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Minireview

Molecules and Cells

Physiological Consequences of Programmed Necrosis, an Alternative form of Cell Demise

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Cell death occurs spontaneously or in response to external stimuli, and can be largely subdivided into apoptosis and necrosis by the distinct morphological and biochemical features. Unlike apoptosis, necrosis was recognized as the passive and unwanted cell demise committed in a non-regulated and disorganized manner. However, under specific conditions such as caspase intervention, necrosis has been proposed to be regulated in a well-orchestrated way as a backup mechanism of apoptosis. The term programmed necrosis has been coined to describe such an alternative cell death. Recently, at least some regulators governing programmed necrosis have been identified and demonstrated to be interconnected via a wide network of signal pathways by further extensive studies. There is growing evidence that programmed necrosis is not only associated with pathophysiological diseases, but also provides innate immune response to viral infection. Here, we will introduce recent updates on the molecular mechanism and physiological significance of programmed necrosis.

INTRODUCTION

Cell survival and cell death are the central events in regulating homeostasis and development of metazoan (Hsu et al., 2009). Over the past few decades, intensive research has identified the signaling networks leading to apoptotic cell death (He et al., 2009; Joza et al., 2001; Riedl and Salvesen, 2007; Strasser et al., 2000). By comparison, little attention was devoted to an alternative cell death modality, necrosis, because it was generally thought to occur in an unregulated way as a result of overwhelming external stresses including exposures to radioisotope, UV and toxic chemicals. Recent updates on cell demise have proposed that necrosis is not a random process. This is in part due to the discovery of necrosis modulators such as receptor interacting protein1 (RIP1). Terms such as programmed necrosis, caspase-independent cell death or necroptosis have been used to describe this alternative cell death program (Galluzzi and Kroemer, 2008). Later, RIP1 specific small molecular inhibitor necrostain-1 (Nec-1) was developed and found to inhibit necrotic cell death but not apoptotic process. Moreover, a genome wide analysis demonstrated that necrosis was induced by a wide network of signal pathways and that some core necrotic factors were specifically involved in death processes triggered by tumor necrosis factor alpha (TNFα) (Hitomi et al., 2008). More recently, breakthrough in the search for novel modulators of cell necrosis has been made by 3 different groups with the identification of another necrosis regulator RIP3 through interference RNA screening. Cho et al. and He et al. demonstrate that RIP3 is on the bifurcate branch of the cell death fate mediated by death receptor (DR) such as tumor necrosis factor receptor (TNFR) and that RIP1-RIP3 pro-necrotic complex formation is required to drive necrotic cell death. Another group suggests that RIP3 plays a key role as a energy metabolism regulator, leading to programmed necrosis through the generation of reactive oxygen species (ROS) (Zhang et al., 2009). Diseases such as septic shock, acute pancreatitis, ischemic neuronal disorder and myocardial infarction have been suggested to be associated with programmed necrosis. Therefore, strategies targeting necrosis modulator or pro-necrotic complex (RIP1-RIP3) will be useful to develop the drug for metabolic diseases and neurodegenerative diseases. In this review, we will discuss the molecular mechanisms of programmed necrosis and its physiological function in details.

Differential features between necrosis and apoptosis

Cell death is largely subclassified into apoptosis and necrosis based on the cell morphology and biochemical features. Two different cell deaths show the significant contrast in phenotypes and molecular traits as described in Table 1. Morphologically, apoptosis shows shrinkage in whole cell and organelles while necrotic cell displays a swollen shape without significant changes of organelles. The distinctive morphological features including chromatic condensation and preserved plasma membrane integrity are more prominent in apoptosis. In contrast, cells undergoing necrosis do not display a typical morphology except for the early rupture of plasma membrane. Besides morphological changes, apoptosis and necrosis exhibit noticeable differences in the biochemical and molecular traits. DNA laddering is indicative of cells undergoing apoptosis, but genomic DNA extracted from DNA damage-induced necrotic cells is smeared on agarose gel. Moreover, caspase activation is a prerequisite for executing

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Table 1. Morphological, biochemical and molecular features discriminating apoptosis and necrosis

	Apoptosis	Necrosis
Cell morphology	Shrinkage	Swelling
Membrane integrity	Preservation	Loss
DNA	Fragmentation	Random digestion
Caspase activation	Caspase dependent	No involvement of caspase
RIP1 requirement	Not necessary	Required
Immune system	No inflammatory response	Significant inflammatory response

apoptosis while necrotic protein RIP1 but not caspases is required for programmed necrosis. In terms of physiological effects, the integrity of cell membrane is critical in determining the biological consequences induced by the damaged cells. Cell membrane is well preserved in apoptotic cells, which are cleared through engulfment by immune cells or neighboring cells (Krysko et al., 2006). On the other hand, damaged cells via necrosis release intracellular adjuvants or "danger signals" that stimulate the adaptive immune responses through T cell activation, leading to elimination of impaired cells by inflammation (Eigenbrod et al., 2008).

Necrosis-inducing agents

DNA alkylating agents

The DNA alkylating carcinogen *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) causes DNA damage via alkylation of bases in nucleoside. These damages are recognized and actively repaired by the cell machinery including base excision to preserve the integrity of genetic information in response to chemical exposures. However, overwhelming exposure to MNNG activates poly(ADP-ribose)polymerase-1 (PARP-1), which leads to cell death in a variety of different cell types by sequential signal pathway of RIP1/TRAF2, JNK1 and mitochondrial dysfunction (Xu et al., 2006).

Inorganic compounds

Cadmium (Cd) is an industrial and environmental pollutant. Although it is not involved in enzymatic processes, it affects Zn-dependent cellular functions due to its chemical similarity to Zn (Hartwig et al., 2002). Treatment of Chinese hamster ovary (CHO) with Cd causes a Ca^{2+} dependent necrotic cell death (Yang et al., 2007), and Nec-1 rescues cells from Cd-induced necrotic cell death as well as death receptor (DR)-mediated necrosis (Hsu et al., 2009). It is generally accepted that Nec-1 attenuates TNFα-mediated necrosis via targeting RIP-1 specifically. In Cd-induced necrotic model, Nec-1 did not affect intracellular Ca contents, downstream calpain activity, or reactive oxygen species (ROS) production, but rather counteracted the reduction in mitochondrial membrane potential (MMP). These results suggest that the mitochondria are nec-1 major acting sites. Another example of caspase-independent cell death by inorganic compound was reported in human cervical cancer treated with arsenic trioxide (Kang et al., 2004). As_2O_3 treatment activates ROS-mediated PARP-1, which, in turn, signals apoptosis-inducing factor (AIF) release from mitochondria, eventually leading to caspase-independent cell death. It remains to be discovered whether cell death triggered by As_2O_3 requires RIP-1 engagement and pro-necrotic complex formation as demonstrated in DR-mediated necrosis.

Shikonin

Shikonin is a naphthoquinone derivative found in the Chinese herb Shiunko. Shikonin and its analogues induced cell death with necrotic features that is inhibitable by Nec-1 (Han et al., 2009). In some cell lines such as MCF-7 and HEK293, shikonin drives dominantly programmed necrosis independently of drug concentrations. On the other hand, other cell lines including HL60 and K562 display differential dose responses with a dominant apoptosis at $\leq$ 2.5 μM, a dominant programmed necrosis $\geq$ 10 μM, and a mixed type at 5 μM. Interestingly, Nec-1 treatment reverses necrosis to apoptosis in cells treated with 10 μM shikonin. It supports strongly the notion that apoptosis and necrosis may act reciprocally to commit cells to die. These results also go against the concept that apoptosis can only be diverted to necrosis when caspases and apoptosis are impaired.

Death receptors

Ligation of death ligands such as TNFα, TNF-related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) to their cognate receptors activates the well-orchestrated extrinsic pathways leading to apoptosis (Petit et al., 2003). Treatment of cells with either the protein synthesis inhibitor cycloheximide (CHX) or Smac mimetic potentiates the TNFα-driven formation of an alternative caspase-8 activating complex comprising of RIP1, Fas-associated death domain (FADD) and caspase-8 (He et al., 2009). However, when apoptosis is interrupted, death receptor-mediated cell death is diverted to programmed necrosis.

Molecular mechanism of programmed necrosis

The past decades of research demonstrated that apoptosis can be triggered by extrinsic or intrinsic factors. The apoptotic signal is amplified in the mitochondria by successive signaling pathways and executed by caspases. In contrast, only recently has it become apparent that necrosis is a specialized pathway of well-orchestrated active cell death that requires a specific serine/threonine kinase RIP1 (Galluzzi and Kroemer, 2008). Recent technical advances in cell biology and molecular biology make it possible to dissect these two different cell death modes with distinct biochemical and morphological features. In particular, the mechanism of programmed necrosis by TNFα has garnered much attention. Figure 1 illustrates the dynamic switch between apoptosis and programmed necrosis in response to TNFα. In brief, once cells are stimulated with death ligands such as TNFα or TRAIL, recruitment of downstream adaptor proteins activates NFκB signaling to the survival pathway. The subsequent modification and internalization of membrane-anchored complex I dissociate TNFR-1 from complex I, and a released RIP1 can associate with caspase 8 and FADD to form cytosolic complex II to execute apoptotic cell death (Micheau and Tschopp, 2003). However, when caspase-8 is inactivated, cells are redirected to programmed necrosis via the formation of pronecrotic complex. Recently, three independent research groups have published that a pronecrotic complex between two kinases, RIP1 and RIP3, mediates programmed necrosis. It has been demonstrated that a molecular switch to necrotic cell death facilitates the necrotic complex formation between RIP1

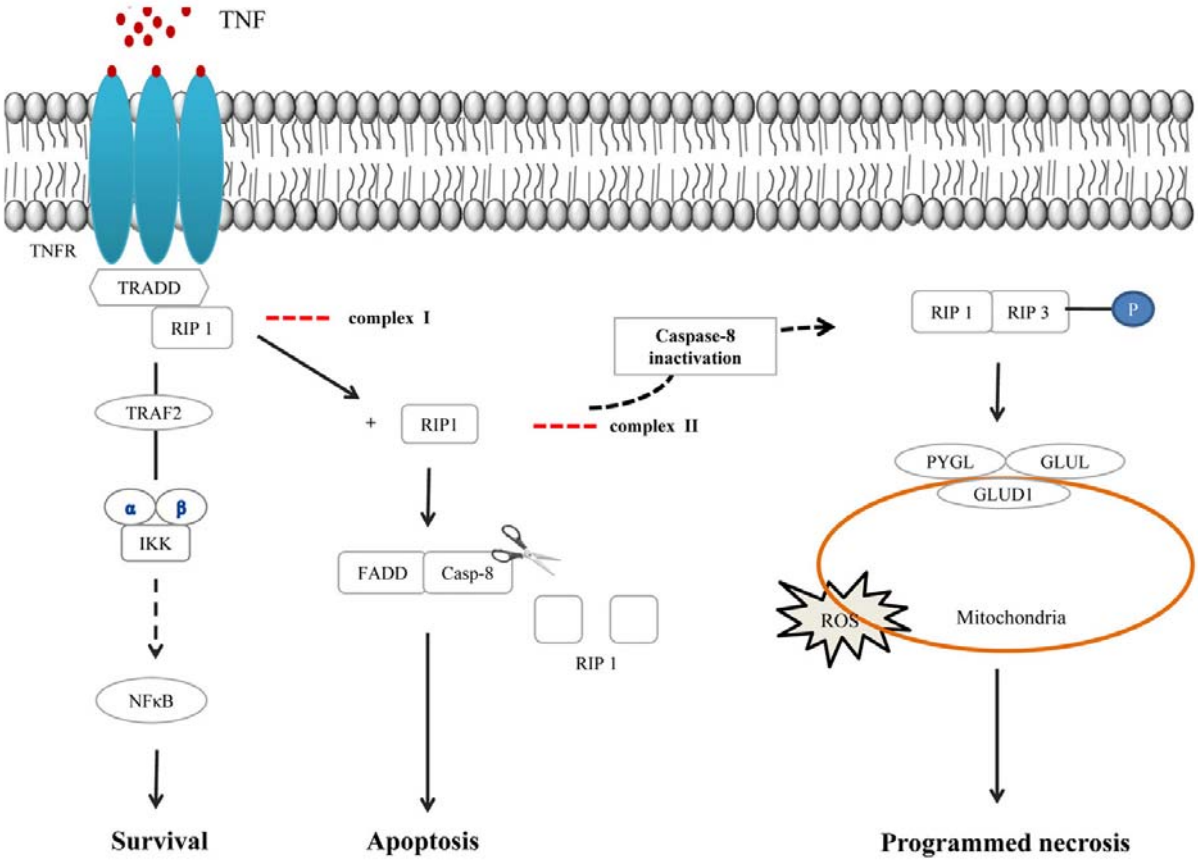

Fig. 1. TNF-mediated signaling pathway of survival, apoptosis and programmed necrosis. Multifaceted TNF treatment leads to cell survival and cell deaths in a tightly regulated way depending on cell types and stimuli contexts. In normal physiological situation, ligation of cells with TNF alone stimulates cell survival rather than cell death via the recruitment of a series of adaptor proteins and consequential NFκB activation. If newly synthesized proteins are blocked by such as CHX and actinomycin D, TNF-mediated cell death termed as apoptosis is mediated through a cascade of caspase activation. Unexpectedly, application of pan caspase inhibitor zVAD to apoptosis-inducing signal redirects cell death modality from apoptosis to programmed necrosis, necroptosis. During the switch of cell demise, pronecrotic complex RIP1-RIP3 interacts with metabolic enzymes PYGL, GLUL and GLUD1 to enhance metabolism accompanied by increased ROS production. From TNF signaling to ROS generation is mediated by RIP3 and is a proposed signal pathway of programmed necrosis linking extracellular activation to mitochondria.

and RIP3 and following mutual phosphorylation within the complex. According to early reports, the crucial significance of ATP was highlighted for determining the cell death modes since apoptosis requires high levels of cellular ATP and necrosis may result from ATP depletion, respectively. However, recent studies suggest that RIP3, but not RIP1, functions as the molecular switch to determine whether cells succumb to apoptosis or necrosis in response to external stimuli. It was supported by the fact that RIP1 drives apoptosis in the absence of RIP3, but that it triggered programmed necrosis only under a condition that RIP3 expression was secured. However, despite such a surprising identification of necrosis modulating protein and partial understanding of molecular mechanism, it still remains elusive whether necrosis is unmasked when essential effectors of apoptosis are impaired or whether necrosis is manifested by newly synthesized proteins and subsequent induction of protein-protein complex formation.

Following death ligand occupation of its corresponding receptor, a series of cytosolic signaling events are transmitted to the mitochondria where membrane permeabilization is differentially altered depending on the cell death modes. Apoptosis and necrosis preferentially cause mitochondrial outer membrane permeabilization (MOMP) and mitochondrial permeability transition (mPT), respectively (Galluzzi and Kroemer, 2008). Both MOMP and mPT are suppressed by anti-apoptotic Bcl-2 proteins. The BH3-only protein Bcl2 modifying factor (Bmf) binds to Bcl-2 and Bcl-xL, and has been implicated in apoptosis. Recently, Bmf was identified as a core component of programmed necrosis (Hitomi, 2008). Although the role of Bmf at the crossroad of apoptosis and necrosis remains to be elucidated, it is tempting to propose that Bmf might promote not only apoptosis by relieving of Bcl-2-mediated suppression of MOMP, but also necrosis by interfering with Bcl-2-mediated blockade of mPT.

In necrosis-prone cells, TNF-activated RIP3 can associate with metabolic enzymes including glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL) and glutamate dehydrogenase 1 (GLUD1) (Zhang et al., 2009). PYGL and GLUL are cytosolic enzymes that catalyze degradation of glycogen and the condensation of glutamate and ammonia to glutamine, respectively. GLUD1 is a mitochondrial matrix enzyme that converts glutamate to α-ketoglutarate. The resulting products glucose-1-phosphate and glutamine are mobilized into mitochondria Krebs cycle to produce ATP production by oxidative phosphorylation. Under necrosis condition, accumulation and enhanced metabolism of energy sources might cause

ROS production, which partly determines the switching of apoptosis and necrosis. Therefore, RIP3 may act as a mediator connecting from TNF-TNFR binding on cytoplasmic membrane to metabolic regulator in mitochondria matrix via activation of cytosolic metabolic enzymes. These results shed light on the new paradigm that programmed necrosis is an active cellular response to death ligands that is driven by the RIP3 kinase.

Identification of programmed necrosis modulating proteins

PARP-1 is a nuclear enzyme that catalyzes covalent linkage of long branched chains of poly (ADP-ribose) (PAR) to a variety of nuclear DNA binding proteins including PARP-1 itself. It plays a role in facilitating DNA repair in response to genomic DNA damage (de Murcia et al., 1994; Lautier et al., 1993). Also, it has been implicated in mediating necrosis through a series of events including ROS generation and ATP depletion when cells were inflicted with massive DNA damage. Another necrosis-relevant protein RIP1 is involved in necrosis but not apoptosis that was caused by TNFα. RIP1 is a serine/threonine kinase with RHIM domain and death domain at the intermediate and c-terminal regions. It is a pleiotropic adaptor that mediates both NFκB activation and programmed necrosis by TNFα (Chan et al., 2003; Holler et al., 2000; Lin et al., 2004). Its kinase activity and RHIM domain are crucial for undergoing the necrotic cell death mediated by death receptors although kinase is dispensable for NFκB activation. Cyclophilin D (CypD) is a mitochondrial member of the cyclophilin family of peptidyl prolyl-*cis*, *trans*-isomerases (PPIases) and has a central role in protein folding. Functionally, it is suggested to be involved in regulating the mPT since cyclosporin A (CsA), a specific inhibitor of Cyp family, blocks mPT. Mice lacking CypD are substantially more tolerable to cardiac and cerebral ischemic injury than their wild-type littermates (Nakagawa et al., 2005). In addition, genome wide siRNA screens show that the necrotic proteins identified are closely interconnected to molecular signaling networks (Hitomi et al., 2008). For instance, it has been demonstrated that sequential activation of PAPR-1, calpains and Bax following excessive alkylating DNA damage is required for apoptosis inducing factor (AIF)-induced necrosis (Moubarak et al., 2007). Hitomi et al demonstrates that a set of genes required for programmed necrosis is enriched in the nervous and immune systems, and that a subset of 32 genes acts downstream as regulators of RIP1 kinase. These results define that there are molecular signaling networks regulating the bifurcation of apoptosis and necrosis pathways (Hitomi et al., 2008).

Physiological significance of programmed necrosis

When apoptosis is triggered, a series of events like DNA laddering and apoptotic body formation ensues. These damaged cells are functionally impaired and are eliminated through phagocytosis by macrophage or neighboring cells. On the other hand, in cells undergoing programmed necrosis, cell membrane integrity is disrupted so that intracellular materials are released into the extracellular milieu. The resulting danger signals elicit the inflammatory responses by immune cells. In general, physical damage, chemical stress or microbial infection will prime cells towards apoptosis (Yang et al., 2007). However, under specific conditions when caspases are genetically impaired or inhibited by various stresses, cells will die by programmed necrosis. Consistently with the inflammatory damage caused by necrosis, caspase inhibition by benzyloxycarbonyl-Val-Ala-Asp (zVAD) exacerbates the TNF-induced shock via oxidative stress and phospholipase A2, demonstrating that pathophysiological conditions developed by TNF is associated with caspase activity (Cauwels et al., 2003).

Intriguingly, many viruses and some intracellular bacteria develop the multiple strategies like caspase suppression or apoptosis signaling intervention to facilitate evasion of pathogens from immune surveillance by host (Pardo et al., 2009). However, virus-infected host or functionally impaired cells, under circumstances that apoptotic machinery does not function properly by such as viral caspase inhibitors, are forced to activate backup mechanism of cell death like necrosis to eliminate damaged cells. For instance, vaccinia virus, a prototype of poxvirus family, expresses the caspase-1/8 inhibitor B13R/Spi2, and therefore, VV infection into host cells might skew the cell death toward necrosis over apoptosis (Cho et al., 2009). In contrast, VV infected RIP3-/- mouse embryonic fibroblasts (MEF) were blunted from TNF-induced programmed necrosis, indicating that RIP3 has a central role in necrotic cell death. Furthermore, like most poxvirus, VV infection into animal model elicits inflammation which is caused by necrotic tissue damage. Intraperitoneal injection of VV into wild type and RIP3-/- mice exhibits differential responses with respect to viral replication and inflammation. Failure to evoke inflammation in RIP3-/- mice enhances viral replication, eventually leading to motality of mice. In contrast, wild mice exhibit necrotic tissue damage in liver and fat fad, resulting in an inflammatory response that limits viral replication and spread. Therefore, RIP3 plays a physiological role in mediating innate immune response against pathogens that inhibit apoptosis. Based on these results, we propose a physiological role of programmed necrosis against apoptosis-overriding pathogen (Fig. 2). Alternative form of cell death is also found in macrophage infected by virulent mycobacterium tuberculosis (MTb) (Porcelli and Jacobs, 2008). Avirulent mycobacteria stimulate the macrophage to undergo apoptosis, resulting in sealing of the bacteria within impermeable envelope that prevents the bacteria from escaping. On the other hand, virulent bacteria cause necrotic death of host, which leads to membrane rupture that enables bacteria to escape and spread. Moreover, necrotic cell death of host cell was demonstrated in *Shigella*-infected non-myeloid cells (Galluzzi and Kroemer, 2009). Shigella kills non-myeloid cells via programmed necrosis and its signaling pathway depends on the BH3-only protein BNIP as well as on CypD. Besides pathogen-induced programmed necrosis and ensuing inflammation, RIP3 might also play more comprehensive role in controlling chemical-induced inflammation. In fact, RIP3-/- mice were more resistant to cerulein-induced pancreatitis (He et al., 2009; Zhang et al., 2009). These results demonstrate that RIP3-dependent programmed necrosis is important for regulating inflammation in certain pathological conditions.

In addition to necrosis activation via death receptor, other conditions such as heavy metal, DNA alkylating agent and shikonin can induce necrotic cell death distinct from apoptotic cell death. Interestingly, shikonin in a specific cell line determines cell death modes depending on its concentrations, and the presence of Nec-1 switches programmed necrosis to apoptosis, suggesting that conversion between programmed necrosis and apoptosis may not be uni-directional but may be interconvertible depending on cell lines and stimulus context. Taken together, these recent results indicate that while apoptosis is a classical cell death program preserved in evolution, programmed necrosis is an alternative cell death response with physiological significance in cell injury induced by chemicals or microbial infection.

Programmed necrosis-associated diseases

Although the mechanistic details remain to be clarified, necrosis has been implicated in septic shock, ischemic brain injury and

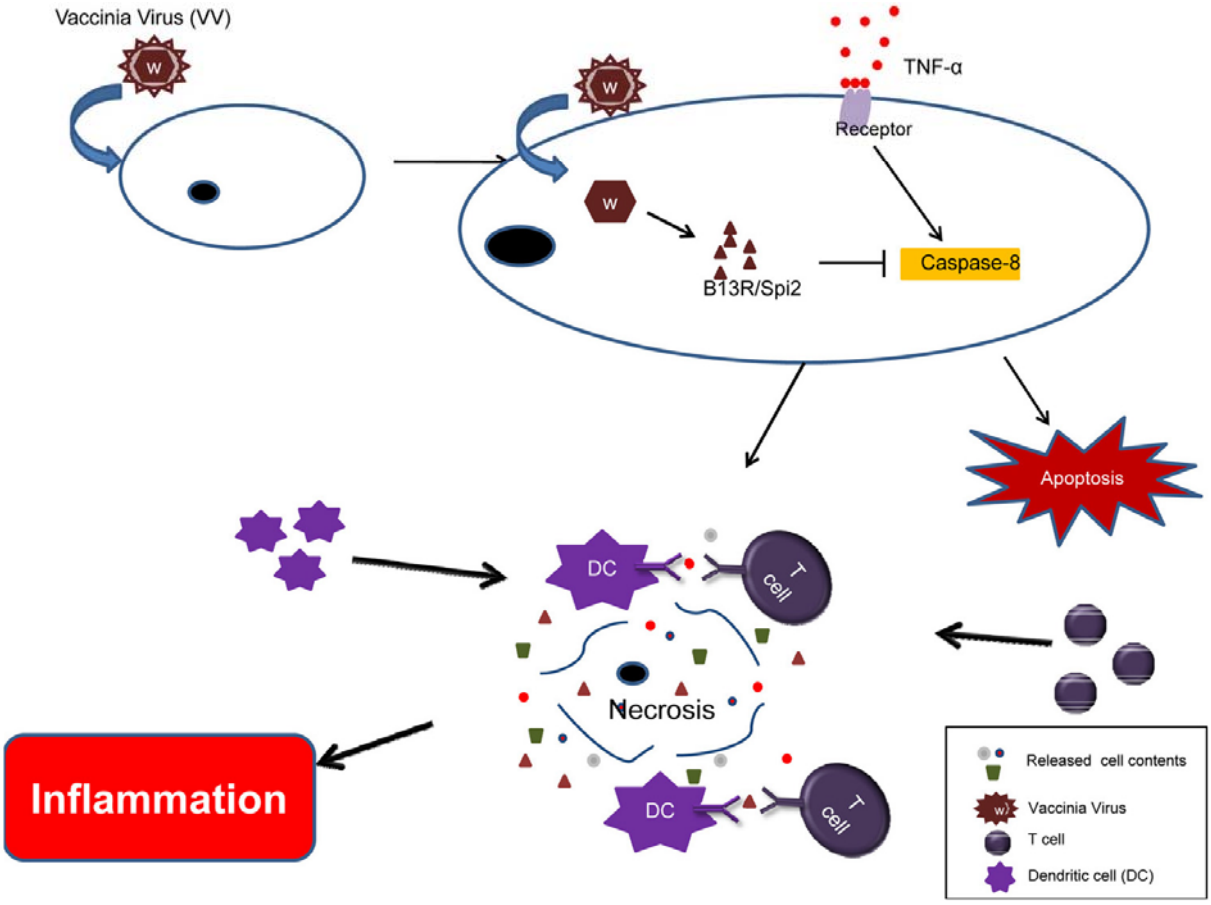

Fig. 2. The proposed model that programmed necrosis is implicated in the innate immune defense against vaccinia virus infection. In general, TNF has been known to be an important cytokine engaged in innate immune defense mechanism against bacterial and viral infections. Upon VV infection, TNF expression is highly induced in multiple cells including fat and liver tissues, but under such a condition, TNF-mediated apoptosis is abrogated by virus-encoded protein B13R/Spi2, caspase-1/8 inhibitor. However, infected cells in which apoptosis is impaired activate alternative backup cell death programmed necrosis to prevent virus spread. Subsequently, cells undergoing necrosis leaks intracellular contents called danger signals, which can induce inflammation by consecutive dendritic cell (DC) recruitment and T cell response. Therefore, programmed necrosis plays a key role in controlling viral infection via elimination of viral factory and promotion of the subsequent virus-induced inflammation.

acute pancreatitis. It is not easy to define the correlation between necrosis and pathological disorders because of the lack of defined markers and the fact that mixed types of cell death are often found during the pathogenesis of diseases. Dysregulated apoptosis is linked to certain pathological conditions. Thus, caspase inhibition has been proposed to be a useful therapeutic strategy to treat those disease like neurodegenerative disorders and cancers. Contrary to this notion, caspase inhibition causes hyperacute shock in response to TNFα, indicating that tissue damage develops independently of caspase (Cauwels et al., 2003). Importantly, Degterev et al discovered a potent small molecule Nec-1 that inhibits necrosis mediated by death receptor. Nec-1 delayed ischemic brain injury in mice, suggesting that it is a promising therapeutic drug for the treatment of stroke (Degterev et al., 2008). It will be interesting to determine if administration of Nec-1 can similarly prevent the development of other necrosis-related diseases.

Another example is the acute pancreatitis, which is characterized by acute inflammation and necrosis of pancreas parenchyma, focal enzymatic necrosis of pancreatic fat and vessels necrosis (Cinquepalmi et al., 2006). In about 85% of patients, acute pancreatitis is a mild disease and is usually associated with a rapid recovery within a few days of illness onset. However, in about 15-20% of patients, severe damage to the pancreas may lead to life threatening illness. Severe acute pancreatitis usually develops when parts of the pancreas become necrotic from the acute inflammation**.** Interestingly, RIP3 plays an essential role in experimental model of pancreatitis (He et al., 2009; Zhang et al., 2009). In addition, RIP3 expression was induced during the early inflammatory phase of wound healing (24 h - 3 days after injury) (Adams et al., 2007). In cancers, programmed necrosis may lead to harmful pathological cell loss through the ensuing local inflammation that promotes tumor growth. However, necrotic cell death may be exploited to eliminate cancer cells. For example, by therapeutic treatment of alkylating DNA damaging agents (Zong et al., 2004) and tumor-targeted photosensitizing molecules (Agostinis et al., 2004) induce necrosis through ROS production.

CONCLUSION

A growing body of evidence clearly establish that cell death by necrosis is not an accidental and unregulated event. Rather, it is an active process orchestrated by specific molecules such as the RIP kinases. Unlike apoptosis, necrotic cell death exhibits

substantial loss in membrane integrity. The release of danger signals or endogenous adjuvants into the surrounding tissues provokes the inflammatory responses. A switch from apoptosis to programmed necrosis was observed in DR-mediated cell death under conditions of caspase inhibition (Zhang et al., 2009). Additionally, other necrosis-inducing agents like heavy metals and DNA-damaging chemicals have been recently reported to induce RIP1-dependent necrosis. Comprehensive identification of proteins specific for necrosis and further understanding of molecular mechanisms governing necrosis will be useful for drug development targeting programmed necrosis-associated diseases.

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