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Role of apoptotic and necrotic cell death under physiologic conditions

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Apoptosis is considered to be a programmed and controlled mode of cell death, whereas necrosis has long been described as uncontrolled and accidental cell death resulting from extremely harsh conditions. In the following review, we will discuss the features and physiological meanings as well as recent advances in the elucidation of the signaling pathways of both apoptotic cell death and programmed necrotic cell death.
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Features of apoptosis and necrosis

The term of apoptosis, which is taken from the Greek word for falling down in Greek, was first used by Kerr et al. in 1972 to indicate a novel type of cell death that was observed in the liver and involved several characteristics that distinguished it from necrotic cell death (1). Specifically, they found that apoptotic cells showed distinct morphological features, such as the presence of membrane bodies, nuclear pyknosis (chromatin condensation), and karyorhexis (nuclear fragmentation). In addition, because cells that died through apoptotic cell death can be engulfed by neighboring phagocytic cells, no leakage of toxic cellular materials or inflammation around the dead cells was observed (1). It was later revealed that this type of cell death also occurred under normal development and pathological conditions, and it is now accepted as a fine-tuned mode of cell death that allows elimination of harmful, seriously damaged, unnecessary, or virus- or bacteria-infected cells, as well as maintains tissue homeostasis by removing unnecessary cells in multi-cellular organisms. In addition, a vast amount of evidence has shown that abnormal apoptotic cell death contributes to various diseases such as cancer (disabled apoptosis) and septic shock (massive apoptosis) (2).

Necrosis, however, is characterized morphologically by cellular swelling and disruption of the plasma membrane, which leads a rapid release of the cellular cytoplasmic content (e.g., a nuclear protein high mobility group box 1 (HMGB1)) into the cell environment. This release, in turn, results in massive inflammatory responses in the physiological environment around the dying cell (3-6). Moreover, it has been reported that necrotic cell death increases the probability of proto-oncogenic mutation, and necrotic cell death has been implicated in various pathological conditions, such as stroke, ischemia, and several neurodegenerative diseases (7, 8). Furthermore, necrosis is often considered to be a causative factor of tumor promotion because excessive and prolonged inflammatory responses are very closely related to the promotion of tumor growth, angiogenesis, and invasion (9, 10).

Death receptor-mediated apoptotic signaling pathways

Apoptotic cell death can be triggered by various death stimuli, including death ligands such as tumor necrosis factor-alpha (TNF-α), FasL/CD95/Apo1, and TNF-related apoptosis-inducing ligand (TRAIL), as well as chemotherapeutic or chemical agents such as cisplatin, doxorubicin, and 5′FU. These death stimuli can activate defined cellular death machineries that can best be described as extrinsic and intrinsic. In the extrinsic pathway, which is also known as the receptor-mediated death pathway, death ligands induce apoptosis by activating the death receptors at the cell surface, whereas in the intrinsic pathway, which is also known as the mitochondria-mediated death pathway, chemical agents directly or indirectly damage the mitochondrial function to induce apoptotic cell death (11-15).

TNF-α was originally identified as an anticancer factor that induces apoptosis in tumor cells (16-18); however, many studies have shown that the major function of TNF-α is stimulation of inflammation through the activation of NF-κB and c-Jun (19, 20). The binding of TNF-α to TNF-α receptor-1 (TNF-R1) recruits the cellular adaptor protein TRADD to the receptor, which then initiates a series of intracellular signaling events (21, 22). To initiate death signaling, it is necessary for TRADD to recruit another adaptor protein, FADD, as well as caspase-8, which then forms the death-inducing signaling complexes (DISC) (21, 23). Although formation of this apoptosis-triggering DISC complex has generally been considered to be an event that occurs at the cell surface, recent findings have shown that

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DISC complexes are formed within the cell after the receptor-containing plasma membrane is internalized by clathrin-mediated endocytosis (24, 25). However, the initial TRADD-containing membrane-bound receptor complex, which does not contain FADD or capase-8, can recruit TRAF2 and RIP to activate NF-κB and c-Jun, respectively (25). This membrane-bound receptor complex can be formed without an internalization step, as is demonstrated by the use of an internalization-deficient TNF receptor. Therefore, this complex is not responsible for the induction of apoptosis (24, 25).

Procaspase-8 activation within the DISC complex triggers downstream signaling cascades that are mitochondria-dependent, as well as mitochondria-independent pathways. In the mitochondria-independent pathways, caspase-8 can directly and fully activate procaspase-3 into active caspase-3, which is sufficient to lead to cell death in type I cells such as BJAB or SKW6.4 (26). However, in type II cells, such as Jurkat cells or hepatocytes in the liver, caspase-8 activation is not sufficient to fully activate the downstream caspases, therefore mitochondrial damage must occur in order for the downstream caspases to be fully activated (26, 27). Caspase-8, however, initiates mitochondrial damage by cleaving Bid into tBid, which then translocates to the mitochondria and causes an efflux of death-promoting proteins such as cytochrome c (28, 29).

TRAIL and FasL are "professional" death ligands that have a limited ability to activate NF-κB and induce apoptosis by binding to their cognate receptors, DR4/DR5 and Fas, respectively (14, 15, 30-33). As seen in TNF-R1 signaling, Fas also transmits death signals by recruiting FADD and caspase-8 to form the DISC complex (23, 34, 35). Recent studies have shown that formation of the DISC complex is closely associated with receptor internalization, which is mediated by clathrin and its accessory proteins in type I cells, but not in type II cells (36). Lee et al. showed that in type I cells, the apoptosis-triggering DISC complex is formed after Fas internalization by clathrin-dependent endocytosis, which can be inhibited by siRNAs of clathrin heavy chain or assembly proteins. However, they also found that the membrane-bound signaling complex at the cell surface is disabled to internalize the Fas receptor, which in turn activates the Erk and NF-κB signaling pathways (36). Interestingly, although TRAIL and its receptors are rapidly internalized by clathrin-mediated endocytosis and clathrin-independent endocytosis, TRAIL receptor internalization does not affect the apoptosis induced by TRAIL in type I and II cells (37). This finding is supported by the results of studies that have shown that TRAIL can still induce apoptotic cell death at 4°C, a condition under which endocytosis is blocked (37,38), and that TRAIL causes cleavage of the clathrin adaptor protein, AP2α, resulting in inhibition of transferrin endocytosis, a well-known example of clathrin-mediated endocytosis. Moreover, the blocking of endocytosis can be induced by temperature-sensitive dynamin-1 mutant enhanced TRAIL-induced apoptosis (38), indicating that DR4/DR5 internalization may not be a necessary event to induce apoptotic cell death. Although TRAIL and FasL appear to share most of the death-inducing signaling molecules, including FADD, caspase-8, and Bid (14, 30, 33, 39, 40), the difference in the internalization of TRAIL receptors and FasL receptors may be useful for differentiating the functional difference between these two pathways to cell death.

Mitochondria-mediated apoptotic death pathways

Although it is well known that mitochondria is an energy-generating sub-cellular organelle, the function of mitochondria has been shown to be more complex since it was found to be closely and deeply involved in apoptotic cell death (41). Although the involvement of mitochondria in apoptosis was suspected by the finding of the mitochondrial localization of anti-apoptotic protein Bcl-2 (42, 43), the most exciting finding for the critical role of mitochondria in apoptosis was that it is able to release the electron-transfer protein cytochrome c in the respiratory chain to cytosol from mitochondria, which in turn activate caspases during apoptosis (44). Additionally, it has been shown that many mitochondrial proteins that are normally localized in the intermembrane space (IMS) between the mitochondrial inner membrane (MIM) and the mitochondrial outer membrane (MOM) are released into the cytosol during apoptosis. These IMS proteins, which include Smac/DIABLO (inhibitor of IAPs) (45, 46), Omi/HtrA2 (47, 48), AIF (49), and EndoG (50, 51), are associated with the activation of caspases or the modification of DNA during apoptosis. To release these IMS proteins, mitochondrial outer membrane permeabilization (MOMP) must occur during apoptosis, and numerous studies have shown that MOMP occurs during the early stages of apoptosis that has been triggered by various death stimuli (41).

In so-called type I cells, direct activation of caspase-3 by caspase-8 is sufficient to induce apoptosis, which indicates that type I cells can die without a mitochondrial death signaling event (26). However, in type II cells, such as primary hepatocytes, the mitochondrial death signaling event must have occurred for apoptosis to be completed (26). This finding is further supported by the fact that Bid-deficient primary hepatocytes are resistant to FasL-induced apoptosis since the mitochondrial death signaling event is blocked in these cells by the lack of Bid protein, which is the most potent inducer of MOMP (27).

The integrity of the mitochondrial membrane has been shown to be controlled by the Bcl-2 family of proteins, which include at least 17 or more members in mammalian cells (52). Bcl-2 proteins share at least one conserved Bcl-2 homology (BH) domains and are divided into two main groups, anti-apoptotic and pro-apoptotic. Anti-apoptotic proteins, such as Bcl-2 and Bcl-x_L, possess four BH domains (BH1 to BH4), whereas pro-apoptotic proteins, such as Bax and Bak, have three BH domains (BH1 to BH3). BH3-only proteins, including Bid, Bim, Bad, Noxa, and PUMA, promote apoptosis by activating pro-apoptotic proteins, such as Bax and Bak, or by re-

pressing anti-apoptotic proteins such as Bcl-2 and Mcl-1 (2, 52). Although some Bcl-2 proteins are known to reside in endoplasmic reticulum (ER), where they regulate ER function (53-56), they primarily act on MOMP, and it is generally accepted that anti-apoptotic Bcl-2 family proteins block MOMP, whereas pro-apoptotic Bcl-2 family proteins promote MOMP (41).

In healthy cells, Bak is localized at the site of the MOM, whereas Bax resides in the cytosol. When they are induced by death stimuli, these proteins self-oligomerize in the MOM to permeabilize the MOM (57-59), thereby allowing the release of IMS proteins, such as cytochrome c, to the cytosol (60, 61). Fibroblasts isolated from mice that are double-deficient for Bax and Bak have been shown to be highly resistant against MOMP, whereas fibroblasts from Bax-deficient or Bak-deficient mice were still susceptible to MOMP, which indicates that one of these two proteins (Bax or Bak) is required for MOMP (61). However, the mitochondria from Bax/Bak double knockout fibroblasts can be permeabilized by Ca^{2+}, which can induce the formation of a permeability transition pore opening through the voltage dependent anion channel (VDAC) (62). As mentioned above, Bax is a cytosolic protein that is translocated onto the MOM after being induced by various death stimuli. Bax translocation to the MOM is associated with conformational changes of Bax, which may be associated with self-oligomerization and formation of protein-permeable pores, possibly with other proteins such as Bak, Bid, and Bad (63). Formation of these pore complexes can be inhibited by Bcl-x_L (63). Taken together, these findings indicate that enhancement of Bax relocalization can promote apoptotic cell death. Indeed, several proteins, including humanin and ASC have been shown to promote apoptosis by enhancing Bax translocation into the mitochondria (64,65). Conversely, if Bax protein is retained in the cytosol, mitochondrial damage and apoptosis should be inhibited. This case was, indeed, supported by finding DNA repair protein Ku70, which blocks the mitochondrial translocation of Bax as well as Bax-mediated apoptosis (66, 67). However, the mechanism by which MOM is mediated by Bax and Bak causes the release of death-promoting IMS proteins such as cytochrome c is not well understood.

It is generally assumed that BH3-only proteins can promiscuously bind to all their anti-apoptotic partners. However, recent biochemical studies conducted using BH3 peptides indicate that the binding activities of different BH3 domains appear to be highly selective. For example, Noxa BH3 domain is critical for the ability of a protein to selectively bind to the BH3 domains of Mcl-1 and A1/Bfl-1, whereas Bad BH3 domain only engages to bind to the BH3 domain of Bcl-2, Bcl-x_L, and Bcl-w. In addition, the BH3 domains of Bid, Bim, and Puma can bind to each of the anti-apoptotic members (68-71). In addition, studies using BH3 peptides showed that only some BH3-only proteins (e.g., Bid and Bim) can directly activate Bax and Bak to initiate MOMP, which occurs either by stimulating the translocation of Bax to the MOM or by triggering the oligomerization of Bak. Other BH3-only proteins (e.g., Noxa and Bad) can not directly activate Bax and Bak, however, they can be made sensitive to promote MOMP by binding to anti-apoptotic Bcl-2 family proteins and then dissociating them from Bax and Bak (72). Based on these findings, it can be assumed that some tumor cells have growth advantages as a result of the expression of Mcl-1 or A1/Bfl-1, whereas others gain an advantage as a result of the expression of Bcl-2, Bcl-x_L, or Bcl-w. This indicates that tumor cells that express high levels of Mcl-1 or A1/Bfl-1 can be efficiently killed by the induction of Noxa, whereas those that express high levels of Bcl-2, Bcl-x_L, or Bcl-w can be efficiently killed by Bad expression. Indeed, 18 lymphoma cell lines can be categorized into three groups based on their BH3-profilings, which makes it possible to predict their sensitivity to ABT-737 (73). Therefore, examination of the BH3-profiles of cancer cells directly isolated from cancer patients may allow prediction of the sensitivity to specific chemotherapeutic agents, possibly providing a logical background for personalized cancer therapy. In addition, this concept provides a basis for the development of small molecules, so-called BH3-mimectic drugs, which mimic Bad, Noxa or Bid/Bim. A promising cancer drug, BH3-mimetic ABT-737, which behaves in a fashion similar to Bad, has been shown to efficiently kill tumor cells that have survival advantages as a result of the expression of Bcl-2, Bcl-x_L, or Bcl-w, however this drug does not kill tumor cells that express high levels of Mcl-1 or A1/Bfl-1 (74, 75).

The morphology of mitochondria in healthy cells maintains the interconnected tubular networks by a dynamic process of mitochondrial fusion and fission (76, 77). This balanced process appears to move toward one direction in response to various environmental conditions, resulting in a fused or fragmented mitochondrial morphology. Recent studies have demonstrated that interconnected mitochondria are disintegrated into small and fragmented mitochondria during the early stages of apoptotic cell death (78), and that this process is tightly regulated by the Bcl-2 family of proteins (76, 79, 80). For example, the pro-apoptotic protein, Bax, has been shown to induce mitochondrial fragmentation by changing its intracellular location during the early stages of apoptosis to focal cluster sites during mitochondrial fission, where they co-localize with the mitochondrial fission mediator, Drp1 (79). Drp1 belongs to the dynamin family, which mediates membrane remodeling in eukaryotic cells and has a GTPase domain that drives mitochondrial fission (81, 82). Ectopic expression of a dominant-negative mutant, Drp1 K38A, which harbors an inactive GTPase domain, induces mitochondrial fusion, resulting in the formation of elongated and highly interconnected mitochondrial networks (81, 83). In this process, Drp1 that normally exists in the cytosol moves to fission sites on the MOM during cell death, where it binds to hFis1, a MOM protein that is evenly distributed on the surface of the mitochondria (84). RNA interference-mediated depletion of hFis1 or Drp1 results in an elongated mitochondrial morphology similar to what is observed in Drp1 K38A-overexpressed cells (85), and in-

hibition of mitochondrial fission delays the caspase activation and cytochrome c release, as well as the process of apoptosis (78, 79, 82, 86). Conversely, overexpression of either mitochondrial fission protein Drp1 or hFis1, as well as down-regulation of mitochondrial fusion protein Opa1 promotes apoptosis in response to various death stimuli including staurosporine and etoposide (84, 87-89). Consistent with these findings, overexpression of MFN1 and MFN2, which are the mitochondrial fusion mediators, inhibits apoptosis (90). Together, these findings indicate that mitochondrial fragmentation is a prerequisite step for apoptosis. However, it should be noted that there are many compelling observations that contradict these findings. For example, several recent studies have shown that the release of cytochrome c occurs several minutes before mitochondrial fragmentation, which suggests that mitochondrial fragmentation might not cause the release of cytochrome c, and that it is instead just associated with it (88, 91). In addition, over expression of Drp1 in HeLa cells induces mitochondrial fragmentation with no apparent apoptosis (92), as does overexpression of mutant hFis1 (93), indicating that mitochondrial fragmentation can occur without being associated with apoptosis.

MIM protein Opa1 (optic atrophia 1), which is another mitochondrial fusion mediator that cooperates with MFN1 (94), is processed by presenilin-associated rhomboid-like protein (PARL) to generate a shorter and soluble intermembrane space (IMS), Opa1 (95). Soluble IMS Opa1 produces hetero-oligomers that contain inner membrane Opa1 protein, which tighten the cristae junctions (95). These hetero-oligomeric Opa1 complexes are disrupted by Bid and BIK to open up the junction region of the mitochondrial cristae, thereby allowing the release of the majority (>80%) of cytochrome c that is normally present within the closed cristae structures and leading to apoptotic cell death (87, 96, 97). Overexpression of Opa1 protects cells from apoptosis induced by etoposide or H_2O_2. This protection against apoptosis by Opa1 is primarily due to the prevention of cytochrome c release from the mitochondria; however, it occurs independently of MFN1-dependent Opa1 mitochondrial fusion activity since Opa1 can still protect MFN1-deficient cells from staurosporine and H_2O_2 (87).

Evidence of programmed necrosis

Despite the significant effects of necrotic cell death under pathological conditions, the molecular mechanisms underlying necrotic cell death are poorly understood. In some cases, the most widely accepted way to define necrotic cell death is by demonstrating a lack of apoptotic features. Because necrosis is believed to be an uncontrollable and passive form of cell death (98, 99), researchers may be less interested in the subject. However, recent studies have demonstrated that there exists not only accidental necrosis, but also normal physiological and programmed necrosis (100, 101). For example, a form of necrosis that is regulated by cyclophilin D-dependent mitochondrial permeability transition is stimulated by many different stimuli, including alkylating DNA damage (102), tumor necrosis factor (103, 104), and cell-cell contracts (105, 106). In addition, caspase-dependent necrosis has been reported (107, 108), and protein synthesis persists during necrosis (109). Furthermore, FADD mediates differential signaling leading to apoptotic and necrotic cell death (104), and disruption of hsp90 function switches TNF-induced necrosis to apoptosis (110). Moreover, activation of the PKC-ERK1/2 signal pathway and administration of ethyl pyruvate switches glucose depletion induced-necrosis to apoptosis, while preventing the release of HMGB1 in lung cancer cell lines by preventing excessive generation of reactive oxygen species (ROS) (111, 112). Several studies have also shown that certain types of cell death share both apoptotic and necrotic mechanisms in a process newly named as "necrapoptosis" (113). These studies have also shown that several common points that induce the necrotic and apoptotic pathway exist, which indicates that the signaling cross point is modulated. Therefore the mode of cell death can be changed from apoptosis to programmed necrosis and vice versa, which further supports the idea that necrosis is programmed and controllable.

The Physiological significance of necrosis

Depending on the endogenous and exogenous cellular factors involved, cells will respond to stressful stimuli in different ways, including senescence, cycle arrest, apoptosis, or necrosis. It is generally accepted that programmed necrosis does not act as a simple alternative cell death path to apoptosis, but that it instead plays an important role in the maintenance of physiologic conditions. The evolutionary advantage conferred by necrosis might be an inflammatory response, because necrosis, unlike apoptosis that results in a quiet and clean type of death, provokes strong inflammatory responses and cytokine production. Zong and Thompson explained that necrosis may serve to maintain tissue and organism integrity by initiating these adaptive and reparative responses in the host (114). TNF-α, as well as several cytokines, heat shock proteins (HSP), and nuclear proteins such as HMGB1 are well known factors that are released from necrotic dying cells and play a role as recruiters of inflammatory cells to the site where tissue damage occurs (3, 115-117). HSP70, which is a well known chaperone protein, has a cytoprotective role and prevents apoptosis or necrosis caused by various stimuli such as heat shock, oxidative stress, UV, and anticancer drugs in the cell (118). However, it has been reported that elevated levels of HSP70 in necrotic tumor cells lead to increased immunogenicity (119). Moreover, HSP70 also acts as a specific signal transducer for the immune system and can enhance the immunogenicity of some other macromolecular antigens (120). HMGB1 is another potent inflammatory mediator that is released from necrotic cells and promotes the recruitment of inflammatory cells such as mononuclear cells. In addition, extracellular HMGB1 acts as a sig-

naling molecule that informs neighboring cells that tissue repair is required (121).

In adults, cancer is frequently preceded by a long period of subclinical inflammatory diseases and micronecrosis. Convertsely, cancers in children most likely develop as a result of a combination of inherited genetic mutations and genetic alterations that can be acquired during embryogenesis. Cancers associated with chronic inflammation include lung, eosophageal, gastric, pancreatic, cervical, bladder, prostate, and colorectal cancers (122-126), and recognized precancerous inflammatory conditions can be triggered by various factors such as viral infection, irradiation, tobacco smoke or chemicals (127, 128). While most pathogens provoke an acute inflammatory reaction that results in complete clearance of the microorganism, some inflammatory agents can induce a continuous inflammatory process without clearance. If this state persists for a prolonged period, it results in a suitable environment for the development of hyperplasia, dysplasia, and frequently malignant neoplasia, which enriches the tumor environment more aggressively through the mechanisms involved in the immune cell response, such as tumor associate macrophages (TAM) and the epigenetic regulation of tumor suppressor genes (129).

Regulatory factors of necrosis

One of the critical features of necrosis is early rupture of the plasma membrane, which is considered a result of ATP depletion since reduction in the function of the ATP-dependent ion pumps on the plasma membrane due to energy depletion may disturb the intracellular homeostasis. Perturbation of intracellular homeostasis would, in turn, lead to opening of the ion channel, resulting in cytoplasmic membrane swelling and disruption.

Several membrane receptors have been implicated in the triggering of both apoptosis and necrosis. For example, TRAIL induces necrotic-like cell death at a lower extracellular pH in human HT29 colon carcinoma and HepG2 hepatocarcinoma cell lines (130). In addition, FADD deficiency sensitizes Jurkat T cells to TNF-α-dependent necrosis during activation-induced cell death (131), and ligation of DR4 and DR5 causes necrosis (132). Taken together, these results indicate that necrotic programs may share part of the apoptotic pathway.

Among the secondary messengers, it has been reported that Ca^{2+} participates in receptor-mediated necrosis. Increased Ca^{2+} influx induces necrosis through MEC-4(d) channel-activated Ca^{2+} release from the ER in neuron cells (133). The role that the Ca^{2+} increase plays in both apoptosis and necrosis has been widely reported, and determination of whether the mode of death is necrosis or apoptosis appears to be regulated by the Ca^{2+} concentration, with a relatively higher concentration of Ca^{2+} inducing necrosis and moderate Ca^{2+} levels triggering apoptosis (134). It is interesting to note that caspase, which is activated during apoptosis, cleaves the plasma membrane Ca^{2+}pump, which results in necrosis through Ca^{2+} overload (135). This result suggests that a more intensive Ca^{2+} increase is required to induce necrosis.

Living cells constantly generate ROS during their respiration and metabolism. However, excessive production of ROS is another factor that triggers necrosis, even though its role in the induction of apoptosis has also been reported. Extreme hepatectomy-induced liver failure occurs due to necrosis as a result of the production of massive oxidative injury; however, this condition can be recovered through IL-6 overexpression or administration, or by reducing oxidative stress and maintaining mitochondrial function (136). In addition, suppression of ROS production through the blocking of SOD1 release by PKC activation or through the addition of antioxidants switches glucose depletion induced-necrosis to apoptosis (111). Furthermore a novel TNF-induced signaling complex that contains TRADD, RIP1, and Rac1 has been reported to play a key role in TNF-induced necrotic cell death by generating ROS through NADPH oxidase (Nox1) (137). In some cases, a specific type of ROS appears to be important for determination of the mode of cell death. For example, superoxide induces apoptosis, whereas hydrogen peroxide induces necrosis in hepatocytes (138) and hydrogen peroxide induces non-apoptotic cell death through JNK1-mediated PARP-1 activation (139). Currently, although it is not clear why ROS generation leads to apoptosis in some cells but necrosis in others, the amounts or the types of ROS are believed to be a critical factor involved in determining the mode of cell death. However, other regulatory factors can not be excluded.

Conclusion

This review has concentrated on the recent advances in our understanding of apoptotic signaling at the level of receptor-mediated and mitochondria-mediated events, as well as on recent findings regarding the programmed necrosis signaling mode. Although recent studies in these fields have led to significant progress in the understanding of signaling events involved in apoptosis and necrosis, further studies are required for a more refined understanding of the molecular elements involved in determining the mode of cell death that occurs in response to various death stimuli. A better understanding of the mechanisms under which the mode of cell death is determined will also contribute to the development of more efficient strategies for the treatment of necrosis- and apoptosis-related human disorders, including cancer.

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