH/NAD^+ is eventually sensed by the mitochondrion and NAD^+ depletion is therefore a trigger for MPTP and loss of membrane potential, which further results in loss of ATP production. The triggering of this pore may be partially the result of a loss of SIRT3 activity, which also requires NAD^+ as a substrate, and which deacetylates CypD, typically preventing CypD-mediated opening of the pore. Lastly, and importantly, PARP overactivation results in the formation a large amount of the PAR, which is toxic to the cell through the action at the mitochondrion. The release of AIF from the mitochondria requires AIF binding PAR. The release of AIF is also thought to be influenced by the permeability transition pore/loss of membrane potential and the Bax protein, although the parthanatic triggers for these events and their role in AIF release are not well characterized. The release of AIF from the mitochondria allows it to translocate to the nucleus, where it can cause DNA breaks and chromatin condensation.

- Apoptosis is classified into intrinsic (which involves mitochondrial permeabilization) and extrinsic (which involves death receptors) pathways. The primary executioners of apoptosis are the caspase family of cysteine proteases.
- Autophagic cell death, or cell death mediated by the autophagic machinery, under the strictest definition is quite rare, although cells dying from apoptosis or necrosis often have autophagic characteristics.
- Recent *in vivo* studies suggest that programmed necrosis is important for both physiological and for pathological processes.
- The necrosome complex, regulated by the RIP1 and RIP3 kinases, is the primary cellular complex that regulates programmed necrosis. However, there are a number of cellular pathways that also contribute to programmed necrosis. Chief of these is the parthanatic pathway, which requires the activation of PARP and release of AIF from the mitochondria.
- The mitochondria, including the opening of the MPTP, play a major role in programmed necrotic death. The MLKL, PGAM5, and Drp1 proteins may connect the necrosome to the mitochondria. In many cases, ROS and JNK may also be involved both upstream and downstream of the mitochondria.

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