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Review

Necrosis, a well-orchestrated form of cell demise: Signalling cascades, important mediators and concomitant immune response

Nele Festjens, Tom Vanden Berghe, Peter Vandenabeele *

Molecular Signalling and Cell Death Unit, Department for Molecular Biomedical Research, VIB and Ghent University, Fiers-Schell-Van Montagu Building, Technologiepark 927, B-9052 Ghent, Belgium

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Abstract

Necrosis has long been described as a consequence of physico-chemical stress and thus accidental and uncontrolled. Recently, it is becoming clear that necrotic cell death is as well controlled and programmed as caspase-dependent apoptosis, and that it may be an important cell death mode that is both pathologically and physiologically relevant. Necrotic cell death is not the result of one well-described signalling cascade but is the consequence of extensive crosstalk between several biochemical and molecular events at different cellular levels. Recent data indicate that serine/threonine kinase RIP1, which contains a death domain, may act as a central initiator. Calcium and reactive oxygen species (ROS) are main players during the propagation and execution phases of necrotic cell death, directly or indirectly provoking damage to proteins, lipids and DNA, which culminates in disruption of organelle and cell integrity. Necrotically dying cells initiate pro-inflammatory signalling cascades by actively releasing inflammatory cytokines and by spilling their contents when they lyse. Unravelling the signalling cascades contributing to necrotic cell death will permit us to develop tools to specifically interfere with necrosis at certain levels of signalling. Necrosis occurs in both physiological and pathophysiological processes, and is capable of killing tumour cells that have developed strategies to evade apoptosis. Thus detailed knowledge of necrosis may be exploited in therapeutic strategies.

Keywords: Calcium; Inflammation; Mitochondria; Phospholipases; Proteases; ROS

Abbreviations: AA, arachidonic acid; AC, acid ceramidase; AGE, advanced glycation end product; AIF, apoptosis inducing factor; ANT, adenine nucleotide translocase; APC, antigen presenting cell; A-SMase, acid sphingomyelinase; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CA, cornu ammonis; $cPLA_2$, Ca^{2+}-dependent phospholipase A_2; Cu/ZnSOD, Cu/Zn-containing superoxide dismutase; CypD, Cyclophilin D; DC, dendritic cell; DD, death domain; DED, death effector domain; eNOS, endothelial NOS; ER, endoplasmic reticulum; ETC, electron transport chain; FADD, Fas receptor associated death domain; GA, geldanamycin; GPx, glutathione peroxidase; HMGB1, high mobility group box I; Hsp 90, heat shock protein 90; IFN, interferon; IKK, IκB kinase; iNOS, inducible NOS; $InsP_3R$, inositol 1,4,5 triphosphate receptor; IRAK-M, IL-1 receptor associated kinase-M; LMP, lysosomal membrane permeability; LOX, lipoxygenase; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MEF, murine embryonic fibroblast; MG, methylglyoxal; MSDH, O-methyl-serine dodecylamide hydrochloride; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; MnSOD, Manganese superoxide dismutase; MPT, mitochondrial permeability transition; NMDA, N-methyl-D-aspartate; nNOS, neuronal NOS; NOS, nitric oxide synthase; Op18, oncoprotein 18; PARP-1, poly-(ADP-ribose) polymerase-1; PCD, programmed cell death; PHGPx, phospholipids hydroperoxide glutathione peroxidase; PLC, phospholipase C; RAGE, receptor for advanced glycated end products; RC, radicicol; RNS, reactive nitrogen species; ROS, reactive oxygen species; RyR, ryanodine receptor; SMase, sphingomyelinase; TLR, toll-like receptor

* Corresponding author.

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1. Introduction

Programmed cell death (PCD) is a genetically encoded form of cell suicide that is central to the development and homeostasis of multicellular organisms. In the context of embryonic development, three main morphologies of PCD have been described [1,2]. Type I apoptotic cell death is characterized by cell shrinkage and extensive chromatin condensation. Formation of autophagic vacuoles inside the dying cell is typical of autophagic or type II cell death, whereas type III PCD is distinguished by rapid loss of plasma membrane integrity and spillage of the intracellular content. The type of cell death selected depends on the stimulus and the cellular context because every cell death program is a net result of self-propagating signals and others that suppress the other cell death programs [1].

Apoptosis is the major cell death pathway used to remove unwanted and harmful cells in a "clean or silent" manner during embryonic development, tissue homeostasis and immune regulation [3]. In addition, most anti-cancer therapies are based on activation of apoptotic pathways. An evolutionarily conserved family of cystein proteases, called caspases, is responsible for most of the observed morphological changes during apoptosis [4]. Two distinct pathways initiate apoptosis: the extrinsic apoptotic pathway starting with the aggregation of death receptors [5], and the intrinsic apoptotic pathway starting with the release of mitochondrial factors in response to various stimuli, such as growth factor withdrawal, UV irradiation and cytotoxic drugs [6]. The extrinsic apoptotic pathway can also impinge on the mitochondrial pathway, which serves to amplify the apoptotic process. The molecules that integrate the signalling of these different cell death stimuli and converge on the release of mitochondrial factors are the BH3-only members of the Bcl-2 family. Apoptosis and the role of mitochondria and Bcl-2 family members therein have been amply described [6,7].

Increasing evidence supports the existence of caspase-independent cell death pathways that induce cell death in a manner that is as well controlled and programmed as in caspase-dependent PCD [8]. Caspase-independent cell death can provide a backup suicide mechanism if the classical apoptosis machinery fails [9,10]. Cell death induced under such conditions lacks the typical features of apoptosis and instead resembles necrosis [11–13]. Necrosis is characterized by cytoplasmic swelling, irreversible plasma membrane damage, and organelle breakdown [10,14]. DNA in apoptotic cells is degraded specifically, giving rise to the characteristic ladder pattern on the gels, whereas DNA in necrotic cells is usually degraded randomly by extracellular DNAse I present in culture serum that has not been heat-inactivated [15], or by lysosomal DNAse II [16], giving rise to a smear of DNA [17]. In necrosis, the cellular contents leak into the extracellular environment, where they may act as a "danger signal", and consequently necrosis is usually associated with inflammation [18]. However, this issue is still controversial because we and others showed that exposure of macrophages to necrotic cells is not sufficient to trigger macrophage activation and the concomitant induction of proinflammatory cytokine expression [19,20]. Rather, necrotic cells trigger an increase in the secretion of proinflammatory cytokines from independently activated macrophages [20]. It is also conceivable that the release of cytokines or other factors from the necrotic cells themselves [21] may be crucial for an inflammatory response.

Autophagy is evolutionarily conserved, and is probably initiated as a survival response to cellular stress-associated damage or nutrient deprivation. Its primary function is to recycle proteins from engulfed cytoplasm or damaged organelles. Autophagy is recognized by the formation of autophagosomes, double membrane autophagic vacuoles that eventually fuse with lysosomes to form autolysosomes. Engulfed contents and the inner membrane of the autophagosome are subsequently degraded by lysosomal hydrolases [22]. Various forms of environmental stress induce autophagy, which eventually results in either caspase-dependent [23,24] or caspase-independent cell death [24–26].

In this review, we will focus on necrotic cell death. Cellular signalling pathways are characterized by three consecutive phases: initiation, propagation and execution. However, our present knowledge of necrotic cell death does not enable us to clearly distinguish between these phases, particularly between propagation and execution events. Many cellular events are initiated and occur at the same time. They may interplay or they may be just bystander consequences of cellular damage. However, despite the actual premature state in definition of a necrotic cell death pathway, we will discuss data illustrating possible signal transduction cascades leading to necrosis with emphasis on important mediators of the propagation and execution steps of necrotic cell death. It will also become apparent that necrosis is not just accidental cell death but fulfils an important role in physiology and pathology.

2. Signal transduction cascades leading to necrotic cell death

Death receptors belong to the TNF receptor superfamily. When they bind their extracellular ligands they aggregate and initiate a signalling pathway that results either in cell survival or in death [27]. Depending on the cellular context, cells die by apoptosis or necrosis (Fig. 1). TNFα, a pleiotropic cytokine produced primarily by macrophages, induces apoptosis in many cells, but it can induce necrosis in the L929 mouse fibrosarcoma cell line [14,28]. Addition of the pan-caspase inhibitor zVAD-fmk or CrmA further sensitizes L929 cells to TNFα-induced necrotic cell death [29]. Likewise, Fas ligand in the presence of zVAD-fmk leads to necrosis of this cell line [11]. Similarly, triggering of TNF-R1, Fas or TRAIL-R in Jurkat cells in the presence of zVAD-fmk or in Jurkat cells deficient in FADD or caspase-8 results in necrosis [12,13,30]. Besides that, TNFα in the presence of caspase inhibitors can induce caspase-independent cell death in murine embryonic fibroblasts (MEFs) [31]. These results indicate that necrotic cell death may function as a backup cell death pathway when caspases are blocked or the caspase-dependent pathways cannot be properly activated.

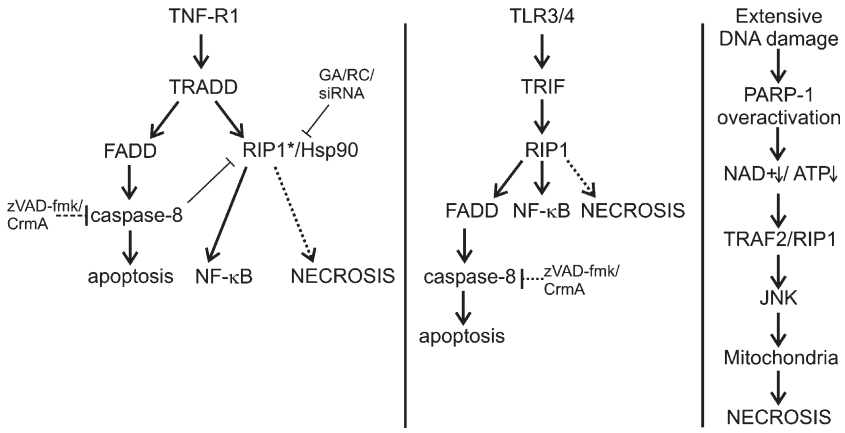

Fig. 1. Signal transduction cascades leading to necrotic cell death and the role of RIP1. Triggering death receptors and TLR3/4 initiates a signalling pathway that leads to either cell survival or death. Depending on the cellular context (presence (dashed line) or absence (full line) of caspase inhibitors), cells die by apoptosis or necrosis. FADD is an important adaptor in death receptor signalling upstream of RIP1. Studies in RIP1$^{-/-}$ Jurkat cells demonstrated that propagation of Fas/TNF-R/TRAIL-R-induced necrosis depends on the presence of kinase active RIP1 (*). Caspase-8 mediated cleavage of RIP1 during apoptosis mediated by TNF-R1, Fas or TRAIL-R suppresses necrotic and anti-apoptotic pathways. Necrosis can be blocked by adding Hsp 90 inhibitors geldanamycin (GA) or radicicol (RC), which are responsible for strong down-regulation of RIP1 levels, and RIP1 RNAi. RIP1 is also a crucial adaptor kinase in TRIF-dependent TLR3/4 signalling (MyD88 signalling is omitted for clarity). A FADD/caspase-8 mediated apoptotic pathway can be initiated downstream of RIP1. TLR3/4-induced necrosis occurs independently of FADD. Besides, DNA damage (e.g. MNNG) can result in overactivation of PARP-1, which catalyzes the hydrolysis of NAD^+ into nicotinamide and poly-ADP ribose, causing depletion of NAD^+ and resulting in a profound drop of ATP. MNNG-induced cell death depends on RIP1 and TRAF2, which function downstream of PARP-1 and are crucial for the activation of JNK. This kinase then impairs mitochondrial membrane integrity, causing release of mitochondrial intermembrane space proteins, and consequent necrosis. (See text for details).

At the crossroad of apoptotic and necrotic signalling pathways is the crucial adaptor molecule FADD (Fig. 1). It contains both a death domain (DD) crucial for initiating necrotic signalling, and a death effector domain (DED) that can propagate apoptotic cell death [32,33]. In Fas and TRAIL-R-induced signalling, FADD is recruited to the receptor and can initiate downstream signalling cascades, such as apoptosis and activation of NF-κB and MAPKs [5,34]. Impeding caspase activation switches cell death from apoptosis to necrosis. In contrast to the triggering of TRAIL-R and Fas, engagement of TNF-R1 does not result in recruitment of FADD to the receptor [35–37]. At the plasma membrane, formation of complex I, which consists of TNF-R1, TRAF2 and RIP1, leads to rapid activation of NF-κB [38,39] and MAPKs, such as p38 MAPK, JNK and ERK [21,40]. Following receptor endocytosis, a second complex is formed, in which TRADD recruits FADD and procaspase-8 or -10 [41,42]. Schneider-Brachert and co-workers showed that the endocytic vesicles fuse with trans-Golgi vesicles containing pro-acid-sphingomyelinase (pro-A-SMase) and pre-pro-cathepsin D. This leads to formation of multivesicular endosomes in which acid-sphingomyelinase and cathepsin D are activated [36]. If complex I does not succeed in inducing sufficient expression of antiapoptotic proteins, caspase-8 is activated, initiating apoptosis. However, if caspases are blocked, necrotic death ensues [29,43]. The importance of FADD in TNFα-induced caspase-independent signalling is controversial: FADD seems necessary for TNFα-induced death in MEFs [31], but it is dispensable for TNFα-induced death of Jurkat cells [12,37,43]. TNF-R2 is not essential for TNFα-induced necrosis but it seems to potentiate the process [31,43].

Studies in RIP1$^{-/-}$ Jurkat cells demonstrated that propagation of necrosis induced by triggering of Fas/TNF-R/TRAIL-R depends on the presence of kinase active RIP1 [12,37,43] (Fig. 1). Caspase-8 mediated cleavage of RIP1 during TNF-R1, Fas and TRAIL-R-mediated apoptosis suppresses necrotic and anti-apoptotic pathways [43,44], also demonstrating that full-length RIP1 is required for necrosis. Moreover, the C-terminal RIP1 cleavage fragment containing the DD sensitizes cells to apoptosis by inhibiting NF-κB activation [44,45]. Studies on the heat shock protein (Hsp) 90, a cytosolic chaperone for many kinases, including RIP1 [46], have also revealed the importance of RIP1 in necrotic signalling. Fas- and TNF-R1-induced necrosis are inhibited by Hsp90 inhibitors geldanamycin (GA) and radicicol (RC), which are responsible for a strong down-regulation of RIP1 levels [12,47]. Moreover, knockdown of RIP1 in L929 cells protects the cells against necrosis induced by TNFα/zVAD-fmk or FasL/zVAD-fmk (Vanden Berghe et al., unpublished results).

Besides death receptor-induced necrosis, triggering of toll-like receptor (TLR) 4 and TLR3 can also lead to necrosis (Fig. 1). When caspase-8 activation is suppressed by IETD-fmk, CrmA or zVAD-fmk, LPS induces a RIP1-dependent, non-apoptotic death of macrophages [48]. The presence of dsRNA in mammalian cells is a hallmark of viral infection, as most viruses produce dsRNA during their replication. Both viral and synthetic dsRNA were shown to kill cells, predominantly by FADD/caspase-8 mediated apoptosis [49]. Synthetic dsRNA, however, induces necrosis in human Jurkat cells and murine L929 fibrosarcoma cells in a caspase-8 and FADD-independent way, and type I and II-interferons (IFNs) can sensitize for this necrosis [13].

We should remark that in some studies following addition of zVAD-fmk or specific RNAi-mediated caspase-8 knockdown in L929 cells, RIP1-dependent autophagic cell death instead of

necrosis was observed [50,51]. However, death kinetics need to be very extended to detect autophagy whereas the death receptor-induced necrosis in this model system proceeds very fast (24–36 h respectively 5–6 h). Additionally, there is some controversy about the induction of necrosis or autophagic death of macrophages following treatment with LPS in the presence of caspase inhibitors [48,52]. Thus whether necrosis or autophagy ensues when apoptosis is inhibited will surely depend on cells and circumstances. Due to the lack of specific markers and clear-cut definitions of necrotic and autophagic cell death, both types of death will frequently get entangled.

Some pathophysiological processes, such as ischemia–reperfusion, inflammation, ROS-induced injury and glutamate excitotoxicity, are accompanied by poly-(ADP-ribose) polymerase-1 (PARP-1)-mediated cell death [53–55] (Fig. 1). Stimuli that directly or indirectly affect mitochondria, such as H_2O_2 and the DNA-alkylating agent N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), also induce cell death mediated by PARP-1 [56,57]. Activation of PARP-1 catalyzes the hydrolysis of NAD^+ into nicotinamide and poly-ADP ribose, causing depletion of NAD^+ [58]. This results in cellular energy failure and caspase-independent death of different cell types [59,60]. MNNG-induced cell death depends on RIP1 and TRAF2, which function downstream of PARP-1 and are crucial for JNK activation. JNK in turn affects mitochondrial membrane integrity, with consequent release of proteins of the mitochondrial intermembrane space, and necrosis [61]. It is not clear how JNK induces mitochondrial membrane depolarization, but it is plausible that it occurs through modifications of Bcl-2 family members [62,63], or via caspase-independent JNK-mediated processing of Bid [64]. PARP-mediated cell death induced by H_2O_2 [65] also depends on a TRAF2/RIP1/JNK-mediated signalling cascade [57]. How intracellular molecules such as RIP1 and TRAF2 sense PARP-1 activation remains elusive.

3. Important mediators involved in the propagation and execution of necrotic cell death

3.1. Reactive oxygen species (ROS)

3.1.1. ROS in physiology and oxidative stress

ROS are produced during normal physiological cellular events, and are involved in various biological processes, including regulation of proliferation, activation of gene expression and cellular response to cytokines [66–68]. Mitochondria are the major source of ROS within the cell [69,70]. Electrons frequently escape along the electron transport chain (ETC), most usually at complex I [71] and complex III [72]. The reaction of the renegade electron with molecular oxygen produces an oxygen radical, which is normally converted into H_2O_2 or other ROS, including hydroxyl radicals and superoxide anions, before being eliminated [73]. To counteract the production of ROS, mitochondria and cells in general, possess numerous ROS defence systems. The redox state of the cell is controlled by different redox buffers and specific antioxidant enzymes confined to specific subcellular locations. These enzymes include glutathione peroxidase (GPx), phospholipid hydroperoxide glutathione peroxidase (PHGPx: cytosolic, mitochondrial and nuclear isoforms), Mn-superoxide dismutase (MnSOD: mitochondrial matrix), Cu/Zn-containing dismutase (Cu/ZnSOD: cytoplasm and mitochondrial intermembrane space), and catalase (peroxisomes) [74,75].

The source of oxidative stress is not the ROS generation *per se* but spatiotemporal imbalance of ROS production and detoxification. However, in some physiological and pathophysiological conditions, the balance between free radicals and their scavengers is disturbed, and overproduction of the former can damage biomolecules, including DNA [76], proteins [77] and lipids [78]. This cellular damage can be associated with apoptosis, inflammation, septic shock [79], neurodegeneration [80], ischemia/reperfusion injury [81], tumour promotion [82] or necrosis.

3.1.2. Mitochondrial and glycolyis-derived ROS and necrosis

Several independent studies have reported the involvement of mitochondria-produced ROS in necrotic killing of L929 fibrosarcoma cells by TNFα [83,84] (Fig. 2). These cells could not be protected by hydrophilic reducing agents, or by overexpression of antioxidant enzymes in the mitochondrial matrix [85]. However, treatment with the lipophilic chemical antioxidants butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) clearly delayed TNFα-induced necrotic cell death [10,86]. These results supported the idea that ROS formed and acting in the hydrophobic environment of the inner mitochondrial membrane play an important role in TNFα-induced necrotic cytotoxicity. This is also confirmed by the inhibitory effect of the complex I inhibitor rotenone on TNFα-induced ROS production and toxicity *in vitro* and *in vivo* [86,87]. ROS were also shown to occur and were suggested to be involved in dsRNA-induced necrosis [13]. Although BHA and BHT caused similar reductions in ROS levels upon treatment of L929 cells with TNFα or dsRNA, BHA was far more effective than BHT in protecting cells against TNFα-induced necrosis [86]. Moreover, BHA protects L929 cells from dsRNA-induced necrosis and shifts the response to apoptosis, whereas BHT does not modulate cell death type [13,86]. These observations support the hypothesis that players other than mitochondrial ROS are also necessary for executing necrosis [86]. Two of these mediators were identified as phospholipase A_2 and lipoxygenases (see below).

TNFα treatment of L929 cells also increases phosphorylation of glyoxalase I, which is mediated by protein kinase A and required for cell death. Concomitantly, a substantial increase in intracellular levels of methylglyoxal (MG) leads to the formation of a specific MG-derived advanced glycation end product (AGE) (Fig. 2) [88,89]. MG is a cytotoxic metabolite produced primarily as a by-product of glycolysis, by non-enzymatic phosphate elimination from the glycolytic pathway intermediates dihydroxyacetone phosphate and glyceraldehyde 3-phosphate [90]. Under normal physiological conditions, most MG is bound to cellular proteins as adducts formed with Lys, Arg and Cys residues [91]. Although the reaction with Cys is

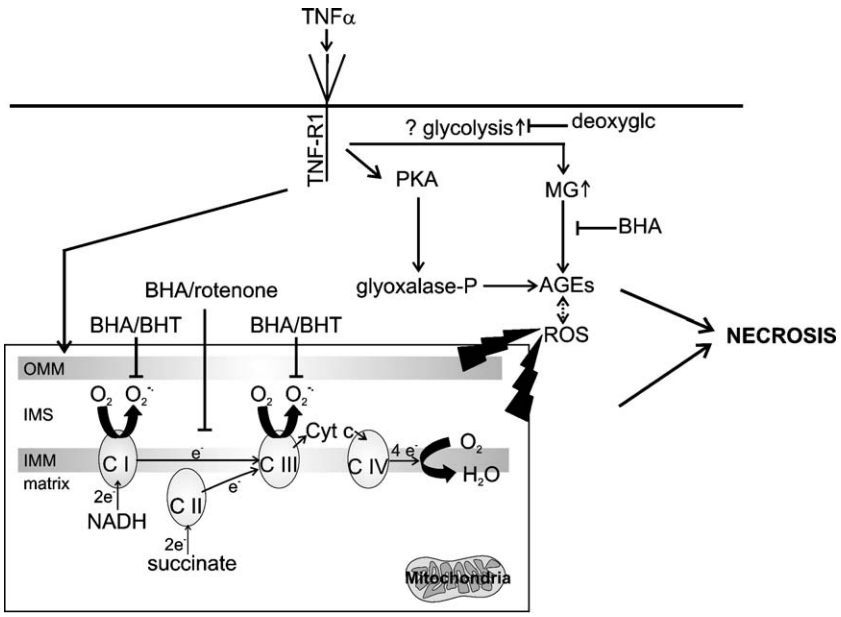

Fig. 2. Mitochondria- and glycolysis-derived ROS and necrosis. Mitochondria are the major source of ROS in the cell. Electrons frequently escape along the electron transport chain (ETC), most usually at complexes I and III. The reaction of the renegade electron with molecular oxygen produces an oxygen radical. ROS formed and acting in the hydrophobic environment of the inner mitochondrial membrane play an important role in TNFα-induced necrotic cytotoxicity. BHA can scavenge ROS and block complex I, thus protecting from necrotic cell death. Further, TNFα induces increased phosphorylation of glyoxalase I by protein kinase A, a process that is required for cell death. Concomitantly, intracellular levels of methylglyoxal (MG) are substantially increased, probably due to enhanced glycolysis. MG leads to the formation of a specific MG-derived advanced glycation end product (AGE). BHA blocks the formation of TNFα-induced MG-derived AGEs, demonstrating that their formation is consequent to increased ROS production. OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; IMS, intermembrane space.

considered reversible, elevated concentrations of MG can lead to irreversible modifications of Lys and Arg residues through formation of AGEs [92]. Because AGEs induce protein cross-links and oxidative stress within cells and tissues, they were thought to contribute to several pathophysiological conditions, such as tissue damage after ischemia/reperfusion [93], as well as aging [94]. TNFα-induced MG-derived AGEs are mainly Arg-modified, and their formation may be a consequence of increased ROS production, as it is blocked by BHA [89]. It is thus plausible that ROS produced by the mitochondria contribute to AGE-formation, and that MG acts as a signalling molecule in the regulation of necrotic cell death.

The contribution of mitochondria to TNFα-induced caspase-independent death is confirmed by the fact that overexpression of Bcl-2 can delay the onset of TNFα-induced toxicity in L929 cells [95,96]. As there is no early release of cytochrome c in necrosis [96], Bcl-2 must exert its protective effect by another molecular mechanism. In this respect, it was suggested that Bcl-2 might act by binding BNIP3, a cytotoxic Bcl-2 family member [97], causing increased production of ROS and decreased mitochondrial membrane potential, without release of inter-membrane space proteins [98,99]. Alternatively, Bcl-2 might prolong the integrity of mitochondrial oxidative phosphorylation [100]. It has also been reported that Bcl-2 has direct anti-oxidant functions, but the molecular mechanism is still unclear [101,102]. It has been shown that overexpression of Bcl-2, although it elevated the basal levels of hydrogen peroxide, nevertheless restricted the excessive production of hydrogen peroxide induced by TNFα [103].

3.2. Calcium

3.2.1. Calcium homeostasis and mitochondria

The endoplasmic reticulum (ER) is the major intracellular Ca^{2+} store, and regulated Ca^{2+} release from this organelle is essential for cellular signalling. The primary role of mitochondrial Ca^{2+} is the stimulation of oxidative phosphorylation, which can occur at different levels [104,105]. As Ca^{2+} is a global positive effector of mitochondrial function, any perturbation in mitochondrial or cytosolic Ca^{2+} homeostasis will have profound implications for cellular function [106] (Fig. 3). Mitochondrial Ca^{2+} overload will cause excessive stimulation of the tricarboxylic acid (TCA) cycle, enhancing electron flow into the respiratory chain, with concomitant overproduction of ROS. Moreover, Ca^{2+} stimulation of nitric oxide synthase (NOS) and subsequent nitric oxide (NO) generation can also affect mitochondrial respiration and increase ROS production (see below). On the other hand, mitochondrial dysfunction and production of ROS can theoretically modulate extramitochondrial Ca^{2+} pools. A direct consequence of ischemic injury is a drastic decrease in the available amount of glucose and oxygen. Anoxic and ischemic damage can thus result in the uncoupling of oxidative phosphorylation [107–109]. According to Lemasters et al., this may result in ROS production and uncontrolled hydrolysis of ATP by the inner membrane ATPase [110]. To maintain ATP production during ischemic conditions, cells make use of remaining glucose in the surrounding tissue and bring into play glycogen stores by anaerobic glycolysis [111,112]. The latter results in tissue

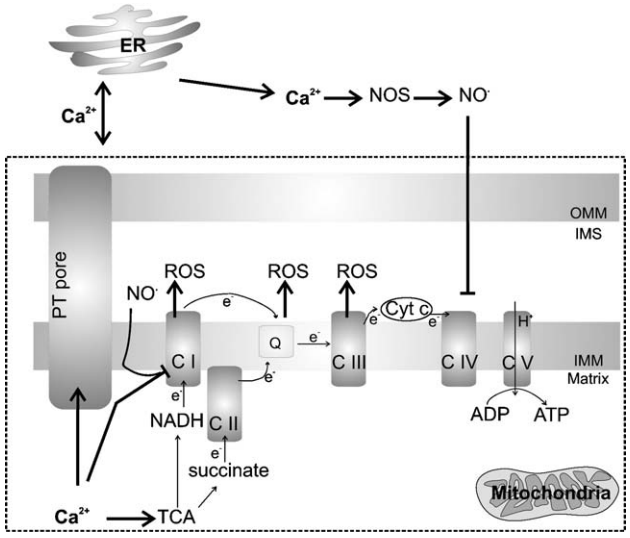

Fig. 3. Mechanism for Ca^{2+} stimulation of mitochondrial ROS generation. Ca^{2+} stimulation of the TCA cycle enhances electron flow into the respiratory chain, and Ca^{2+} stimulation of NOS and subsequent NO generation inhibit respiration at complex IV. These events enhance ROS production from the Q cycle. In addition, $NO^{\cdot}$ and Ca^{2+} can inhibit complex I, possibly enhancing its production of ROS. Ca^{2+} at high concentrations triggers the opening of the PTP (permeability transition pore). OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; IMS, intermembrane space.

acidosis, which in turn increases Ca^{2+} influx by activating acid-sensing ion channels [113]. Sustained Ca^{2+} can stimulate mitochondrial dehydrogenases with concomitant increase in NADH levels. The augmented NADH concentration contributes to an increased mitochondrial ROS production following reperfusion, which can lead to disruption of mitochondrial inner membrane integrity and again loss of the ability to generate ATP [114].

Besides, and as mentioned above, processes such as ischemia–reperfusion, inflammation, ROS-induced injury and glutamate excitotoxicity are also accompanied by a decrease in ATP due to overactivation of PARP-1 [53–55]. Generally, energy depletion slows active transmembrane ion transport systems [115], leading to increased cell membrane permeability, altered distribution of K^+, Na^+ and Ca^{2+} ions, and decreased mitochondrial transmembrane electrical potential [116]. If the onset of mitochondrial permeability transition (MPT) is widespread and involves most mitochondria in a cell, and glycolytic sources of ATP are unavailable, cells become profoundly ATP depleted. This will ultimately lead to organelle disruption and cell lysis. Adenine nucleotide translocase (ANT)-deficient mitochondria still display MPT in response to calcium ionophores, demonstrating that ANT may not be essential for MPT [117]. Ca^{2+}-induced death of $ANT^{-/-}$ hepatocytes occurs via a caspase-independent pathway that can be blocked by cyclosporine A, indicating that cyclophylin D (CypD) is a key regulator of MPT-related necrotic cell death [117]. This is corroborated by the resistance of CypD-deficient cells to necrotic cell death induced by H_2O_2 and Ca^{2+} overload. In addition, CypD-deficient mice are highly resistant to cardiac injury induced by ischemia/reperfusion, confirming that CypD-dependent MPT regulates some forms of necrotic death [118–121].

It is important to mention that TNFα-induced necrosis of L929 cells proceeds with normal amounts of ATP [122], while Ca^{2+} chelators are clearly protective (Denecker, G. and Vanden Berghe, T., unpublished data), demonstrating that ATP- and Ca^{2+}-mediated signalling processes are not always interrelated. The fact that translation persists during necrosis of Jurkat and L929 cells also argues against a massive drop in ATP concentrations during necrotic cell death [123].

The important role of calcium during necrotic cell death is underscored by the fact that necrotic cell death in *C. elegans* seems to be influenced by at least four ER proteins regulating intracellular Ca^{2+} levels, namely the Ca^{2+} channels $InsP_3R$ and ryanodine receptor (RyR), and the Ca^{2+}-binding chaperones calreticulin and calnexin [124]. Besides calcium's effect on mitochondrial homeostasis, Ca^{2+}-dependent activation of phospholipases, lysosomal hydrolases and proteases leads to rapid loss of proteins, DNA and RNA (see below).

3.2.2. Calcium stimulation of nitric oxide synthase (NOS)

Oxidative stress arises not only from increased levels of ROS; a significant increase in concentrations of reactive nitrogen species (RNS), such as nitric oxide (NO) and peroxynitrite ($ONOO^-$), also enhance oxidative stress (Fig. 3). Calmodulin, the first protein shown to interact with NOS, is necessary for the enzymatic activity of all three isoforms [125]. The Ca^{2+} dependence of NO synthesis distinguishes the NOS isoforms, with nNOS (neuronal isoform) and eNOS (endothelial isoform) requiring much more Ca^{2+} than iNOS (inducible isoform) [126]. Excessive influx of Ca^{2+} can lead to enhanced activation of NOS and disproportionate production of NO. The latter can inhibit complex IV of the ETC, which would in turn enhance ROS production, and impair mitochondrial ATP synthesis and cell function [127]. In addition, a combination of NO and high concentrations of mitochondrial Ca^{2+} can inhibit complex I [128], possibly enhancing ROS generation by this complex. As NO can impede caspase-3 activity by nitrosylation [129], it may directly inhibit apoptosis and thereby promote necrosis.

Activation of nNOS and production of NO play important roles in excitotoxicity, a phenomenon in which neuronal cells undergo necrosis or apoptosis due to excessive release of neurotransmitters or in response to overexposure to excitatory amino acids, such as N-methyl-D-aspartate (NMDA) and glutamate [130]. In particular, prolonged activation of the NMDA receptor leads to massive Ca^{2+} influx, resulting in cytosolic Ca^{2+} overload and cell death. Ca^{2+} influx, besides activating lipases and proteases and affecting mitochondrial respiration, will activate nNOS and lead to excessive NO production [131], which in the presence of $O_2^{-\cdot}$ can generate $ONOO^{-\cdot}$. The latter is a potent trigger of MPT, and damages DNA, with consequent activation of PARP-1 [132]. Hyperactivation of PARP-1 results in caspase-independent death [60] (see above). Excitotoxicity plays an important role in

many neurodegenerative disorders such as Alzheimer's and Huntington's disease and central nervous system disorders such as seizure.

3.3. Phospholipases and lipoxygenases

Phospholipase A_2 (PLA_2) encompasses a family of esterases that are responsible for the liberation of fatty acids from the sn-2 position of phospholipids. Several distinct mammalian PLA_2 enzymes have been identified which are classified into three major subfamilies. Ca^{2+}-independent PLA_2 ($iPLA_2$) is thought to be mainly involved in the maintenance of the composition of membrane phospholipids. Secretory PLA_2 ($sPLA_2$) is an extracellular low molecular mass enzyme that shows antibacterial and inflammation-inducing properties and which activation requires millimolar amounts of Ca^{2+}. Cytosolic PLA_2 ($cPLA_2$) is an intracellular enzyme preferentially hydrolyzing arachidonate-containing phospholipids and thus mainly responsible for the release of arachidonic acid (AA) [133]. The translocation of $cPLA_2$ to the membranes of the nucleus, ER and Golgi apparatus, which enables it to interact with its substrates, is essential for $cPLA_2$-mediated release of AA from membranes [134]. Ca^{2+} is indispensable for the translocation of $cPLA_2$ but not for its activity [135]. On the other hand, phosphorylation of four serine residues is essential for both $cPLA_2$-translocation and activity. MAPKs, in particular p38 MAPK, MAPK activated protein kinases, and Ca^{2+}/calmodulin kinase-II, are responsible for $cPLA_2$ phosphorylation [133] (Fig. 4). Once released, the AA can be further converted by one of three major enzymatic pathways: the cyclooxygenase, the lipoxygenase or the cytochrome P450 mono-oxygenase pathway which are responsible for the production of eicosanoids [82]. Besides their role in inflammatory signalling pathways, lipoxygenases (LOXs) are also involved in the mobilization of lipids and peroxidation reactions [136]. LOXs constitute a family of monomeric non-heme, non-sulphur iron dioxygenases that catalyze the conversion of polyunsaturated fatty acids into conjugated hydroperoxides [136]. Nevertheless, the main substrate of LOXs in mammals is arachidonic acid, either in esterified or free form depending on the type of LOX. LOX activity depends on the presence of Ca^{2+} which concentration increases following the generation of free fatty acids as this causes a change in cytosolic pH with concomitant activation of acid-sensing ion channels and subsequent influx of Ca^{2+} [113] (Fig. 4). LOX activity is also regulated by the hydroperoxide tone of the cells as the presence of some hydroperoxides is necessary for converting the non-heme iron from the resting Fe^{2+} to the active Fe^{3+} oxidation state [137]. Lipid peroxidation can be switched from an enzymatic to a nonenzymatic form by inactivating LOXs. Liberated iron ions then react with lipid hydroperoxides, starting a chain reaction. Nonenzymatic lipid peroxidation can also be initiated by hydroxyl radicals generated by dysfunctional mitochondria. These radicals attack unsaturated fatty acids, generating lipid peroxides [138].

Lipid hydroperoxidation may lead to disruption of organelle and plasma membranes [138–140], key features of necrosis. The involvement of LOXs in TNFα-induced

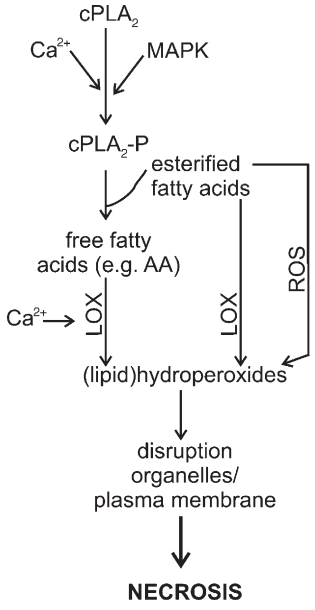

Fig. 4. The roles of phospholipases A_2 and lipoxygenases in necrosis. Phospholipases A_2 (PLA_2) are responsible for liberating arachidonic acid (AA) from the sn-2 position of phospholipids. Translocation of $cPLA_2$ to the membranes, which enables it to interact with its substrates, is essential for the release of AA from membranes. Ca^{2+} is essential for the translocation of $cPLA_2$ but not for its activity. On the other hand, phosphorylation of four serine residues is essential for both the translocation and activity of $cPLA_2$. MAPKs are responsible for $cPLA_2$ phosphorylation. Lipoxygenases (LOXs) catalyze the conversion of polyunsaturated fatty acids into conjugated hydroperoxides. AA is the main substrate of LOXs, either in its esterified or free form, depending on the type of enzyme, and PLA_2s are therefore considered upstream components of the LOX signalling pathway. Generation of lipid hydroperoxides can also occur by nonenzymatic lipid peroxidation processes. Dysfunction of mitochondria can cause the generation of hydroxyl radicals, which attack unsaturated fatty acids to generate lipid peroxides. Lipid hydroperoxidation may disrupt organelle and plasma membranes.

apoptosis and other apoptotic pathways has been reported several times [138,141]. Treatment of L929 cells with TNFα leads to activation of PLA_2, and overexpression of $cPLA_2$ sensitized TNFα-resistant L929 variants to TNFα-induced necrosis [142,143]. $cPLA_2$ was also shown to play a major role in TNFα-induced necrosis of MCF7 cells [144] and in chemically-induced and oxidant-induced renal epithelial cell necrosis [145,146]. A contribution of $cPLA_2$ to TNFα-induced lethal shock was also proven *in vivo* [87]. Additionally, injury after brain ischemia was proven to be decreased in $cPLA_2^{-/-}$ mice [147]. Besides a role for $cPLA_2$ in necrosis, a contribution of $iPLA_2$ has been demonstrated in several caspase-independent cell death signalling pathways leading to nuclear shrinkage [147]. We recently showed that activation of a PLA_2/LOX pathway contributes to TNFα-induced necrotic death of L929 cells [86].

3.4. Proteases

Calpains are intracellular cysteine proteases ubiquitously and constitutively expressed in mammalian cells. They are

believed to participate in various signalling pathways mediated by Ca^{2+} by modulating the activities and/or functions of other proteins [148]. They are present in the cytosol as inactive precursors that are activated by increased cytosolic Ca^{2+} [149,150]. Extreme conditions often result in elevation of cytosolic Ca^{2+} levels and overactivation of calpains. The latter then cleave the Na^{+}/Ca^{2+} exchanger, which is required for extrusion of Ca^{2+}, thus leading to a sustained increase in intracellular Ca^{2+} [151]. Calpains were shown to fulfil important roles in necrotic cell death in neurons of *C. elegans* [152] and in necrosis of dystrophin-deficient muscles [153]. Direct evidence for calpain activation preceding ischemic neuronal death was also obtained from studies on cornu ammonis (CA) 1 neuronal death in monkeys [154]. Immunoelectron microscopy showed that activated μ-calpain was localized at the vacuolated or disrupted lysosomal membrane [155,156]. In addition, this type of necrotic cell death was attended by lysosomal leakage of cathepsin B [157]. Lysosomal membrane permeability (LMP) does not seem to be a secondary response, as inhibitors of cathepsins B and L significantly reduced neuronal death [155,157]. Yamashima and colleagues therefore postulated a "calpain–cathepsin hypothesis", suggesting that necrotic insults causing excessive Ca^{2+} overload lead to calpain-mediated lysosomal disruption with consecutive release of cathepsins B and L [157]. Cathepsin B was also shown to be involved in caspase-independent cell death induced by death receptor ligands [158]. Increased activity of calpains is also observed in certain neurodegenerative diseases, such as Parkinson's [159] and Alzheimer's diseases [160,161]. Whether this activity is strictly linked to the occurrence of necrosis during these pathologies remains unclear.

In addition, oxidative stress induces the release of lysosomal enzymes both *in vitro* [162] and *in situ* [163], and depending on the severity of the insult, apoptotic or necrotic cell death can ensue [164]. Addition of low concentrations of O-methyl-serine dodecylamide hydrochloride (MSDH), a lysosomotropic agent [165], results in partial LMP, activation of caspases, and apoptosis. At high concentrations, MSDH causes extensive LMP and necrosis. LMP can also activate MPT, and thus lead to cell death [166]. Besides the release of cathepsins, LMP is also associated with the activation of PLA_2, which in turn can again increase the production of ROS [167], thus establishing a vicious cycle of oxidative stress and damage. Thus it is likely that not only calpain proteolysis but also oxidative stress is capable of provoking lysosomal membrane damage leading to neuronal death.

The Nec protein is a serine protease inhibitor controlling the Toll-mediated immune response of *Drosophila* [168]. Mutations in *nec* cause the formation of patches of epithelial necrosis and consequent death of the fly [169], indicating that serine proteases may be involved in the execution phase of necrotic cell death. This is in line with the inhibition of TNFα-induced necrosis of L929 cells in the presence of serine protease inhibitors [28,96,170].

3.5. Ceramide

Ceramide, which is considered a second messenger, is generated mainly by hydrolysis of membrane sphingophospholipid sphingomyelin (SM) by sphingomyelinase (SMase), a SM-specific form of phospholipase C (PLC). Ceramide was also shown to play a pivotal role in the TNFα-induced caspase-independent death of L929 cells [171,172]. $cPLA_2$ activity seemed to be necessary for ceramide generation [172] (Fig. 5). TNFα in combination with zVAD-fmk even induced a much more pronounced increase of intracellular ceramide than TNFα alone [173], and was associated with a much more rapid killing of L929 cells [29]. In line with a causative role for ceramide in these cell death processes, L929 clones overexpressing acid ceramidase (AC) were more resistant to TNFα/zVAD-fmk than parental cells. Acid ceramidase degrades ceramide generated by acid sphingomyelinase (A-SMase) in response to TNFα [171]. Sphingomyelinase-deficient cells were also pronouncedly more resistant to TNFα/zVAD-fmk than WT cells, and pharmacological agents inhibiting A-SMase activity also enhanced resistance [173]. Comparable results were obtained in murine NIH3T3 fibroblasts, in human leukemic Jurkat T cells stimulated with TNFα/zVAD-fmk, and in FADD-deficient Jurkat cells [173]. Ceramide accumulation starts well before the onset of cell death, indicating that ceramide represents a cause rather than a consequence of caspase-independent death.

Interfering with RIP1 by different methods, such as addition of GA or RC or RIP1 RNAi, conferred protection against TNFα/zVAD-fmk-induced generation of ceramide and caspase-independent death in all types of cells studied [173]. RIP1

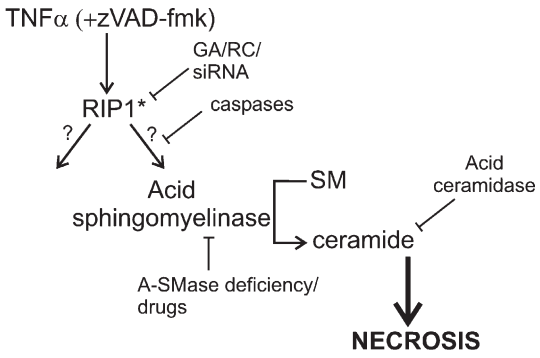

Fig. 5. Ceramide, an important player in necrotic signalling pathways. Ceramide is generated mainly by hydrolysis of the membrane sphingophospholipid sphingomyelin (SM) through the action of a sphingomyelinase (SMase), a SM-specific form of phospholipase C. Ceramide plays a pivotal role in TNFα-induced caspase-independent death of L929 cells. TNFα in combination with zVAD-fmk induced a much more pronounced increase of intracellular ceramide than TNFα alone. Acid ceramidase-overexpressing cells and SMase-deficient cells are more resistant to TNFα/zVAD-fmk than parental cells. Pharmacological agents inhibiting A-SMase activity also enhanced resistance (D609: inhibits PLC, an enzyme upstream of A-SMase, thus indirectly inhibiting TNFα-dependent activation of A-SMase [238]; desipramine: inhibits A-SMase by inducing proteolytic degradation of the enzyme [239]). Interference with RIP1 by different means, such as addition of GA, RC or RIP1 RNAi, and the use of RIP1 deficient cells uniformly conferred protection against TNFα/zVAD-fmk-induced ceramide generation. RIP1 is thus essential for TNFα- and RIP1-induced ceramide production. (See text for details).

deficient cells were also resistant. RIP1 is thus essential for induction of ceramide production by TNFα, and both RIP1 and ceramide mediate their death signals through the same pathway, which is conserved across different cell types. As RIP1 appears to be a central initiator of necrosis, one may speculate that RIP1 phosphorylates and activates $cPLA_2$, which in turn mediates the generation of ceramide. Because inhibition of ceramide accumulation clearly diminished caspase-independent PCD but not as completely as inhibition of RIP1, ceramide obviously represents a central factor, but most likely not the only one, transmitting the death signals generated by RIP1 in response to TNFα. Besides, addition of ceramide analogues or induction of intracellular ceramide accumulation were shown to mediate caspase-independent death in several cell types other than L929, Jurkat and NIH3T3 cells [174,175]. The fact that BHA protects against TNFα-induced cytotoxicity in L929 and NIH3T3 cells [86,173,176] but has no effect in Jurkat and U937 cells [173,177] indicates that mechanisms of ceramide-induced death may differ between cell types.

4. Involvement of the cytoskeleton in the execution of necrosis

Redistribution of mitochondria from a dispersed state to perinuclear clustering has been associated with TNFα-induced necrosis [178]. Disrupting microtubules with nocodazole and inhibiting the motor protein kinesin can each block TNFα-induced perinuclear redistribution of mitochondria in necrosis [178]. TNFα also induces persistent phosphorylation of oncoprotein 18 (Op18), a phosphorylation-responsive regulator of microtubule dynamics [179]. Hyperphosphorylation of Op18 promotes cell death. Since hyperphosphorylation diminishes the ability of Op18 to destabilize microtubules, stabilization of the microtubule network is believed to be associated with TNFα-induced necrosis [179].

The cytoskeletal protein filament β-actin appears to function downstream of TNF-R1 and upstream of TNFα-induced mitochondrial changes during TNFα-induced necrosis of L929 cells, as mutations in β-actin impeded perinuclear redistribution of mitochondria. Moreover, ROS production in TNFα-treated $actin^{Mut}$ cells was much less than in parental cells [180]. As zVAD-fmk does not have any effect on perinuclear distribution of mitochondria but dramatically enhances TNFα-induced ROS production [178], it can be suggested that ROS production is a later or separate event of perinuclear redistribution of mitochondria. Actin mutation in L929 cells did not lead to a global change in TNFα signalling: TNFα-induced activation of NF-κB and p38 was normal in the mutant [180].

5. Immune response concomitant with necrotic cell death

5.1. Activation of inflammatory signalling pathways by necrotic cells

Externalization of phosphatidylserine, the hallmark of apoptosis, designates a cell with an “eat-me” label and is a very early feature of apoptotic death [181]. In contrast, necrotizing cells are phagocytosed only after loss of membrane integrity by a macropinocytotic mechanism involving formation of multiple ruffles directed towards necrotic debris [182]. This means that uptake is delayed and less efficient [19]. Observations of PS exposure before loss of cell membrane permeability in necrotic death [183,184] indicate that PS exposure may be important for recognition, but additional factors may be necessary for uptake. This idea is supported by the observation that PS exposure is not sufficient to ensure clearance [185,186], and that the PS receptor is not required for recognition and clearance of apoptotic cells [187]. The late uptake of necrotic cells allows the dying cells to activate pro-inflammatory and immuno-stimulatory responses [18] whereas apoptotic cell death is immunologically and inflammatorily silent [188].

Exposed or released intracellular components are potential sources of autoantigens that might be processed and presented to initiate an autoimmune reaction [189]. During cell death, several post-translational modifications occur, such as hyperphosphorylation, (de)ubiquitination, methylation, citrullination, transglutaminase crosslinking and proteolytic cleavage [190,191]. These modifications can increase the risk of an autoimmune response, especially when repeatedly presented to the immune system in a proinflammatory context [192]. On the other hand, increased transglutaminase activity might also assure tissue integrity maintenance, limiting massive necrosis and reducing the inflammatory response [193]. Further studies are necessary to clearly demonstrate the function of increased transglutaminase activities during necrotic cell death processes.

Spillage of the contents of necrotic cells into the surrounding tissue activates inflammatory signalling pathways. Depending on molecular signals from necrotic cells, diverse types of immune cells (neutrophils, macrophages, dendritic cells) become involved in the immune response. In contrast, apoptotic cells induce antigen presenting cells (APCs) to secrete cytokines that inhibit Th1 responses [194]. Immature dendritic cells efficiently phagocytose a variety of apoptotic and necrotic tumour cells, but only the latter induce maturation and optimal presentation of tumour antigens [195,196].

High mobility group box I (HMGB1) is one of the proinflammatory molecules passively released by necrotic cells [197]. It is both a nuclear factor and a secreted protein. In the nucleus it acts as an architectural chromatin-binding factor that bends DNA, stabilizes nucleosomes and facilitates transcription [198]. Outside the cell, it binds with high affinity to receptor for advanced glycated end products (RAGE), TLR2 and TLR4, and is a potent mediator of inflammation, cell migration and metastasis [199–201]. HMGB1 was shown to play a major role in sepsis and synovial inflammation in arthritis [202]. Apoptotic cells do not release HMGB1, which may help avoid inflammation even if phagocytic clearance fails [197]. However, a recent study performed in our laboratory showed that both primary and secondary necrotic cells (L929 and HeLa) release HMGB1, indicating that HMGB1 is released passively following membrane rupture (Vanden Berghe T., unpublished data). The reason for this discrepancy is not clear yet.

Moreover, Hsp70, Hsp90, gp96 and calreticulin specifically released by necrotic cells [195], activate APCs, including DCs

[196,203–205]. mRNA released from necrotic cells is a potent host-derived activator of TLR3, inducing the activation of NF-κB and the expression of TNFα, IRF-1 and IL-1 receptor associated kinase-M (IRAK-M) in DCs [206]. This results in DC activation with concomitant secretion of IFNα.

However, the activation of macrophages by "danger signals" present in the extracellular environment due to spillage of necrotic debris remains controversial. We and others showed that exposure of macrophages to necrotic cells is not sufficient to trigger macrophage activation and the concomitant induction of proinflammatory cytokines [19,20]. Rather, necrotic cells trigger the enhanced secretion of proinflammatory cytokines from independently activated macrophages [20].

It is also conceivable that the active release of proinflammatory cytokines from necrotic cells due to the activation of NF-κB and MAPKs [21] (see below) may be crucial for an inflammatory response. In contrast, binding of apoptotic cells to macrophages immediately leads to inhibition of proinflammatory cytokine gene transcription [188], underscoring the innate immune discrimination of apoptotic cells. These observations are in agreement with the inhibition of translation during apoptosis, but not during necrosis [123,207,208]. Apoptotic signalling thus negatively regulates the release of inflammatory cytokines by down-regulating the transcription and translation machineries.

5.2. Activation of proinflammatory pathways can be uncoupled from the activation of necrotic cell death

The role of MAPKs in cell death signalling pathways is not yet clear [209]. It was suggested that transient activation of p38 MAPK or JNK can protect from cytotoxicity [210], whereas their prolonged activation may induce apoptotic death [211]. Sustained activation of both p38 MAPK and JNK is observed following treatment of MEF cells with the DNA-alkylating agent N-methyl-N′-nitro-N-nitrosoguanidine (MNNG). However, only JNK contributes to the MNNG-induced necrosis, as demonstrated by the survival of JNK1$^{-/-}$ and JNK2$^{-/-}$ MEFs, but not p38$^{-/-}$ MEFs, following MNNG treatment [61]. During TNFα-, TNFα/zVAD-fmk and IFNβ/dsRNA-induced necrosis, early and transient activation of p38 MAPK and JNK could be seen. However, this was not the case for FasL/zVAD-fmk-induced necrosis [21], indicating that these pathways are not absolutely required. TNFα also induced a secondary prolonged activation of p38 MAPK, which was absent for JNK. Prolonged activation of JNK has been attributed to accumulation of ROS. The ROS hamper the function of phosphatases that are important for inactivating MKK, which is involved in the phosphorylation of JNK [212,213]. NF-κB counteracts this process [212,213]. This may explain why a secondary peak of JNK activation does not occur during necrotic death of L929 cells, which is associated with high levels of NF-κB [21]. Inhibition of NF-κB activation blocked IL-6 production but did not block TNFα-induced necrosis, instead it sensitized for it. This observation supports the finding that NF-κB may act to inhibit ROS accumulation [212,213] and thus may have anti-necrotic properties [31]. Necrotic cell death was not affected by inhibition of p38 MAPK [21]. These data clearly show that activation of proinflammatory pathways could be uncoupled from activation of necrotic cell death following certain stimuli.

6. Occurrence of necrosis in physiological and pathophysiological conditions

Necrotic cell death has long been described as a consequence of physico-chemical stress, such as freeze–thawing or severe hyperthermia [14], and thus as accidental and uncontrolled [214]. However, necrotic cell death has emerged as an important and physiologically relevant signalling process that seems to contribute to ovulation [215], immune defence [216,217], death of chondrocytes controlling the longitudinal growth of bones [218], and cellular turnover in the intestine [219]. In addition, removal of interdigital cells in the paws of Apaf 1$^{-/-}$ mice during embryogenesis occurs by a caspase-independent necrotic-like process [220]. Noteworthy, the occurrence of necrosis in these *in vivo* models was mostly defined morphologically. Several reports also illustrate the occurrence of necrotic cell death during viral and bacterial infections. HIV-1 was shown to kill CD4^{+} T lymphocytes by necrosis [221–223] and *Shigella* and *Salmonella* can induce necrotic cell death of infected neutrophils and macrophages, respectively [224,225]. Caspase-independent death is also strongly induced in ischemia–reperfusion injury after cerebral ischemia and myocardial infarction, ROS-induced injury, and glutamate excitotoxicity

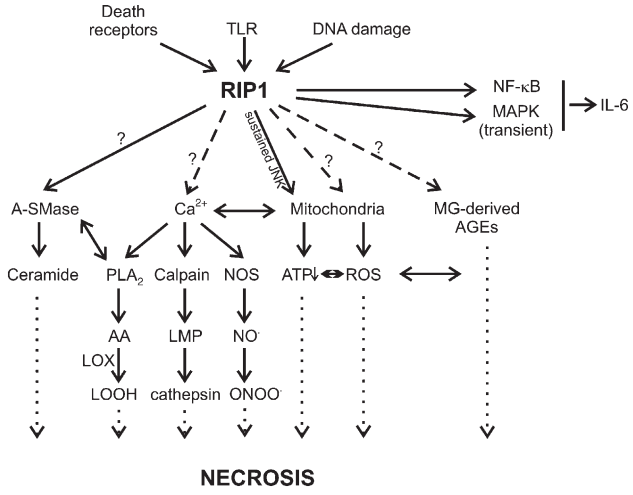

Fig. 6. Necrotic cell death is the result of interplay between several signalling cascades. RIP1 appears to be a central initiator of necrosis. RIP1 has been implicated in the generation of ceramide upon TNFα-induced necrosis, and its kinase activity was shown to be crucial for Fas-, TNF-R1- and TRAIL-R-induced necrosis. Necrotically dying cells can actively release inflammatory cytokines due to the activation of NF-κB and MAPKs, a process in which RIP1 is also implicated. ROS and Ca^{2+} are main players during the propagation and execution phases of necrotic cell death. ROS can be produced in the cytosol when the glycolytic rate is high, but mitochondria are the main producers of ROS. An increase in cytosolic Ca^{2+} concentrations can increase oxidative stress by activating NOS, or by affecting mitochondrial respiration. On the other hand, Ca^{2+} contributes to the activation of proteases, $cPLA_2$ or lipoxygenases, which in turn disrupt organelle and cell integrity by damaging protein structures or lipid bilayers. (See text for details).

[53–55,226]. Besides, diseases such as epilepsy [227], Alzheimer's disease [228] and other inflammatory injuries [229] are also attended by necrosis.

7. Conclusion

Necrotic cell death is not due to one well-described signalling cascade but is the result of interplay between several signalling pathways. With our present knowledge of necrotic cell death, it is impossible to clearly distinguish between the initiation, propagation and execution phases of necrotic cell death (Fig. 6). In particular, differences between propagation and execution events are not always clear-cut, for example, ROS can either act as propagators by activating lipoxygenases [137,141], or as executioners by directly modifying organelle or plasma membranes [138] or proteins [77]. RIP1 appears to be a central initiator of necrosis. It has been implicated in the generation of ceramide during TNFα-induced necrosis [173], and its kinase activity has been shown to be crucial for Fas-, TNF-R1- and TRAIL-R-induced necrosis [12,43]. Identifying substrates of RIP1 will thus give more insights into downstream signalling pathways contributing to death receptor-induced necrotic cell death. Additionally, RIP1 is involved in necrotic cell death induced by TLR stimulation and DNA damage [48,57,61]. Besides the capacity of necrotically dying cells to induce an inflammatory response upon lysis and spillage of their contents, they can also actively release inflammatory cytokines due to the activation of NF-κB and MAPKs [21], a process that also involves RIP1 [38–40,230–232].

ROS and Ca^{2+} are two main players during the propagation and execution phases of necrotic cell death. ROS can be produced in the cytosol if the glycolytic rate is high, but they are produced mainly by the mitochondria. Higher cytosolic Ca^{2+} concentrations can increase oxidative stress by activating NOS or by affecting mitochondrial respiration. On the other hand, Ca^{2+} contributes to the activation of proteases, $cPLA_2$ and lipoxygenases, which in turn disrupt organelle and cell integrity by damaging protein structures or lipid bilayers [106]. Calcium thus acts mainly as an amplifier of necrotic signalling cascades.

Induction of necrotic cell death may be of utmost importance upon, for example, viral or bacterial infection. In order to avoid host cell apoptosis, several viruses encode caspase inhibitors. Induction of necrotic cell death under these circumstances will activate innate and adaptive immune responses, which are crucial for conquering the infection and promoting recovery of the host. On the other hand, inefficient clearance of necrotic cells may contribute to the spread of a pathogen and generalized infection. Moreover, stimulation of a caspase-independent pathway may also contribute to eradication of certain tumour cells, which often evade apoptosis by increasing expression of anti-apoptotic proteins (e.g. XIAP, IAP1, IAP2, Bcl-2, Bcl-x_L) or accumulating mutations in crucial pro-apoptotic proteins (e.g. p53 and Bax) [233,234]. Approaches for inducing necrotic cell death in cancer cells include administering alkylating DNA-damaging agents [235] and photosensitizing molecules that preferentially accumulate in tumour cells and generate ROS following excitation with light from various spectra [236,237].

Unravelling in more detail several caspase-independent death pathways may thus help us to develop new therapeutic options for preventing cell death in neurodegenerative diseases, killing tumour cells, coping with pathogenic infections, and various other applications.

Acknowledgments

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