REVIEW

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The mechanism of necroptosis in normal and cancer cells

Simone Fulda

Institute for Experimental Cancer Research in Pediatrics; Goethe-University; Frankfurt, Germany

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Abbreviations: AIF, apoptotic inducible factor; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ATP, adenosine 5′-triphosphate; cIAP, cellular inhibitor of apoptosis; CLL, chronic lymphocytic leukemia; CYLD, cylindromatotis; DAMP, danger-associated molecular pattern; DRP, dynamin-related protein; EDAR, ectodermal dysplasia receptor; FADD, FAS-associated death domain protein; IAP, inhibitor of apoptosis; JNK, c-Jun N-terminal kinase; LEF1, lymphoid enhancer-binding factor 1; LTβ, lymphotoxin beta; MLKL, mixed-lineage kinase domain-like protein; MMP, mitochondrial membrane potential; NFκB, nuclear factor kappaB; PARP, poly (ADP-ribose) polymerase; PGAM, phosphoglycerate mutase; RIP, receptor-interacting protein; ROS, reactive oxygen species; Smac, second mitochondrial activator of caspases; SNP, single nucleotide polymorphism; TNFR1, tumor necrosis factor receptor 1; TNFα, tumor necrosis factor alpha; TRADD, TNFR-associated death domain; TRAF, TNF receptor-associated factor; TRAIL, tumor-necrosis-factor-related apoptosis-inducing ligand; TWEAK, TNF-related weak inducer of apoptosis

Programmed cell death is a basic cellular process that is critical to maintain tissue homeostasis. Besides apoptosis, necroptosis has more recently been discovered as another form of regulated cell death. Necroptosis plays a pivotal role during normal development and has also been implicated in the pathogenesis of a variety of human diseases. The control of necroptosis by defined signal transduction pathways offers the opportunity to target this cellular process for therapeutic purposes. For example, in cancer necroptosis is often impaired during tumorigenesis and can be engaged by targeted pharmacological approaches. Further insights into the signaling networks involved in the regulation of necroptosis will likely have important implications for the exploitation of this form of programmed cell death for the diagnosis or treatment of many diseases.

Introduction

Cell death represents a key physiological process that is critical for maintaining tissue homeostasis, since a tight balance between cell growth on one side and cell death on the other side is pivotal for various functions in our body.[1] There exist a variety of subroutines which finally can lead to cell death in mammalian cells.[2] In addition, these processes and mechanisms are evolutionary highly conserved across species, ranging from yeast, flies, and worms to mammals and humans, underscoring the importance of these basic cellular mechanisms for multicellular organisms. As far as programmed cell death is concerned, distinct modes can be distinguished.[2] Among them, apoptosis has been identified several decades ago and has since then been characterized in numerous studies.[3]

Far less well understood is necroptosis, a more recently identified form of programmed cell death.[2] While necrosis has for long been considered as an accidental mode of cell death, it was recently recognized that a form of cell death with morphological criteria of necrosis can also be regulated in a programmed manner via defined signal transduction pathways.[4] Since its identification, there is increasing evidence indicating that necroptosis is involved in the regulation of a variety of normal physiological processes, for examples during development. In addition, the picture emerges that deregulated signaling pathways involved in the control of necroptosis form the basis of many human diseases, e.g., during viral or bacterial infections, ischemia–reperfusion injury, neurodegeneration or cancer. Against this background, it is a timely and important question to review necroptosis and in particular its implications in cancer. However, it is also important to note that, besides necroptosis, there are additional forms of non-apoptotic programmed cell death, for example, autophagic cell death.

Signaling Pathways to Necroptosis

The molecular pathways that eventually lead to necroptosis have been intensively studied during the last years. In principle, a multitude of different stimuli can initiate the necroptotic cell death machinery which then comprises different phases of signal transduction, including an initiation and an execution phase, to finally fuel into cell death associated with the loss of cell and organelle integrity. The many different stimuli that can lead to the induction of necroptosis can be classified according to their nature of origin and to the regulatory mechanisms involved. One of the most extensively investigated models of necroptosis is the one induced by the tumor necrosis factor receptor 1 (TNFR1)/

Correspondence to: Simone Fulda; Email: simone.fulda@kgu.de
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tumor necrosis factor α (TNFα) receptor ligand system that occurs in a receptor-interacting protein (RIP)1- and/or RIP3-dependent manner.[5-10] However, the TNFR1/TNFα signaling is not the only death receptor/ligand system that can engage the necroptotic machinery. Additional death receptors of the TNFR superfamily, including CD95 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors, have also been reported to trigger a form of non-apoptotic cell death consistent with necroptosis.[5,11-13] Furthermore, TNF-related weak inducer of apoptosis (TWEAK), lymphotoxin β (LTβ), and ectodermal dysplasia receptor (EDAR) have been implicated in the induction of necroptosis.[14-18] Besides ligand-/cytokine-mediated necroptosis, several viral or bacterial pathogens have been described to initiate the necroptosis machinery.[19-25] Furthermore, different kinds of physical-chemical stress stimuli can initiate necroptosis, including reactive oxygen species (ROS), ischemia–reperfusion injury, anticancer drugs, in particular DNA-damaging agents, ionizing radiation, photodynamic therapy, glutamate, and calcium overload.[6,10,26-37] This list of necroptotic triggers is not exhaustive and steadily growing.

These different stimuli can enter the necroptotic cell death program by distinct entry sites. As an example, the prototypic TNFR/TNFα necroptotic signaling pathway will be discussed in the following. Ligation of the plasma surface receptor TNFR1 by its natural ligand TNFα or by agonistic antibodies leads to oligomerization of receptors into aggregates and recruitment of various proteins including TNFR-associated death domain (TRADD), RIP1, cellular inhibitor of apoptosis (cIAP) proteins, TNF receptor-associated factor (TRAF)2, and TRAF5 into a multimeric protein complex at the plasma membrane, the so-called TNFR1 complex I.[4] Within this complex, RIP1 is polyubiquitinated via K63-linked ubiquitin chains by cIAP proteins which in turn leads to the activation of the classical (canonical) signaling pathway of the transcription factor nuclear factor kappa B (NFκB). Furthermore, TNFR is rapidly internalized upon ligand binding, which results in alterations in the composition and posttranslational modification of receptor-associated proteins. For example, RIP1 becomes deubiquitinated by the deubiquitinase cylindromatotis (CYLD), which in turn reduces its ability to interact with components of canonical NFκB signaling and instead increases its affinity to proteins involved in cell death signaling. This so-called cytoplasmic cell death complex II comprises as key components RIP1, TRADD, FAS-associated death domain protein (FADD), and caspase-8 and drives the activation of caspase-8 via a cytoplasmic cell death signaling platform. However, when caspase activation is inhibited due to genetic or pharmacological inhibition, RIP1 forms instead a complex together with RIP3 to fuel into the necroptotic signal transduction pathway.[10,38] This RIP1-/RIP3-containing cytoplasmic necroptotic protein complex is called necrosome and constitutes a key molecular platform of necroptosis. This involves the reciprocal phosphorylation of RIP1 and RIP3 in an autocrine/paracrine manner, leading to activation of their kinase activity.[6-8] Also, the mixed-lineage kinase domain-like protein (MLKL) has been identified as a substrate that is phosphorylated by RIP3 and shown to play an important role in the transduction of the necroptotic signal to cell death.[39,40] Accordingly, knockdown of MLKL was shown to result in inhibition of TNFα-mediated necroptosis. In addition to MLKL, phosphoglycerate mutase (PGAM)5L has also been reported to become phosphorylated upon activation of RIP3.[41] PGAM5L then interacts with PGAM5S on the mitochondrial membrane to initiate the dephosphorylation of dynamin-related protein (DRP)1, a mitochondrial fission regulator, which leads to mitochondrial fission and mitochondrial fragmentation.[41]

Signaling to necroptosis may involve metabolic alterations including mitochondria-associated processes and the overproduction of ROS which may damage different macromolecules including lipids, proteins, and DNA, thereby contributing to the execution of necroptosis.[6] Furthermore, calcium-mediated activation of calcium-regulated enzymes such as calpain can contribute to the destruction of cellular components, for example by promoting permeabilization of lysosomal membrane and the release of lysosomal enzymes into the cytosol.[4,42,43] Moreover, sphingomyelinases have been described to be activated during the execution phase of necroptosis and may promote necroptosis by enhancing the generation of sphingosine as a second messenger that contributes to lysosomal membrane permeabilization.[4,44-46] In addition, the RIP1/RIP3 necrosome may promote activation of the stress kinase c-Jun N-terminal kinase (JNK) which can contribute to necroptosis by altering the iron storage compartment.[4,47] Mitochondria-associated alterations during necroptosis can also alter cellular levels of adenosine 5′-triphosphate (ATP).[4,48,49] Accordingly, many examples of necroptotic cell death are eventually associated with a bioenergetic breakdown of the cells with profound drop of intracellular ATP pools. It is important to note that the final consequences of necroptotic cell death can be distinguished from apoptotic cell death, as the plasma membrane typically ruptures, resulting in the release of the intracellular content into the microenvironment, for example danger-associated molecular patterns (DAMPs). This in turn leads to inflammatory and immunogenic responses to the dying cell. Furthermore, MLKL has been identified as a substrate that is phosphorylated by RIP3 and shown to play an important role in the transduction of the necroptotic signal to cell death.[39,40] Accordingly, knockdown of MLKL was shown to result in inhibition of TNFα-mediated necroptosis. In addition to MLKL, PGAM5L has also been reported to become phosphorylated upon activation of RIP3.[41] PGAM5L then interacts with PGAM5S on the mitochondrial membrane to initiate the dephosphorylation of DRP1, a mitochondrial fission regulator, which leads to mitochondrial fission and mitochondrial fragmentation.[41]

Necroptosis and Cancer

There is mounting evidence showing that necroptosis can be disturbed in human cancers. For example, chronic lymphocytic leukemia (CLL) cells have recently been reported to harbor defects in key signal transduction components that are involved in the regulation of necroptosis, as RIP3 and the deubiquitination enzyme CYLD are frequently downregulated in CLL.[50] Mechanistically, CYLD was shown to be suppressed at the transcriptional level in CLL cells by lymphoid enhancer-binding

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factor (LEF)1, a downstream effector of the Wnt/β-catenin pathway.[50] By identifying LEF1 as a transcriptional repressor of necroptosis signaling constituents, these results imply that therapeutic strategies to block LEF1 may also restore the necroptotic pathway in CLL.

Furthermore, procaspase-8, a key regulator of apoptotic cell death, has recently been described to paradoxically also promote cell survival under certain conditions.[51] In a mouse model of T lymphoma, downregulation of caspase-8 was reported to increase rather than decrease cell death and also to reduce cell growth via its protease activity.[51] This form of cell death was associated with the production of ROS and inhibited upon addition of ROS scavengers or pharmacological or genetic inhibition of RIP1 or RIP3.[51] In addition to these features of non-apoptotic cell death, the protease activity of caspase-8 was also shown to regulate apoptotic signaling events in this model of T lymphoma involving caspase activation and caspase-mediated cell death.[51] These findings indicate that inhibition of both apoptotic and non-apoptotic forms of cell death including necroptosis might be involved in T lymphoma.

In non-Hodgkin lymphoma, single nucleotide polymorphisms (SNPs) in the RIP3 gene were identified in a large cohort of 458 patients and correlated with increased risk of non-Hodgkin lymphoma.[52] These results indicate that genetic variations in the RIP3 gene may contribute to the etiology of this cancer.

Necroptosis and Cancer Therapy

There is a growing list of compounds and anticancer drugs with various primary mechanisms of action that have been shown to initiate necroptosis in cancer cells. For example, Shikonin, a naturally occurring naphthoquinone, has been reported to induce necroptotic cell death in cancer cells including drug- and apoptosis-resistant cancer cells with overexpression of P-glycoprotein, MRP1, BCRP, Bcl-2, or Bcl-x_L.[53,54] FTY720, a sphingolipid analog drug that mimics ceramide, was shown to target the I2PP2A/SET oncoprotein, resulting in PP2A reactivation and induction of necroptotic cell death.[55] Derivatives of amiloride such as 5′-benzylglycinyl-amiloride (UCD38B) and glycinyl-amiloride (UCD74A) were reported to trigger necroptosis via loss of mitochondrial membrane potential (MMP) and the release of apoptotic inducible factor (AIF) from mitochondria into the cytosol followed by its nuclear translocation.[56] BI2536, a small-molecule inhibitor of the mitotic kinase Plk1, has been described to engage necroptosis in androgen-resistant prostate cancer cells.[57] Staurosporine, an inhibitor of protein kinases, has been reported to trigger necroptosis in leukemia cells, when caspase activation is inhibited.[57] This form of necroptosis was described to be blocked by several pharmacological inhibitors including RIP1 inhibitor necrostatin-1, HSP90 inhibitor geldanamycin, MLKL inhibitor necrosulfonamide, and a cathepsin inhibitor.[58]

The death receptor ligand TRAIL was recently described to engage a form of necroptosis via RIPK1-/RIPK3-dependent poly (ADP-ribose) polymerase (PARP)1 activation and depletion of cellular ATP levels, thereby placing PARP1 activation as an effector mechanism downstream of RIP1.[59] This study comprised both in vitro cell culture models of carcinoma cells, including colon carcinoma and hepatocellular carcinoma, and an in vivo mouse model of concanavalian A-induced hepatitis.[59] Also, necroptosis was found to contribute in part to radiotherapy-mediated cell death besides apoptosis in thyroid and adrenocortical cancers.[60]

Obatoclax (GX15-070), a small-molecule inhibitor of anti-apoptotic Bcl-2 proteins, was shown to induce autophagy-dependent necroptosis in glucocorticoid-resistant childhood acute lymphoblastic leukemia (ALL).[61] This mechanism bypassed the block in mitochondrial apoptosis of glucocorticoid-resistant leukemic blasts, thereby overcoming resistance toward glucocorticoids, one of the central pillars of treatment protocols for ALL.[61] Furthermore, obatoclax was recently reported to stimulate the assembly of the necrosome on autophagosomal membranes, thereby connecting obatoclax-triggered autophagy to necroptosis signaling pathways.[62] As shown by coimmunoprecipitation studies, obatoclax triggered the physical interaction of Atg5, a component of autophagosomal membranes, together with RIP1 and RIP3 as key constituents of the necrosome.[62]

Furthermore, a diphtheria-based toxin targeted against acute myeloid leukemia (AML) was demonstrated to engage caspase-independent necroptosis in AML via inhibition of protein synthesis, although the exact molecular mechanisms remain to be defined.[63] Of note, this engagement of necroptotic cell death even occurred in apoptosis-resistant AML cells, indicating that necroptosis may open new perspectives for cancer drug development in AML.[63]

Besides regulating apoptosis, small-molecule inhibitors targeting inhibitor of apoptosis (IAP) proteins such as second mitochondrial activator of caspases (Smac) mimetics have been reported to engage necroptotic cell death. To this end, the bivalent Smac mimetic BV6 that antagonizes XIAP, cIAP1, and cIAP2 has been shown to sensitize apoptosis-resistant leukemia cells that lack essential regulators of apoptosis signaling, such as FADD or caspase-8, for TNFα-triggered necroptosis in a highly synergistic fashion.[64] This synergistic induction of necroptosis by Smac mimetic and TNFα was not only found in leukemic cell lines but also in primary, patient-derived ALL cells.[64] This Smac mimetic-/TNFα-triggered necroptosis occurred in a RIP1-dependent but caspase-independent manner in FADD- or caspase-8-deficient leukemia cells.[64] In sharp contrast, the same cotreatment of Smac mimetic and TNFα induced apoptotic cell death in FADD- or caspase-8-proficient leukemia cells.[64] This illustrates that Smac mimetic can prime leukemia cells to TNFα-mediated cell death in a context-dependent fashion via either necroptosis or apoptosis depending on the cellular context. Similar to leukemia cells, fibrosarcoma cells were sensitized to TNFα-mediated necroptosis upon the addition of the Smac mimetic BV6.[65] This Smac mimetic-mediated enhancement of TNFα-induced cell death was associated with increased production of ROS and elevated activity of RIP1 kinase.[65] Pharmacological or genetic interference with RIP1 or RIP3 inhibited both ROS production and cell death induction, underlining that this form of necroptosis occurs in a RIP1-/RIP3-dependent manner. Furthermore, recent evidence indicates that Smac mimetic in combination with demethylating agents can circumvent apoptosis resistance of AML cells

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by engaging necroptosis as an alternative cell death program.[66] To this end, the Smac mimetic BV6 synergized with demethylating agents such as 5-azacytidine or 5-aza-2'-deoxycytidine to induce cell death in AML cells.[66] While this form of cell death was associated with activation of caspases, a broad-range caspase inhibitor failed to rescue Smac mimetic-/demethylating agents-mediated cell death.[66] By comparison, the RIP1 inhibitor necrostatin or the MLKL inhibitor necrosulfonamide significantly reduced cell death upon combination treatment with Smac mimetic and decitabine.[66]

Furthermore, photodynamic therapy using the pharmacological agent 5-aminolevulinic acid was reported to trigger a form of RIP3-mediated necrosis in glioblastoma cells.[67] In response to 5-aminolevulinic acid-based photodynamic therapy, the authors reported the formation of a RIP3-/RIP1-containing complex in the cytosol that was critical for the induction of cell death. Formation of this complex was initiated upon photodynamic therapy-induced production of singlet oxygen, pointing to the involvement of ROS generation in the engagement of this form of programmed cell death.

Conclusions

Necroptosis can be engaged as alternative form of programmed cell death even under conditions when apoptosis is blocked. In fact, necroptosis and apoptosis are intricately linked programs of cell death. In many circumstances, inhibition of caspase activity, one of the hallmarks of apoptosis, has been shown to enhance rather than inhibit necroptosis, since caspase activity negatively regulates necroptosis by cleaving RIP1 and RIP3, two key components of necroptosis signaling. Since apoptosis is frequently impaired in human cancers, engagement of necroptosis as an alternative mode of programmed cell death opens new opportunities to kill cancer cells, even in treatment-resistant forms of cancer. The challenge will be to selectively initiate necroptosis in tumor cells by pharmacological means, while sparing normal, non-malignant cells. In addition, novel tools are required for specific detection of necroptosis in tissue or tumor samples. Further elucidation of necroptosis signaling pathways and their regulators will likely further advance the development of diagnostic and therapeutic strategies to exploit this form of programmed cell death for the diagnosis and treatment of human diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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