Exploitation of necroptosis for treatment of caspase-compromised cancers (Review)

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Abstract. Programmed necrosis, or necroptosis, is a type of specialized cell death with necrotic characteristics, including the loss of membrane integrity and swollen organelles in dying cells. However, unlike simple necrosis, it may be induced as an alternative form of cell death when apoptosis is blocked and it is mediated in an orchestrated manner, similar to apoptosis, by a series of signaling molecules. Necroptosis-associated proteins and their specific small molecules have been extensively identified in order to illuminate the underlying mechanisms by which necroptosis is activated through a novel signaling pathway. However, the biological significance of necroptosis, which is known as a secondary route of apoptosis, remains under debate. Concurrent with these concerns, the clinical application of necroptosis has been cautiously proposed to treat necroptosis-associated diseases, and to overcome resistance to anticancer drugs. Accordingly, the present review will highlight the harnessing of necroptosis for anticancer therapy. To this end, the state-of-the art technique of necroptosis as a cancer therapy will be briefly described, and then its potential for clinical purposes will be delineated. For a further understanding of necroptosis, the present review begins with a basic introduction to necroptosis and its multifaceted physiological consequences.

Contents

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1. Introduction

To maintain cellular homeostasis, cell survival is in a state of constant equilibrium with cell death (1). When cell demise occurs in an ordered and controlled way to cause programmed cell death, this is termed apoptosis. As well as being part of normal tissue turnover, apoptosis is an essential process for development, differentiation and immune responses (2-4). It is known to be actively involved in the removal of useless or severely damaged cells (5). In contrast, necrosis is a type of unrequired cell death that occurs when cells are exposed to overwhelming stresses, including radiation overdose or toxic chemicals. Concurrently, there is an ordered type of necrosis that is executed by signaling pathway of its associated proteins. This type of programmed cell death, which exhibits necrotic features, is termed programmed necrosis or necroptosis. It was initially considered to be a specialized and regulated form of necrotic cell death (6). At present, necroptotic cell death is known to serve a central function in cell development, immunity, cancer and degenerative diseases (7-10). It exhibits typical necrotic characteristics and is under the control of a well-defined signaling pathway. The present review begins with a description of necroptosis-derived features, and updated information on necroptosis regulators and their specific inhibitors.

A list of features discriminating between apoptosis and necroptosis are summarized in Table I. Cells undergoing apoptosis are morphologically shrunken with condensed cytoplasm, while necroptotic cells and nuclei are swollen. Membrane integrity is a definite determinant parameter to discriminate apoptosis and necroptosis (11). Cells that are dying by apoptosis or necroptosis exhibit intact or disintegrated membranes, respectively. At the molecular level, a cascade of signaling pathways leading to caspase activation is required for the mediation of apoptosis via intrinsic or extrinsic pathways, but necroptosis is achieved by the formation of the receptor-interacting protein kinase (RIP)1-RIP3 necrosome complex (12). Necroptosis is different from apoptosis as the former exhibits more marked physiological effects compared with the latter. Specifically, necroptotic cells release intracellular danger signaling molecules into the media to provoke inflammation and immune responses. These endogenous molecules are referred to as damage-associated molecular patterns (DAMPs), which include high mobility group box 1 (HMGB1), DNA fragments and mitochondrial contents. In particular, HMGB1 is a major DAMP protein derived from necroptotic cells, and serves a pivotal role in

triggering inflammatory responses (11). Apoptotic cells are completely cleared by macrophages or neighboring cells, therefore presenting no apparent physiological responses when compared with the consequences of necroptosis. Conclusively, the physical and biochemical parameters that characterize apoptosis or necroptosis contribute to different physiological outcomes in biological systems.

The terminology 'programmed' indicates that each processing step is developed in a well-organized way and specifically regulated in an orchestrated manner. Necroptosis-associated proteins have been extensively identified through RNA interference screening to establish a series of signaling networks (13). Notably, certain key regulators are known to execute tumor necrosis factor α (TNFα)-mediated necroptosis. The signaling pathway leading to necroptosis is summarized in Fig. 1. Upon TNFα ligation to its cognate TNFα receptor (TNFR), RIP1, TNF receptor 1-associated death domain protein, Fas-associated death domain and caspase-8 are assembled to form complex I (14). Subsequently, transition of the membrane-bound complex I to the cytosolic complex II ensues, leaving TNFR (15). Then, a tumor suppressor cylindromatosis (CYLD) protein promotes the deubiquitination of RIP1 in either complex I or complex II (16,17). It is generally hypothesized that necroptosis is mediated by formation of the RIP1-RIP3 complex when caspase is defective (18). Since the identification of RIP3 as a proximal protein of necroptosis, a mitochondrial protein phosphoglycerate mutase family member 5 (PGAM5) and mixed lineage kinase domain-like (MLKL) protein have been additionally identified as downstream proteins of TNFR ligation. The RIP1-RIP3 complex transmits a death signal to its downstream target, MLKL (19,20). MLKL has been suggested to be responsible for reactive oxygen species generation and c-Jun N-terminal kinase activation during TNFα-induced necroptosis (20). PGAM5, an additional protein that interacts with RIP3, has been identified in addition to RIP1 and MLKL (21). The PGAM5 gene encodes two protein isoforms, PGAM5-long form and PGAM5-short form, via alternative splicing (22). One of these splice variants, PGAM5-short form, may recruit mitochondrial fission factor dynamin-related protein 1 to cause mitochondrial fragmentation (21). PGAM5 functions at the convergence point of multiple necrotic death pathways, linking extracellular stimuli derived from TNFα to the mitochondria through ligation of the death receptor and activation of a series of intracellular proteins (21). With the identification of necroptosis-associated proteins, a few small molecules that may specifically modulate necroptosis have been identified via high-throughput screening (23). Necrostatin-1 (Nec-1) and necrosulfonamide are specific inhibitors of RIP1 and MLKL, respectively (23,24). These are valuable chemical probes to confirm the presence of necroptotic cell death and to elucidate the underlying molecular mechanisms. Extensive identification of specific necroptotic proteins with the development of specific small molecules may provide data to fill the gaps in these signaling pathways.

2. Physiological roles and pathophysiological conditions associated with necroptosis

Originally, necroptosis was regarded as an alternative cell death modality to apoptosis upon TNFα stimulation. Its activation and subsequent release of DAMPs may not only serve a physiological function, but also cause a wide range of diseases (Table II). Necroptotic cell death is distinctive from necrosis in the sense that the cells actively respond to death stresses, and is also hypothesized to be a method of cell demise when apoptosis is compromised. Genome-wide small interfering RNA analysis demonstrated that a set of 432 genes were enriched in the immune and nervous systems, and that the cellular response to necroptosis was affected by a signaling network relevant to innate immunity (13). At present, necroptosis is proposed to be the dominant cell death program when apoptosis is inhibited (25). Generally, apoptosis via the intrinsic or extrinsic pathways has been regarded as the primary mechanism for the contraction phase of T cell immunity and the elimination of autoreactive T cells. However, necroptosis serves a key function in maintaining T cell homeostasis with defective B-cell lymphoma-2-like protein 11, which is a crucial effector in the negative selection of autoreactive thymocytes, highlighting that caspase inactivation leads to induction of necroptosis (26). In addition, the death of host cells through necroptosis contributes to the first defense mechanism against infectious pathogens that may suppress or evade apoptotic surveillance. In fact, cells infected with viruses may be removed by apoptotic cell death or the immune response. When a virus disarms the apoptotic machinery of host cells for the proliferation of its progeny, necroptosis may be induced as an alternative form of cell death to inhibit virus propagation (27). In addition, RIP3-mediated necroptosis provides a secondary process to clear pathogens through the induction of inflammation (12). Intracellular pathogens, including *Mycobacterium tuberculosis* and *Salmonella typhimurium*, transduce type I interferon signaling to kill macrophages via the induction of necroptosis (28,29). As demonstrated by the dynamic functions of necroptosis during viral or bacterial infection, the excess of intracellular molecules from cells undergoing necrosis or necroptosis leads to a pro-inflammatory response, which may provoke the immune system to fight against pathogens.

Conversely, excessive necroptosis in peripheral tissues and ischemia reperfusion injury may cause an inflammatory signal that leads to detrimental consequences, which may result from the release of DAMPs from necroptotic cells into the extracellular compartment. In a previous study, 33 out of 432 genes identified were proposed to be implicated in human diseases, including Huntington's disease, although the associations between necroptosis-regulating genes and human diseases have remained elusive (13). There is a growing body of evidence suggesting that necroptosis is associated with pathological conditions including acute pancreatitis, retinal detachment, renal ischemic reperfusion injury, myocardial infarction and traumatic brain injury (Table II) (12,26-36). Necroptotic cell death was identified in cerulein-induced acute pancreatitis, in which RIP3 overexpression was induced in the pancreas but not in other areas (30). In addition, necroptosis of Paneth cells in the terminal ileum was revealed to be associated with the pathogenesis of inflammatory bowel disease (31). There is also marked RIP3 expression in patients with inflammatory disorders (31). In addition, necroptotic cell death is actively induced in photoreceptor cell loss and acute kidney injury (32,33). A notable previous study suggested that the inhibition of necroptosis was protective against

Table I. Key features discriminating apoptosis and necroptosis.

Feature or characteristic	Apoptosis	Necroptosis
Cell & organelles morphology	Shrinkage	Swelling
Membrane integrity	Intact	Disintegrated
DNA ladder fragmentation	Yes	No
Signaling pathway	Intrinsic and extrinsic routes	RIP1/RIP3/MLKL/PGAM5
Molecular complex	Apoptosome	Necrosome
Biological markers	Caspase, Poly (ADP-ribose) polymerase	High mobility group B1
Physiological significance	Clearance of dead cells	Inflammation and innate immunity

RIP, receptor-interacting protein kinase; MLKL, mixed lineage kinase domain-like; PGAM5, phosphoglycerate mutase family member 5.

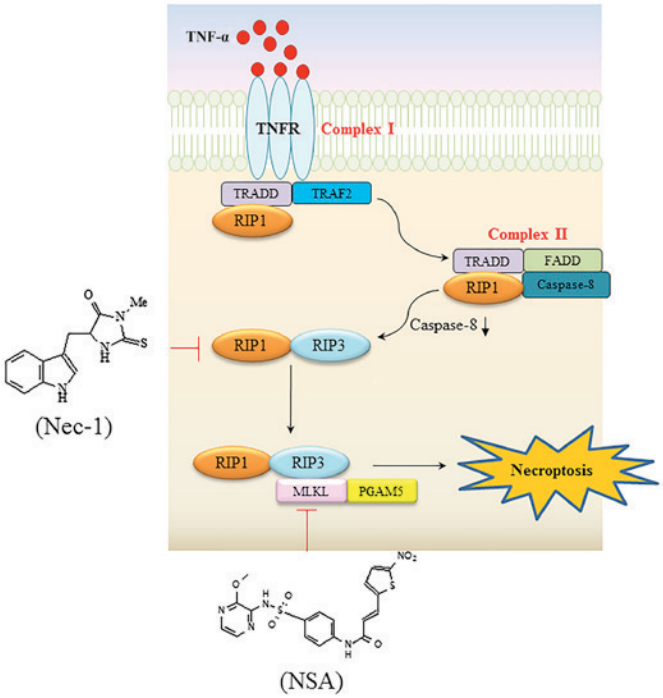

Figure 1. Signaling pathway resulting in TNFα-mediated necroptosis, and specific necroptotic inhibitors targeting RIP1 and MLKL. Upon TNFα binding to its cognate receptor, TRADD, TRAF2 and RIP1 are recruited to form complex I. In the second step, bound proteins dissociate from the receptor when TNFR is engulfed into the cytosol. In turn, TRADD and RIP1 are bound to FADD and caspase 8, eventually forming the cytoplasmic complex II. In situations where caspase is compromised, RIP1 interacts with RIP3 to trigger consecutive downstream signaling events, including the recruitment of MLKL and PGAM5, which transmit cytosolic death signals to the mitochondria. Nec-1 and NSA inhibit RIP1 and MLKL, respectively, with high specificity. TNFα, tumor necrosis factor α; RIP, receptor-interacting protein kinase; MLKL, mixed lineage kinase domain-like; TRADD, TNF receptor 1-associated death domain protein; TRAF2, TNK receptor-associated factor 2; TNFR, TNFα receptor; FADD, Fas-associated death domain; PGAM5, phosphoglycerate mutase family member 5; Nec-1, necrostatin-1; NSA, necrosulfonamide.

liver-associated disease conditions (34). Acetaminophen may induce RIP3 expression along with elevated levels of alanine aminotransferase (ALT) in mice, leading to extensive necrosis. Wild type mice subjected to morpholino antisense targeting RIP3, or RIP3-deficient mice, are protected against acetaminophen-induced liver damage (34). The activation of necroptosis has previously been demonstrated to be a prerequisite for the pathogenesis of multiple sclerosis (35). Kitur *et al* (36) revealed that the toxin derived from *Staphylococcus aureus* caused necroptosis-associated lung damage.

3. Therapeutic exploitation of necroptosis

As described above, necroptosis is a specialized cell death mode that is does not occur in normal homeostasis. It may be induced by external stresses in conjunction with specific circumstances involving the absence of caspase. Necroptosis has previously been documented in multiple diseases, including ischemic brain injury and degenerative diseases (10). In addition, it becomes an alternative cell death mechanism in multicellular organisms when cells are infected with pathogens that are able to evade the apoptotic machinery of the host. This process is associated with the innate immune response, and may be the first line of defense against pathogens, including viruses and bacteria. Understanding the molecular mechanisms by which necroptosis may be activated is of significance for the implementation of a protective strategy against microbial infection. As well as the defensive function of necroptosis in the host, attention has been paid to harnessing alternative cell death pathways to fight tumor cells with acquired resistance to cancer drugs. Along with apoptosis, necroptosis is a promising cell death process for sensitizing tumor cells to anticancer drugs, and its induction is expected to be a therapeutic tool for killing tumor cells, particularly apoptosis-resistant types of cancer. Cancer cells may evolve to multiply by evading chemotherapy-induced apoptosis, whilst remaining inherently susceptible to necroptosis (37). Therefore, exploitation of the induction of necroptosis may be a secondary therapy to counteract types of cancer resistant to apoptosis. The potential induction of necroptosis for cancer therapy has been paradoxically encouraged by the fact that necroptosis is impaired during tumorigenesis (38). Chronic lymphocytic leukemia cells exhibit defects in necroptosis regulators, including RIP3 and CYLD, an enzyme that may regulate RIP1 ubiquitination (39). RIP3 polymorphisms in non-Hodgkin lymphoma have been demonstrated to be correlated with tumor progression (40). Therapeutically, necroptosis should be induced or suppressed for anticancer therapy or the prevention of necroptosis-associated pathological diseases, respectively. For a more selective induction of necroptosis,

Table II. Physiological and pathophysiological significance of necroptosis.

Physiological or pathophysiological condition	Target or pathway	Consequences	Comments	(Refs.)
Viral infection	RIP3-dependent pathway, RIP1/RIP3 complex	Virus clearance	Vaccinia virus	(12,27)
Immune homeostasis	Inactivation of caspase-8 in B-cell lymphoma 2 interacting mediator of cell death$^{-/-}$	T cell homeostasis	Suppression of autoimmunity	(26)
Bacterial infection	Type I interferons-mediated RIP-dependent necroptosis in macrophages	Protection against infection	*Mycobacterium tuberculosis, Salmonella enterica* serovar *typhimurium*	(28,29)
Acute pancreatitis	RIP3	DAMPs-provoked inflammation	DAMPs emission	(30)
Inflammatory bowel disease	RIP3	Crohn's disease	Caspase 8 deficiency in intestine epithelium cells	(31)
Retinal degeneration	RIP3	Photoreceptor cell loss	Caspase 8 inhibition with retinal detachment	(32)
Acute kidney injury	RIP1/RIP3	Acute tubular necrosis	Poly adenosine diphosphate ribose polymerase-calpain axis or mitochondrial permeability transition pathway involved	(33)
Hepatocyte necrosis	RIP3	APAP-induced liver injury	RIP3: an early mediator of APAP hepatotoxicity	(34)
Neurodegenerative disease	RIP1/RIP3 insoluble aggregates	Multiple sclerosis	Tumor necrosis factor.-mediated oligodendrocyte loss	(35)
Staphylococcus aureus-induced necroptosis	Necroptosome complex	Inflammatory necrotizing pneumonia	Pore-formation and inflammasome activation by multiple *S. aureus* toxins	(36)

RIP, receptor-interacting protein kinase; DAMPs, damage-associated molecular patterns.

the construction of signaling pathways connecting necroptosis proteins and the development of small molecules that may target necroptosis regulators are required. However, this becomes difficult when considering the interplay between apoptosis, necrosis and autophagy-associated networks. Accordingly, the intricate molecular crosstalk between death modalities should be fully understood prior to the clinical application of necroptosis.

There are a number of feasible examples in which the induction of necroptosis may be applied as a cancer treatment. For example, a number of incurable cancers, including lung cancer, have evolved to evade or interfere with apoptotic machinery when challenged with repetitive treatment with anticancer drugs (41). Accordingly, as an alternative cell death pathway, necroptosis may be exploited to control cancer cells with acquired anticancer drug resistance. The combined treatment of a caspase inhibitor and an antagonist of inhibitor of apoptosis proteins triggers TNFα-induced necroptosis in various apoptosis-resistant cell lines and patient xenografts (42). Ovarian cancer cells undergoing necroptosis exhibit the formation of a necrosome-like complex with RIP1 (42). This suggests that it is feasible to target the necroptotic signaling pathway identified in ovarian cancer cells in a therapeutic setting. In addition, Table III summarizes the synthetic small molecules or natural products that may trigger necroptotic cell death in cancer cells. Obatoclax-bearing indole bipyrrole moiety is able to induce necroptosis via the formation of the necrosome on the autophagosomal membrane (43). Staurosporin, a protein kinase inhibitor, and B12536, which targets mitotic kinase pololike kinase 1, have been suggested to induce necroptosis (44,45). A synthetic naftopidil analogue, HUHS1015, kills human gastric cancer cells via the induction of necroptosis and caspase-independent apoptosis (46). A amiloride derivative, 5'-betaenzylglycinyl-amiloride, was identified to be an inducer of caspase-independent necroptosis in glioma cells (47). The Chinese medicine shikonin promotes necroptotic cell death of glioma cells in a RIP1-dependent manner (48). Shikonin has also been suggested to exert antitumor effects on osteosarcoma by inducing RIP1- and RIP3-dependent necroptosis (49). The use of an additional natural compound, honokiol, in combination with chemotherapeutic agents synergistically kills drug-resistant cell lines via apoptosis and necroptosis (50). As well as chemotherapy, photodynamic therapy using a photosensitizer talaporfin sodium has been indicated to mediate necroptotic cell death in glioblastoma T98G cells via a signaling pathway consisting of RIP1, RIP3 and MLKL (51). In addition, radiotherapy was performed to induce necroptosis in anaplastic thyroid and adrenocortical cancers (52). Specifically, Nec-1 and zVAD effectively protect cells from radiotherapy, indicating that necroptosis is partly involved in radiation-induced cell death.

4. Perspectives of necroptosis

As aforementioned, necroptosis was initially considered a secondary cell death pathway to TNFα-induced apoptosis under a caspase-deficient condition. At present, it is hypothesized that necroptosis may be triggered to evoke physiological and pathological consequences to diverse stimuli, although its regulatory mechanism remains unknown. From physiological and pathological aspects, its activation may be beneficial or harmful depending on the stimulus context, and on the cell-specific responses to it. Therefore, the induction of necroptosis may not only provide a secondary safety mechanism against pathogenic infection, but may also be associated with various diseases: Apart from innate immune surveillance, an increasing number of diseases associated with necroptosis, including tissue inflammation and degeneration, have been identified. Necroptosis has been demonstrated to be involved in various neurological disorders, including trauma, strokes, multiple sclerosis and Huntington's disease (53). In addition, the conversion of cholesterol to 24(S)-hydroxycholesterol and its consequent passage through blood-brain barrier is hypothesized to induce necroptosis in neuronal cells that are caspase-8-defective (54). Therefore, protection against necroptotic cell death is of primary concern to prevent the pathogenesis of these diseases. Genetic or pharmacological interference with necroptosis signaling results in neuroprotection against ischemic heart or brain injury (53,55). RIP3 deficiency or administration of Nec-1 has been demonstrated to exhibit protective effects on necroptosis-based heart or brain damage (55,56). Apart from RIP3, additional potent target proteins have been identified as regulators of necroptotic cell death, including RIP1, MLKL, PGAM5 and CYLD (17,21,23,57), which comprise a cascade of signaling pathways for necroptosis (Fig. 1). Subsequently, a small number of inhibitors targeting RIP1 or MLKL have been developed to effectively protect against necroptotic cell death (23,57). Hence, additional identification and validation of more potential targets will be crucial for the development of drugs that may improve pathological conditions.

Beyond necroptosis-associated pathological consequences, necroptosis may be exploited as an alternative therapy to overcome drug-resistant types of cancer. It is based on the hypothesis that a failure in cancer management may be caused by the acquired ability of cancer cells to evade cancer drug-induced apoptosis. Therefore, inducing necroptosis may kill cancer cells and improve immune responses to the danger molecules derived from dying cells, although the release of intracellular contents from dying cells may also promote neoplasia. Although not described in the present review, autophagy complicates the processes of cell death or survival depending on the cell types or the context of the stress. For example, exposure to Obatoclax in rhabdomyosarcoma results in substantial autophagy, which in turn causes RIP3 activation and then necroptotic cell death (43). Notably, caspase-8 does not inhibit RIP3 activation in the autophagosome-driven necroptotic process, unlike receptor-mediated necroptosis (43). In this situation, Obatoclax mediates necroptosis by forming necrosome complexes on autophagosomes. These data highlight that understanding the crosstalk between necroptosis and other cell death types is a prerequisite for selecting optimal treatments customized to specific types of cancer and necroptosis-associated diseases. In conclusion, the comprehensive regulation of cell death is expected to provide clinical opportunities to use cell death programs to treat various diseases, including different types of cancer and degenerative disorders.

Table III. Use of necroptosis for anticancer therapy.

Treatment	Target or pathway	Cancer type	(Refs.)
Combination treatment			
zVAD+SMAC memetics	RIP3-dependent	Triggering necroptosis in apoptosis-resistant ovarian carcinoma	(42)
Chemotherapy			
Obatoclax (GX15-070)	Necrosome complex	Inducing non-apoptotic form of cell death in rhabdomyosarcoma cells	(43)
Staurosporin	Nonspecific kinase inhibitor	Necroptotic cell death in caspase-compromised U937	(44)
B12536	Protein kinase Plk1	Plk1 leads to necroptosis in androgen-insensitive prostate cancer cell	(45)
HUHS1015	AMID accumulation	Human gastric cancer cells due to AMID accumulation in the nucleus	(46)
5'-betaenzylglycinyl-amiloride	AIF	AIF-mediated malignant glioma cells	(47)
Shikonin	RIP1, oxidative stress	Glioma cells primarily via necroptosis	(48)
	Induction of RIP1 and RIP3	Osteosarcoma	(49)
Honokiol	Enhanced apoptosis/additional necroptosis	Multidrug resistance breast cancer cells	(50)
Photodynamic therapy			
Using talaporfin sodium	Necroptosis pathway	Glioblastoma T98G	(51)
Radiation-induced cell death			
6Gy	RIP1 dependent	Anaplastic thyroid and adrenocortical cancer	(52)

RIP1, receptor-interacting protein kinase; Plk1, polo-like kinase 1; AMID, mitochondrion-associated inducer of death; AIF, apoptosis-inducing factor.

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