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Invited Mini Review

The role of necroptosis in the treatment of diseases

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Necroptosis is an emerging form of programmed cell death occurring via active and well-regulated necrosis, distinct from apoptosis morphologically, and biochemically. Necroptosis is mainly unmasked when apoptosis is compromised in response to tumor necrosis factor alpha. Unlike apoptotic cells, which are cleared by macrophages or neighboring cells, necrotic cells release danger signals, triggering inflammation, and exacerbating tissue damage. Evidence increasingly suggests that programmed necrosis is not only associated with pathophysiology of disease, but also induces innate immune response to viral infection. Therefore, necroptotic cell death plays both physiological and pathological roles. Physiologically, necroptosis induce an innate immune response as well as premature assembly of viral particles in cells infected with virus that abrogates host apoptotic machinery. On the other hand, necroptosis per se is detrimental, causing various diseases such as sepsis, neurodegenerative diseases and ischemic reperfusion injury. This review discusses the signaling pathways leading to necroptosis, associated necroptotic proteins with target-specific inhibitors and diseases involved. Several studies currently focus on protective approaches to inhibiting necroptotic cell death. In cancer biology, however, anticancer drug resistance severely hampers the efficacy of chemotherapy based on apoptosis. Pharmacological switch of cell death finds therapeutic application in drug- resistant cancers. Therefore, the possible clinical role of necroptosis in cancer control will be discussed in brief. [BMB Reports 2018; 51(5): 219-224]

INTRODUCTION

Cell survival is constantly in dynamic equilibrium with cell death for tissue homeostasis. Aside from cell survival, cell death occurs in various modes such as programmed or random mechanisms upon exposure to various stresses. Programmed cell death or apoptosis is activated via intrinsic or extrinsic pathways in response to death stimuli. Both pathways involve a series of signaling molecules with activated caspases and is termed caspase-dependent cell death. Meanwhile, necroptosis is classified as type III programmed cell death with apoptotic and necrotic features. It is also known as programmed necrosis or caspase-independent cell death according to morphological or molecular features. Initially, necroptosis was considered as undesirable passive cell death. However, it is currently considered as specialized cell death since it is orchestrated under a caspase-compromised condition. Similar to apoptosis, necroptosis is executed via distinctive signaling mechanism comprising a cascade of specified proteins, resulting in regulated necrotic cell death. Despite unknown mechanisms and pathological significance compared with apoptosis, the discovery of pharmacological inhibitors targeting necroptosis has been extensively pursued. Here, we introduce the concept of necroptotic cell death induced under various pathophysiological conditions and delineate the pathological mechanisms unmasking necroptosis in a well-orchestrated fashion. In this review, necroptosis-associated diseases and the underlying pathogenesis will be discussed.

SIGNALING PATHWAYS LEADING TO NECROPTOSIS INDUCTION AND BIOCHEMICAL CHANGES

Typically, necroptosis occurs in cells exposed to extrinsic stimuli such as TNFα, FASL, and TRAIL in combination with compromised caspase-8 (C-8). C-8 inhibition is induced by genetic defects, viral proteins, and treatment with a pan-caspase inhibitor zVAD (1-3). Necroptosis-regulating proteins have been identified and biologically validated comprehensively. Genome-wide siRNA screening yielded a few candidate genes associated with necroptosis (4). In addition to RIP1 as a necroptosis regulator, RIP3, mixed lineage kinase domain-like protein (MLKL) and PGAM5 were identified (5-8). The signaling pathway leading to necroptosis is illustrated in Fig. 1. TNFα ligation to its cognate receptor triggers caspase-dependent cell death as a default cell death mode. Caspase-compromised conditions drive cell death via necroptosis with accompanying RIP1 activation (9). Upon stimulation, RIP1 interacts with RIP3 leading to the formation of a necrosome complex via RIP homotypic interaction motif (RHIM) within cells (10). MLKL pseudokinase was identified as

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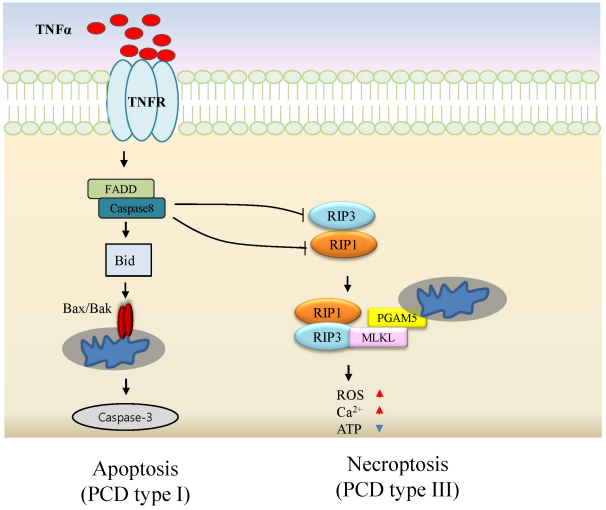

Fig. 1. Necroptosis regulators and signaling pathways in necroptosis activation. Upon TNF receptor ligation, a signal transduction mediated via RIP1, caspase activation, and tBID cleavage occurs, resulting in apoptotic cell death. Active caspase-8 inhibits necroptosis via cleavage of RIP1 and RIP3. Inhibition of caspase leads to the formation of a physical complex of RIP1 with RIP3 to trigger downstream signaling events including MLKL and PGAM5 recruitment, and transduction of cytosolic death signals to mitochondria, resulting in necroptosis.

a substrate of RIP3 under necroptotic condition. Phosphorylated RIP3 induces MLKL phosphorylation at serine or threonine sites. The RIP3-MLKL complex translocates to mitochondria-associated membrane mediated via phosphoglycerate mutase 5 (PGAM5), which is a necrosome-associated protein regulating dynamic-related protein (Drp1) in turn (8). Finally, Drp1 induces mitochondrial fragmentation, a crucial event for necroptosis (11).

Biochemical changes during the early stages of necroptosis include ATP depletion, ROS generation, calcium overload, and loss of mitochondrial permeability transition (12). At the cellular level, necroptosis is characterized by loss of plasma membrane integrity as well as organelle swelling, finally leading to cellular collapse (10). Notably, necroptotic cells release various damage-associated molecular patterns (DAMPs) such as HMGB1, cytokines and histones into extracellular media. Particularly, a disulfide form of HMGB1 by oxidation contributes to the inflammatory response (13). HMGB1 binds to a cognate receptor on endothelial cells and macrophages to transduce cellular responses such as release of proinflammatory cytokines and chemotactic cell migration (14).

NECROPTOSIS: TARGET PROTEINS AND INHIBITORS

As described above, a series of necroptosis-associated proteins were identified and further validated as targets of necroptosis. Subsequently, a few small molecules were successfully discovered, specifically targeting necroptosis proteins. Target-specific small molecules that can modulate necroptosis are listed in Table 1.

Table 1. Target proteins associated with necroptosis and their specific inhibitors

Necroptosis Target proteins	Inhibitors	Mode of action/function
RIP-1	Nec-1	Allosteric inhibitor
	Furo[2,3-d]pyrimidine derivatives	SAR reported
	GSK'963	>10000-fold selective for RIP1
RIP-3	GSK'840, GSK'843, GSK'872	Caspase activity activated
MLKL	Necrosulfonamide	Covalent binding
	TC13172	Covalent binding (nanomolar potency)
	GW806742X	Binds to pseudokinase domain (ATP-mimetic)
Others	Ponatinib & pazopanib	Interfere with signaling proteins upstream of MLKL
	Sorafenib	Target unknown
	TPCK	UCH-L1 activator
	Hydroxyanisole	ROS scavenger
	Diphenyleneiodonium (DPI)	ROS suppression

RIP-1 inhibitors

Necrostatin-1 (Nec-1), 5-(1H-indol-3-ylmethyl)-2-thiohydantoin 1, was first discovered by screening of necroptosis inhibitors and later identified as an allosteric inhibitor of RIP1 via stabilization of an inactive conformation of the kinase domain (KD) (15). It has been widely validated in various necroptosis-associated animal models (16-19). Accordingly, Nec-1 plays a crucial role in prevention or alleviation of necroptotic damage caused by various stimuli via targeting RIP1. Another potent Nec-1 derivative Nec-1s (7-Cl-O-Nec-1) showed greater specificity to RIP1 compared with other kinases. Other inhibitors have been developed as RIP1 inhibitors, including furo[2,3-d]pyrimidines and GSK'963 (20, 21).

RIP3 inhibitors

Recent studies proposed that necroptosis is mediated in a RIP1- or RIP3-dependent manner (22, 23). Unlike RIP1, RIP3 is essential for necroptosis, but not apoptosis, and targeting RIP3 appears to be more specific in controlling necroptosis. Interestingly, silencing of RIP3 substantially protected cells and tissues against necroptosis (24). Small molecules such as GSK'840, GSK'843 and GSK'872 have been reported to suppress RIP3-dependent necroptosis (25). Mechanistically, these molecules form a complex with RIP3 to restore caspase

activity. Consequently, RIP3 inhibitors potentially protect cells from diverse stimuli than RIP1 inhibitors.

MLKL inhibitors

Necrosulfonamide ((E)-N-(4-(N-(3-methoxypyrazin-2-yl) sulfamoyl) phenyl)-3-(5-nitrothiophene-2-yl) acrylamide, NAS) protects cells against TNF-induced necroptosis via covalent modification of a cysteine in MLKL (7). A compound TC13172 is an MLKL inhibitor with single nanomolar potency. It induced covalent binding at Cys-86 of MLKL (26). In addition, GW806742X targets the pseudo-kinase domain of MLKL with nanomolar inhibitory activity, protecting against necroptosis, although it shows off-target effects against other kinases (26).

Additional agents have been developed as inhibitors of necroptosis acting directly via targets that have yet to be designated. Ponatinib and pazopanib (27) are two anti-cancer agents identified from a representative panel of United States Food and Drug Administration (FDA)-approved drugs through phenotypic screening. Both drugs abrogate phosphorylation of MLKL during TNFα-induced necroptosis, suggesting interference with the signaling proteins upstream of MLKL. However, they do not rescue cells from apoptosis. Interestingly, ponatinib inhibits both RIP1 and RIP3 while pazopanib acts against preferential inhibitor of RIP1. Further, sorafenib is a multi-targeting kinase inhibitor with potent inhibitory activity against B-RAK, and is widely used for the treatment of acute leukemia. It blocks signaling target proteins upstream of MLKL such as RIP1 and RIP3, although its real target remains elusive (28). Therefore, sorafenib can be harnessed for fine-tuning of necroptosis-inducing agents.

TPCK

N-tosyl-l-phenylalanine-chloromethyl ketone (TPCK), a serine protease inhibitor, protects cells from TNF-mediated necroptosis. Using a TPCK probe, HtrA2/Omi has been identified as a target and it acts as an activator of ubiquitin C-terminal hydrolase (UCH-L1) resulting in necroptosis (29).

ROS scavengers

Reactive oxygen species (ROS) play a role in diverse signaling pathways, due to high reactivity with biomolecules such as proteins, DNA and lipids. Upon exposure of L929 cells to TNFα, necroptotic signaling generates ROS via mitochondrial transport chain but not cytosolic enzyme (30), which was strongly supported by reports suggesting that butylated hydroxyanisole blocks ROS accumulation and cell death (31). An NADPH oxidase inhibitor diphenyleneiodonium (DPI) protects renal tubular epithelial cells against necroptosis by blocking ROS generation (32).

PHYSIOLOGICAL AND PATHOLOGICAL SIGNIFICANCE OF NECROPTOSIS

Necroptosis was originally considered as undesirable cell death upon exposure to stimuli, inducing tissue damage. Furthermore, it is the alternative cell death induced under conditions of defective apoptosis. However, growing evidence suggests that necroptosis is mediated via an orchestrated and specialized pathway (33-35). Well-regulated cell death occurs in a range of biological phenomena such as development, immunology and differentiation. Further, extrinsic apoptosis and necroptosis contribute to host defense mechanism against microbial infection. Viruses such as adenoviruses, poxviruses, and herpes viruses evade host apoptosis machinery, and perpetuate if host cells only execute caspase-dependent default cell death (36-38). For instance, vaccinia viruses encode a caspase 8 inhibitor to block apoptotic cell death upon infection. Under this caspase-compromised condition, cells are committed to alternative necroptosis (6). The resulting necroptosis is vital to provoke innate immune response by killing virus-infected cells and releasing danger signals from host cells into external milieu. Furthermore, necroptosis in T cells regulates antigen-activated T-cell proliferation and survival. Caspase-8 negatively regulates necroptosis, promoting survival of activated T cells under physiological conditions. In mice lacking caspase-8, T cells fail to show immune response when infected with murine hepatitis virus (39).

Pathologically, evidence suggests that necroptotic cell death leads to various diseases. Sepsis is mainly caused by Gram-negative bacteria, which release endotoxin that elicits systemic inflammation via release of TNFα and IL-1 inflammatory cytokines. Necroptosis occurs in ischemia-reperfusion (I/R) injury and neurodegenerative diseases. During restoration of blood flow into tissues, tissue damage occurs with severe neutrophil infiltration and cytokine production. Furthermore, necroptosis is involved in traumatic brain and spinal cord injuries (19, 40). Excitotoxicity, Huntington's disease and retinal degeneration are closely associated with necroptosis. Exposure to a specific RIP inhibitor Nec-1 effectively protects cells from necroptosis. Nec-1 protects hippocampal HT-22 cells against glutamate-induced oxytosis (41). Inhibition of RIP1 kinase or RIP3 silencing significantly rescues necroptotic cell death (42). Furthermore, Nec-1 reduces or delays necroptotic damage in transgenic mice expressing mutant Huntingtin protein, astrocytes from amytrophic lateral sclerosis, and retinal pigment epithelium (42-44). Interestingly, microbial infection induces necroptosis in host cells. Microbial proliferation occurs by circumventing default programmed cell death in the host. Necroptosis is induced by viral infections such as vaccinia virus (VV). VV expresses an inhibitor of caspase-1 and -8, diverting the host response toward necroptosis in a RIP3-dependent pathway (45). Further, Sendai virus induces necroptosis in neuroblastoma cells (17). Macrophage infected with *S. typhimurium* induces necroptotic cell death in a RIP1- and RIP3-dependent manner.

PERSPECTIVES OF NECROPTOSIS AND CONCLUSIONS

Necroptosis has been recognized as an alternative to apoptosis when cells are exposed to various stimuli under specific conditions.

As mentioned above, it induces a variety of pathological conditions including septic shock, acute pancreatitis and neuronal degeneration. Apoptotic death of damaged cells is removed by phagocytosis in macrophages or neighboring cells. Conversely, because loss of membrane integrity occurs in cells undergoing necroptosis, intracellular substances including heat-shock proteins and HMGB1 are released into extracellular media to provoke inflammation and immune responses.

In response to infectious viruses or intracellular bacterial pathogens escaping apoptotic cell death, host cells actively switch to necroptosis, causing premature assembly of virus particles or bacteria progeny and release of critical components triggering an immune response (46-48).

Similarly, necroptosis was demonstrated under conditions of chronic sterile inflammation such as alcoholic-induced liver injury and atherosclerosis (18, 49). Under these pathological conditions, necroptotic cells release DAMPS to trigger sterile inflammation, via unknown mechanism.

A potent and specific regulation of necroptosis is therefore, needed. Indeed, pharmacological blockade of necroptosis is of primary concern in the treatment of various diseases. Further studies will be extensively undertaken to identify the target molecules mediating the signal transduction leading to necroptosis and facilitate the discovery of mechanism-based inhibitors.

On the other hand, necroptosis induction can be actively harnessed via molecular switch or unmasking. Generally, cancer cells grow in an uncontrolled manner and further acquire mechanisms to evade cell death intrinsically or extrinsically. Chemotherapy or radiotherapy is mainly based on apoptosis through caspase activation. However, many cancers have developed strategies to disarm apoptotic machinery, including dysregulated apoptosis, activation of pro-survival signaling pathways and upregulation of drug transporters. Altered apoptosis contributes to drug resistance of cancer during chemotherapy. To overcome drug-resistant cancers, alternative cell death mechanisms such as necroptosis or autophagy can be considered. In previous reports, cancer cells that are refractory to apoptotic agents were shown to succumb to necroptosis-inducing agents (50). It is conceivable that resistance to apoptosis can be overcome by necroptosis, because necroptotic pathway is distinct from apoptotic mechanisms. Furthermore, several reports suggest that necroptosis contributes to suppression of tumorigenesis. Particularly, mutations in the *CYLD* gene aggressively facilitate carcinoma via upregulation of angiogenic factors (51). Further, *RIP3* gene polymorphisms occur in non-Hodgkin's lymphoma and levels of a spliced variant RIP3-γ are relatively high in colon and lung cancers (52, 53).

Practically, a few inducers of necroptosis have been reported to trigger necroptotic cell death in malignant cancers although their targets remain to be identified. Caspase activation is a prerequisite for apoptosis induction, and apoptotic cell death fails if caspases are blocked or compromised by unknown mechanisms. Under those conditions, cells activate necroptosis in response to cell death stimuli. In fact, a natural product shikonin induces necroptotic cell death in MCF-7 breast cancer cells that express Bcl-2 or Bcl-xL, which acquire multidrug resistance. Obatoclax, an antagonist of Bcl-2 family members, promotes necroptosis based on autophagy in acute lymphoblastic leukemia resistant to glucocorticoids. In addition, combined treatment of pancaspase inhibitors with 5-fluorouracil drives necroptosis in colorectal cancer (54). Furthermore, necroptotic cells trigger adaptive immunity in dendritic cells (DCs), which in turn activate $CD8^+$ T cells for antitumor immunity (55).

However, necroptosis-based cancer therapy still remains elusive. It has been demonstrated that necroptosis in tumor microenvironment contributes to inflammation and cancer metastasis (16). Furthermore, the low expression of key modulators of necroptosis in specific cancers fails to induce necroptosis (56-58), resulting in cancer evasion.

In conclusion, suppression or enhancement of necroptosis is therapeutically effective in specific diseases. A comprehensive insight into the underlying mechanisms is needed to facilitate the diagnosis, biomarkers, and drug development in necroptosis-associated diseases. Most studies have focused on the identification of specific inhibitors targeting necroptosis, and their underlying mechanisms of regulation. As a result, a few small molecules have been discovered from the chemical library, and optimized for further clinical use.

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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