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Review

Programmed cell death and its possible relationship with periodontal disease

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Abstract: Cell death occurs in physiological conditions and as a result of injury or disease. Programmed cell death has an important role in the development and homeostasis of human tissue. Aberrant regulation of this process is thought to cause numerous diseases, including developmental disorders, neurodegenerative disease, and cancer. Apoptosis is the main type of programmed cell death and is well understood. However, recent intensive studies have revealed other types of programmed cell death. Here, we include an overview of three types of programmed cell death: apoptosis, necroptosis, and autophagic cell death. We also provide information on damage-associated molecular patterns (DAMPs), which have pro-inflammatory effects and are reportedly associated with cell death. Finally, we discuss the link between programmed cell death and periodontal disease and propose a hypothetical role for programmed cell death and DAMPs—which are released from cytoplasm of necrotic cells—in periodontal disease initiation. (J Oral Sci 54, 137-149, 2012)

Keywords: programmed cell death; apoptosis; necroptosis, autophagic cell death; damage-associated molecular patterns (DAMPs).

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Introduction

Ever since Kerr et al. described the feature of cell death as apoptosis (1) and studies of *Caenorhabditis elegans* by Horvitz et al. established that apoptosis is genetically programmed and regulated (2), cell death has been functionally categorized as apoptosis or necrosis. In virtually all recent biology textbooks, apoptosis is described as the sole form of programmed cell death, and necrosis is described as passive cell death caused by physical trauma (3). However, intensive research over the last two decades has largely elucidated the apoptotic mechanism and revealed other types of programmed cell death.

The current intense focus on non-apoptotic programmed cell death, e.g., necroptosis and autophagy, is changing the recognition and definition of programmed cell death. Here, we describe current knowledge of the different facets of programmed cell death and propose a possible link between programmed cell death and periodontal disease.

Apoptosis

Apoptosis is a crucial cellular event during development and tissue homeostasis. For example, in the developing human embryo, cells between the fingers apoptose during finger differentiation, which results in separation of the digits. Furthermore, when cells are subjected to various cellular stresses, such as DNA damage, they undergo apoptosis to prevent adverse effects on the host. Apoptotic cells have characteristic morphological features, including rounding of cells and decreased cellular volume, chromatin condensation and

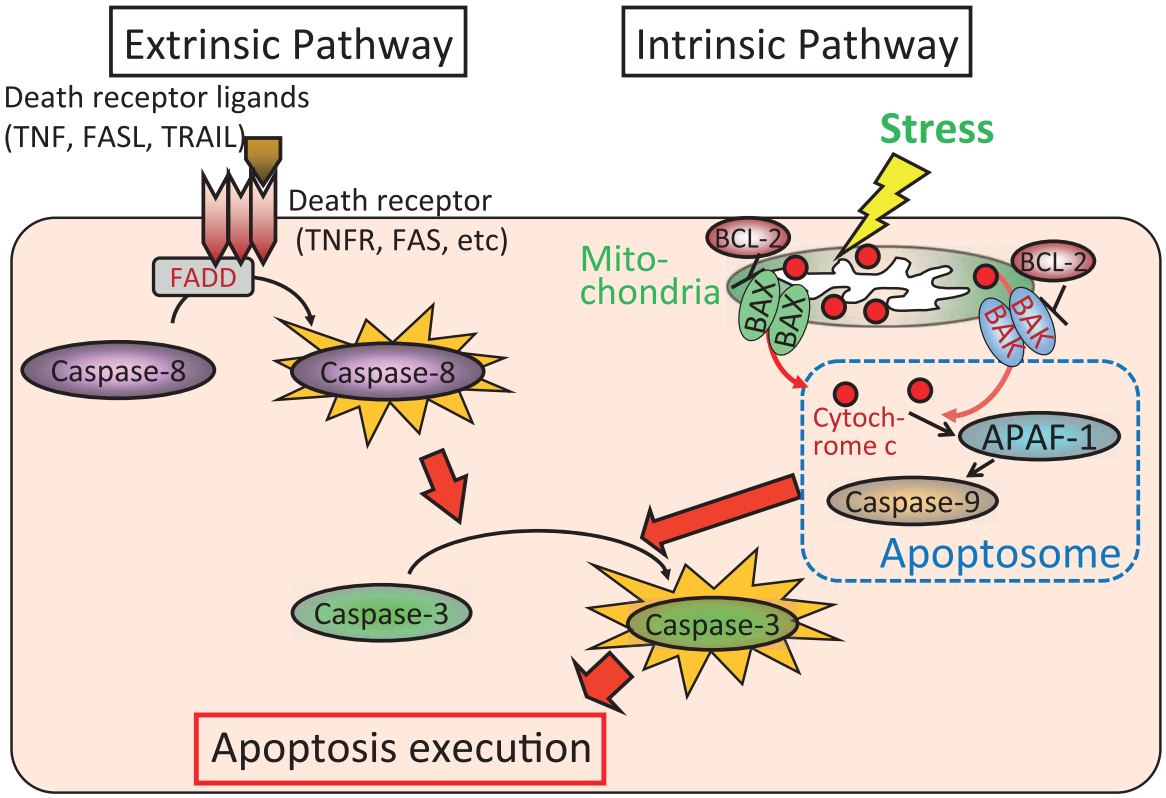

Fig. 1 Schematic representation of two major apoptotic signaling pathways. Apoptosis can be induced in response to various stimuli from inside or outside the cell. In the extrinsic pathway, binding of death ligands (TNF, FASL, and TRAIL) to death receptors (TNFR, FAS, etc) results in recruitment of the adaptor molecule FADD and caspase-8. Caspase-8 is then activated and initiates apoptosis by cleavage of downstream effector caspases, such as caspase-3. The effector caspases initiate apoptosis. Mitochondria are involved in the intrinsic pathway, which can be triggered by a variety of stress stimuli, such as oncogenes, ultraviolet radiation, γ-irradiation, DNA damage, hypoxia, survival factor deprivation, and chemotherapeutic agents. Stresses activate BH3-only proteins, which, in turn, activate BAX and BAK. Active BAX and BAK alter mitochondrial permeability, leading to release of cytochrome c from the intermembrane space of mitochondria to cytoplasm. Released cytochrome c binds to APAF-1 to facilitate formation of apoptosomes, which activate caspase-9. Activated caspase-9 then cleaves caspase-3, resulting in the downstream events involved in apoptosis. Anti-apoptotic BCL-2 family members (BCL-2 and BCL-XL) prevent cytochrome c release, presumably by binding to and thus inhibiting BAX and BAK.
Abbreviations: TNF, tumor necrosis factor; FASL, FAS ligand; TRAIL, TNF-related apoptosis-inducing ligand; TNFR, TNF receptor; FADD, FAS-associated protein with death domain; BH3, BCL-2 homology domain 3; BAX, BCL-2-associated X protein, BAK, BCL-2 homologous antagonist/killer; APAF-1, apoptotic protease-activating factor 1; BCL-2, B-cell lymphoma 2; BCL-XL, B-cell lymphoma-extra large.

nuclear fragmentation, blebbing of the intact plasma membrane, and shedding of vesicles containing intracellular matrix (apoptotic bodies). The apoptotic bodies are then scavenged by phagocytes. Two major signaling pathways (extrinsic and intrinsic) initiate apoptosis (Fig. 1) (3,4). The extrinsic pathway is triggered by the binding of a so-called death ligand, such as FAS ligand (FASL), to its cell surface transmembrane death receptor (FAS). Triggering of the death receptor activates the cysteine protease caspase-8, which cleaves and activates downstream caspase-3, resulting in execution of apoptosis. For example, active caspase-3 activates caspase-activated DNase and causes DNA fragmentation, which is an apoptotic hallmark indicative of chromatin condensation.

The intrinsic pathway involves mitochondria (Fig. 1). In a viable cell, members of the pro-apoptotic B-cell lymphoma 2 (BCL-2) protein family—BAX, BAK, and BH3-only proteins (which contain only a BH3 domain and none of the other domains usually found in proteins with BCL-2 homology domains in the BCL-2 protein family)—are antagonized by anti-apoptotic members such as BCL-2 and B-cell lymphoma-extra large (BCL-

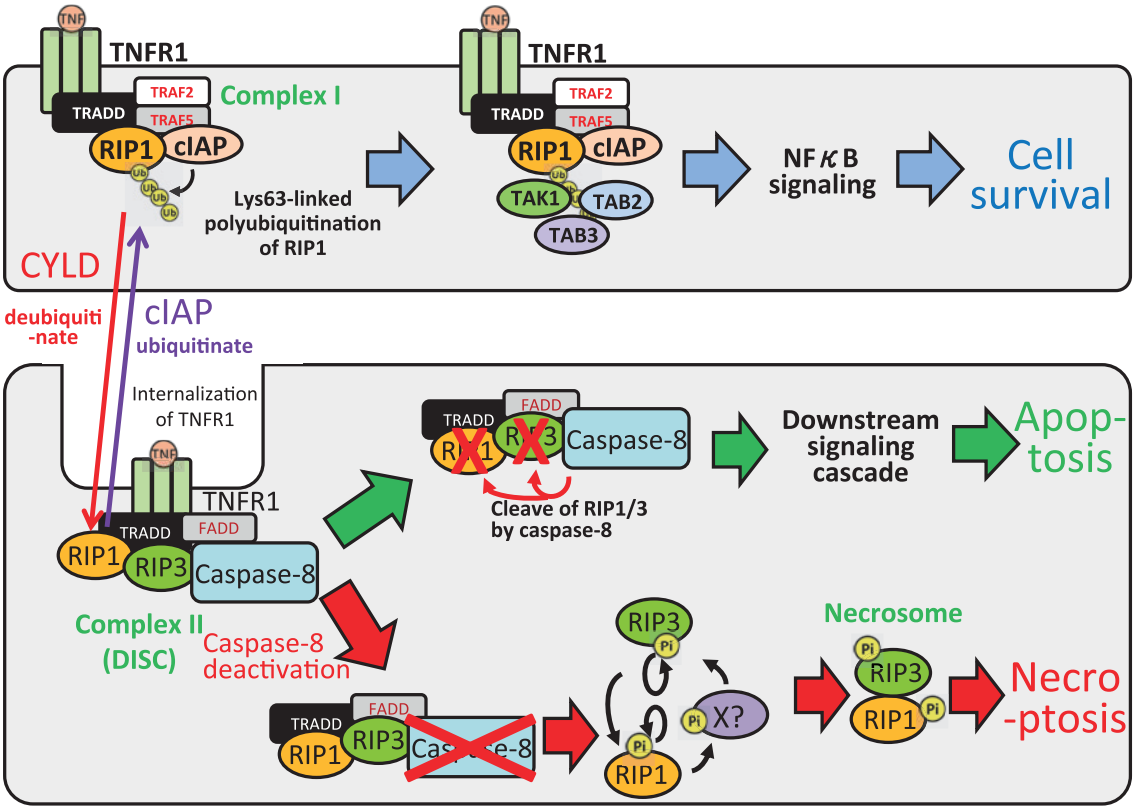

Fig. 2 Molecular signaling pathway of necroptosis. Ligand-bound TNFR1 recruits TRADD, RIP1, TRAF2, TRAF5, and cIAP to the activated receptor at the cell surface, and the recruited proteins form complex I. In the complex, cIAPs (E3 ubiquitin ligases) polyubiquitinate RIP1. TAK1, TAB2, and TAB3 form a complex by binding to polyubiquitinated RIP1, and the complex triggers canonical NF-κB activation, resulting in induction of transcription of cytoprotective genes and subsequent facilitation of cell survival. After ligand binding to TNFR1, complex I is internalized by endocytosis, which leads to rearrangement of complex I to complex II (DISC, comprising FADD, TRADD, RIP1, RIP3, and caspase-8). The deubiquitination status of RIP1 is critical in the transition between complex I and complex II, and this status is modified by cIAP and CYLD; cIAPs polyubiquitinate RIP1, which is deubiquitinated by CYLD. Normally, caspase-8 is activated in complex II, and the active caspase-8 digests RIP1 and RIP3, resulting in apoptosis by activation of downstream apoptotic signaling. When caspase-8 is genetically or pharmacologically blocked, RIP1 and RIP3 are phosphorylated to generate a molecular complex called a necrosome, resulting in necroptosis.
Abbreviations: TRADD, tumor necrosis factor receptor type 1-associated death domain; RIP, receptor-interacting protein; TRAF, TNF receptor-associated factors; cIAP, cellular inhibitors of apoptosis; TAK, transforming growth factor β-activated kinase; TAB, transforming growth factor β-activated kinase-binding protein, NF-κB, nuclear factor-κ B; DISC, death-inducing signaling complex; TNFR, TNF receptor; FADD, FAS-associated protein with death domain; CYLD, cylindromatosis.

XL). Cellular stresses (such as oncogenes, ultraviolet radiation, γ-irradiation, DNA damage, hypoxia, survival factor deprivation, and chemotherapeutic agents) activate BH3-only members, and these activated BH3-only proteins prevent anti-apoptotic BCL-2 members from binding to them. Subsequently, the pro-apoptotic proteins BAX and BAK are thought to change their conformations, oligomerize, and insert themselves into the mitochondrial membrane, where they form pores. The pores alter the permeability of the mitochondrial membrane, which results in cytochrome c release from the mitochondria into the cytosol. Cytochrome c then binds to apoptotic protease activating factor-1 (APAF-1), and this complex binds to caspase-9 to form an apoptosome. In the apoptosome, caspase-9 is activated, and the activated caspase-9 subsequently activates the effector caspase-3, leading to the execution of apoptosis.

The central role of caspase-3 in inducing apoptosis has been revealed by studies of caspase-3 knockout mice. Cells from these mice exhibit defects in apoptosis (5,6). For example, their hepatocytes and thymocytes were much less susceptible than those of wild-type cells

to death receptor-induced apoptosis (5,6). However, the phenotype of caspase-3 knockout mice was restricted: no marked abnormalities were found in the hearts, lungs, livers, or kidneys of embryos, although some effect was seen in the central nervous system (5,6). *bak/bax* double-knockout mice (in which the intrinsic apoptotic signaling pathway is blocked) exhibited a delay of only 1 day in the disappearance of cells between fetal fingers (7). These results suggest that other types of programmed cell death may compensate for the backup mechanisms of apoptosis. Starting in the next section, we present an overview of representative forms of non-apoptotic programmed cell death.

Programmed necrosis (necroptosis)

Necrosis has been defined as incidental cell death caused by external factors, such as severe energy shortage and physical injury (3). Necrotic cells exhibit some common morphological features, including increased cell volume, swelling of organelles (such as mitochondria), random fragmentation of cellular DNA, and bursting of the cytoplasmic membrane (8,9). Moreover, compromising the integrity of cell membranes causes leakage of cytosolic components such as damage-associated molecular patterns (DAMPs), which can induce inflammation and an immune response (10,11). Therefore, necrotic cells induce inflammation in adjacent tissue.

Necrosis was long believed to result only from accidental cell death. However, in 1988 Laster et al. reported that tumor necrosis factor (TNF) induced both apoptosis and necrosis, which suggested the existence of controlled necrosis (12). Stimulation of death receptors (FAS or TNF receptor) by their cognate ligands activates an extrinsic apoptotic pathway that induces sequential activation of multiple caspases (13-18). However, an increasing number of studies have reported that caspase inhibition does not completely abolish cell death (19-21). Holler et al. discovered that FASL, TNF-α, and TNF-related apoptosis-inducing ligand (TRAIL) induced necrotic death of Jurkat cells in the presence of the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp (-OMe)-fluoromethylketone (zVAD-fmk) (21).

Moreover, TNF-α stimulation leads directly to death of Jurkat cells defective in both caspase-8 and FAS-associated protein with death domain (FADD), an adaptor molecule that bridges death receptors to caspase-8 (21). Similar report showed that inhibition of caspases does not block death-ligand-induced cell death and leads to necrosis of fibroblasts and monocytes (22). Holler et al. also found that death-ligand-induced necrotic death of caspase-deficient cells requires the activity of the receptor-interacting protein-1 (RIP1), a serine/threonine kinase (21). In 2003, Chan et al. demonstrated that RIP1 was required for TNF-induced necrotic cell death, which the authors referred to as "programmed necrosis" (23). The term "necroptosis" was first used for the programmed necrosis described by Degterev et al. (24).

The molecular mechanisms responsible for initiating necroptosis are now being identified (Fig. 2). The most extensively investigated model of necroptosis involves activation of TNF receptor 1 (TNFR1) (25). The signaling complex recruited to the activated TNFR1 consists of the TNFR-associated death domain (TRADD), RIP1, TNFR-associated protein 2 (TRAF2), TRAF5, and cellular inhibitor of apoptosis proteins (cIAPs) at the cell surface (complex I) (18,25). The cIAPs (i.e., E3 ubiquitin ligases) catalyze Lys63-linked polyubiquitination of RIP1 at Lys377 (26). The polyubiquitinated RIP1 provides a binding site for transforming growth factor-β-activated kinase 1 (TAK1), TAK1-binding protein 2 (TAB2), and TAB3. The TAK1-TAB2-TAB3 complex triggers canonical nuclear factor-κB (NF-κB) activation (27), and activated NF-κB induces transcription of cytoprotective genes and facilitates cell survival. After ligand binding to TNFR1, the complex is then internalized by endocytosis (28). This internalization leads to rearrangement of complex I composition to complex II (also called death-inducing signaling complex [DISC]), which comprises FADD, TRADD, RIP1, RIP3, and caspase-8 (18,29). The ubiquitination state of RIP1 is critical for the transition from complex I to complex II (30-32). The cylindromatosis (CYLD) protein most potently deubiquitinates RIP1 (33). Normally, procaspase-8 is activated in complex II, and the activated caspase-8 cleaves RIP1 and RIP3 in the complex II to inhibit their activities, resulting in apoptosis via activation of downstream apoptotic signaling (32,34). Cellular FADD-like interleukin-1 β-converting enzyme (FLICE)-inhibitory protein (cFLIP) competitively binds to FADD and inhibits procaspase-8 activation (35). When caspase-8 is blocked by genetic factors (e.g., expression of cytokine response modifier protein A [CMPA]) or pharmacological factors (e.g., treatment with pan-caspase inhibitor, zVAD-fmk), RIP1 and RIP3 are phosphorylated and generate a molecular complex called a necrosome (20,21). The formation of a necrosome initiates necroptosis (20,21). Despite exhaustive research efforts, there are limited data on the molecular events that occur downstream in necroptosis.

Production of reactive oxygen species (ROS) may have a role in necroptosis. Kamata et al. demonstrated that ROS promote TNF-α-induced cell death by sustaining c-Jun N-terminal kinase (JNK) activation (36). ROS

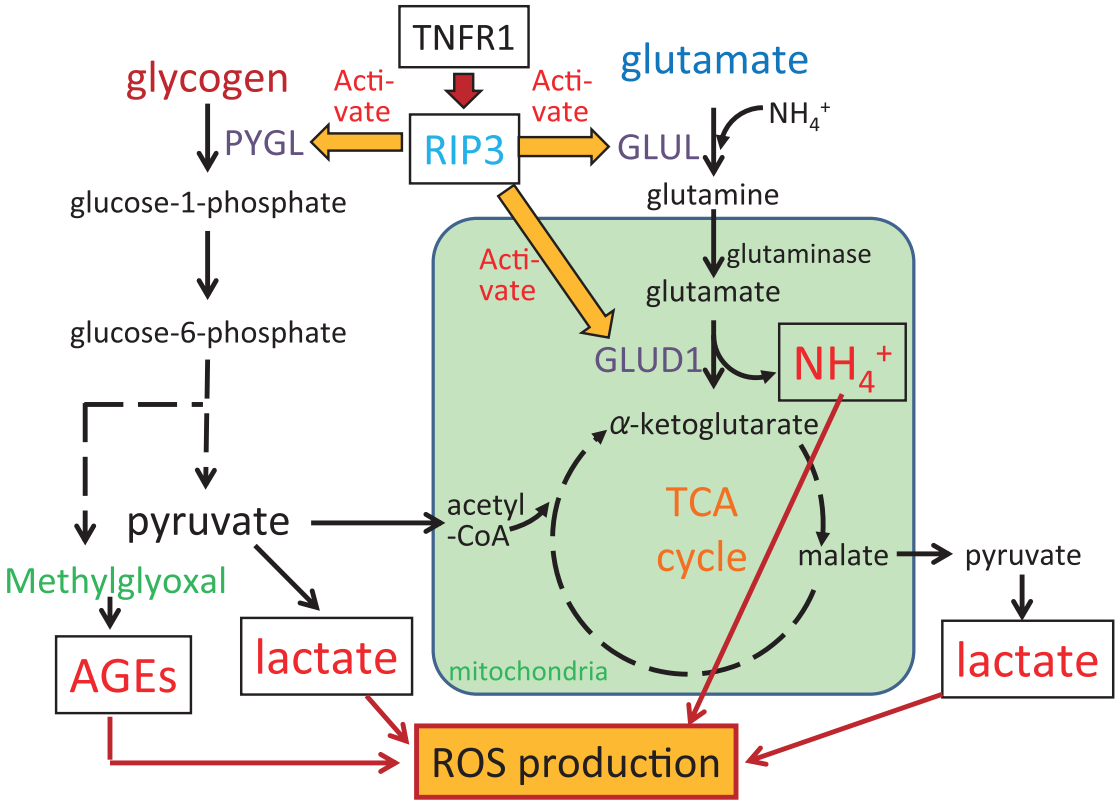

Fig. 3 Possible ROS-mediated execution of necroptosis. It has been proposed that ROS production has a role in necroptosis. TNF stimulation forms a necrosome, and active RIP3 in the complex interacts with and activates GLUL, GLUD1, and PYGL. Activated GLUL catalyzes the formation of glutamine from glutamate and free ammonia (NH_4^+). After internalization of glutamate into mitochondria, the glutamate is reconverted to glutamate. Active GLUD1 catalyzes deamination of glutamate and generates α-ketoglutarate. Free NH_4^+, which is generated during deamination, stimulates ROS production. α-Ketoglutarate is used in the TCA cycle, and pyruvate is generated through malate decarboxylation; pyruvate has the potential to produce lactate, an inducer of ROS. Activated PYGL catalyzes the breakdown of glycogen and generates glucose-6-phosphate through glucose-1-phosphate. Pyruvate produced from glucose-6-phosphate is processed by the TCA cycle and is therefore able to induce ROS as described above. The cytotoxic molecule methylglyoxal is synthesized at a rate commensurate with glycolytic activity and covalently binds to proteins and forms AGEs, which provide a new center for sustained ROS generation.
Abbreviations: ROS, reactive oxygen species; GLUL, glutamate-ammonia ligase; GLUD1, glutamate dehydrogenase 1; PYGL, glycogen phosphorylase; TCA, tricarboxylic-acid; AGE, advanced glycation end-product.

inhibit mitogen-activated protein kinase (MAPK) phosphatase, which inactivates JNK, by oxidizing catalytic cysteine residues of MAPK phosphatase (36). Sustained JNK activation causes subsequent cell death (36). Schulze-Osthoff et al. reported the first evidence that ROS production has a role—through the respiratory function of mitochondria—in the TNF-induced necrotic response (37). Zhang et al. linked TNFR1 complexes to excessive ROS generation (38). Activated RIP3 in the necrosome interacts and activates glutamate-ammonia ligase (GLUL), glutamate dehydrogenase 1 (GLUD1), and glycogen phosphorylase (PYGL) (38). RNAi-mediated knockdown of any of these enzymes decreased ROS production and necroptosis in both TNF- and zVAD-fmk-trerated cells (38).

The relationship between ROS production and enzymes involved in TNFR1 signaling mediated by GLUL, GLUD1, and PYGL was reviewed by Galluzzi et al. and Vandenabeele et al. (Fig. 3) (3,9). GLUL and GLUD1 are enzymes required for glutaminolysis. GLUL catalyzes the formation of glutamine from glutamate and free ammonia. After uptake of glutamine into mitochondria, glutaminase converts glutamine to glutamate, and GLUD1 then converts glutamate into α-ketoglutarate, releasing free ammonia, which induces ROS production (3,9,39). α-Ketoglutarate is used in the tricarboxylic acid (TCA) cycle to generate reduced equivalents (NADH and $FADH_2$) and pyruvate (through malate decarboxyl-

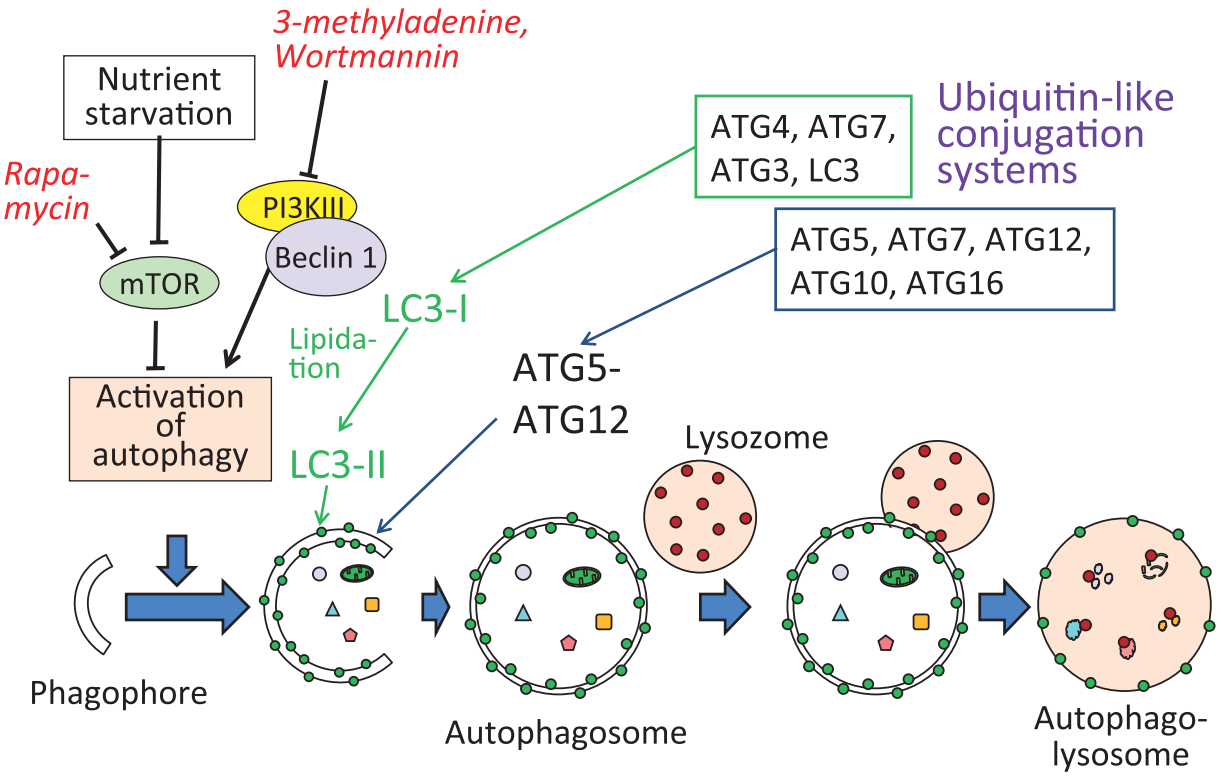

Fig. 4 Molecular mechanisms of autophagy. Formation of phagophores is negatively regulated by mTOR, which can be inhibited by nutrient starvation and rapamycin treatment (induction). Subsequent formation of double-membrane vesicles is controlled by Beclin 1 and PI3KIII, which can be inhibited by 3-methyladenine and wortmannin (membrane nucleation). Engulfment of cytosolic molecules and organelles, and formation of the complete autophagosome, is mediated by two ubiquitin-like conjugation systems: ATG12-conjugation (which is mediated by ATG5, ATG7, ATG 12, ATG 10, and ATG 16) and LC3-conjugation systems, which requires ATG 3, ATG 4, ATG 7, and LC3 (sequestration). During sequestration, LC3-I is conjugated with phosphatidylethanolamine and converted to membrane-bound LC3-II. The generated autophagosome fuses with lysosome (autophagolysosome), and its contents are degraded by lysosomal enzymes (maturation and degradation).
Abbreviations: mTOR, mammalian target of rapamycin; PI3KIII, class III phosphatidylinositol 3-kinase; LC3, microtubule-associated protein 1 light chain 3.

ation). Thus, there is the potential to produce lactate, an inducer of ROS (3,9,39). PYGL catalyzes the breakdown of glycogen into glucose-1-phosphate, which immediately isomerizes to glucose-6-phosphate. The pyruvate produced from glucose-6-phosphate is processed by the TCA cycle and can induce ROS as described above (3,9).

The cytotoxic molecule methylglyoxal is synthesized at a rate commensurate with glycolytic activity and induces dysfunction of mitochondrial respiration and increases ROS production (40). Methylglyoxal covalently binds to proteins and forms advanced glycation end-products (AGEs), which alter protein function and serve as a new center for sustained ROS generation (Fig. 3) (41). Van Herreweghe et al. reported that inhibition of glyoxalase I, an enzyme that has a role in detoxification of methylglyoxal, accelerates TNF-induced cell death (41). Thus, TNF stimulation activates GLUL, GLUD1, and PYGL through RIP3 activation and produces ROS-inducible factors (such as lactate, ammonium, and AGEs), resulting in the execution of TNF-induced necrotic cell death. Activation of NADPH oxidase in response to TNFR1 activation also contributes to TNF-induced necroptosis (42). Riboflavin kinase is a TNFR1-binding kinase that recruits and anchors $p22^{phox}$ and NADPH oxidase 1 (NOX1) to RNFR1. The recruited $p22^{phox}$ and NOX1 constitute an NADPH oxidase complex (43). RIP1 is essential for recruiting an NADPH oxidase complex to the cytoplasmic membrane (42). TNF stimulation activates NOX1 and induces ROS generation (44). A NOX1 siRNA reduced TNF-induced ROS generation and subsequent necrotic cell death (42). Several non-enzymatic reactions also amplify ROS (3,9). The Haber–Weiss and Fenton reac-

tions generate hydroxyl radicals from hydrogen peroxide (3,9). Although some reports show that ROS have a role in the execution of necroptosis, other studies show that treatment with an antioxidant failed to rescue cells from necroptosis, which suggests that ROS participation in necroptosis may depend on cell type, as cell types differ in their production of and response to ROS (24). Thus, the contribution of ROS to the execution of necroptosis must be evaluated in future studies.

In contrast to apoptosis, few reports suggest that necroptosis has a role in healthy development and homeostasis in mammals. Rather, necroptosis is commonly reported in pathological conditions. Necrostatin-1, which inhibits necroptosis by inhibiting RIP1 kinase (45), rescued hippocampal HT-22 cells from glutamate-induced neuronal excitotoxicity (46). Another study showed that necroptosis contributes to rat cortical neuron excitotoxicity induced by N-methyl-D-aspartate (NMDA), which also stimulates NMDA-type glutamate receptor (47). Excitotoxicity occurs in chronic neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease (29). Therefore, necroptosis may have roles in the development of these diseases. Moreover, Degterev et al. showed that necroptosis contributes to delayed mouse ischemic brain injury *in vivo* and that necrostatin-1 significantly decreased infarct size (24).

Autophagic cell death

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved process that is responsible for bulk degradation of cytoplasmic molecules and organelles in healthy cells (48,49) and is induced by various stimuli, including nutrient deprivation. In autophagy, cytoplasmic components and organelles are engulfed by double-membrane vesicles called autophagosomes and fused with lysosomes (autophagolysosomes) (Fig. 4) (4,50). Their cargo is then degraded by lysosomal enzymes (Fig. 4) (4,50) and the degraded components are recycled for organelle synthesis or energy generation (4,50). Moreover, autophagy eliminates damaged organelles and infectious agents from the cytosol (51). Autophagy is thus generally considered to be a pro-survival mechanism (4,50,51).

Identification of the role of *atg* genes in autophagy in yeast (*Saccharomyces cerevisiae*) was a huge breakthrough in the study this process (52). Many orthologues of the *atg* genes have been identified in mammalian cells and were shown to control the dynamic processes of autophagy (52). Numerous molecules, including *atg* genes, orchestrate autophagy, which comprises 4 steps (49), namely, i) induction, which involves formation of the phagophore and is negatively regulated by the mammalian target of rapamycin (mTOR); ii) membrane nucleation, which is associated with the formation of double-membrane vesicles, mediated by a complex containing Beclin 1 and class Ⅲ phosphatidylinositol 3-kinase (PI3KⅢ), and can be inhibited by 3-methyladenine and wortmannin; iii) sequestration, which is critical for formation of the complete autophagosome that engulfs cytosolic molecules and organelles and is controlled by two ubiquitin-like conjugation systems (ATG12-conjugation and microtubule-associated protein 1 light chain 3 [LC3]-conjugation); during sequestration, phosphatidylethanolamine is conjugated to LC3-I (LC3-II), and the product is anchored to the autophagosomal membrane (LC3 lipidation; often used as a marker of autophagy); and iv) maturation and degradation, which involves fusion of the autophagosome and lysosome (autophagolysosome) and the digestion of its contents by lysosomal enzymes.

As mentioned above, autophagy is usually considered to be a pro-survival mechanism, but there is evidence that it also participates in a pro-death mechanism (8,53-56). Berry and Baehrecke et al. demonstrated that autophagic cell death occurs under physiological conditions in *Drosophila melanogaster* (57). During the pupal stage of *Drosophila*, death occurs in midgut cells so as to allow tissue remodeling and metamorphosing of the larva into an adult fly (58). This process depends on autophagy rather than apoptotic signaling (58). Suppression of autophagy by either overexpression of dominant negative mutants or the knockdown of autophagy-driving molecules significant delays midgut regression (58). Although caspase is activated during midgut regression, suppression of caspase activity fails to rescue cells from death (58). These research findings suggest the existence of autophagic cell death *in vivo*.

Most experiments that demonstrated autophagic cell death in mammals were mainly done using cultured cells or cells defective in apoptotic signaling molecules (59-63). Rat hippocampal neural stem cells reportedly undergo autophagic cell death when insulin is withdrawn, which induces autophagic flux and LC3-I conversion to LC3-II (63). *Atg7* knockdown, which suppresses autophagy, reduces induction of autophagic flux and LC3 conversion by insulin withdrawal and also decreases cell death (63). Moreover, cell death is independent of apoptotic caspase activation. Shimizu et al. showed that embryonic fibroblasts from a *bax/bak* double knockout mouse, which is resistant to apoptosis, die in a non-apoptotic manner after treatment with etoposide, an inducer of apoptosis (59). Microscopic analysis revealed increased

Table 1 DAMPs and their pro-inflammatory activity and receptors

DAMPs	Pro-inflammatory activity	Potential receptors
HMGB1	*In vivo*: inflammation in response to liver injury blocked by neutralizing antibody; neutrophil recruitment induced by purified molecule *In vitro*: chemotaxis, cytokine induction	RAGE, TLR2, TLR4
Uric acid	*In vivo*: gout induced by purified molecule; neutrophil recruitment induced by purified molecule *In vitro*: cytokine induction	TLR2, TLR4, CD14
Chromatin nucleosomes and DNA	*In vivo*: neutrophil recruitment induced by purified molecule *In vitro*: cytokine induction; B-cell activation induced by chromatin-IgG complexes	TLR9 (with BCR or Fc receptor)
Heat shock proteins	*In vitro*: cytokine induction	CD14, CD40, CD91, TLR2, TLR4, scavenger receptors
Galectins	*In vivo*: monocyte recruitment induced by purified molecule *In vitro*: chemotaxis	CD2 and others containing β-galactose
S100 protein	*In vivo*: neutrophil recruitment induced by purified molecule *In vitro*: chemotaxis; cytokine induction	RAGE
Cathelicidins	*In vitro*: chemotaxis	FPRL1
N-formylated peptides	*In vivo*: neutrophil recruitment induced by purified molecule *In vitro*: chemotaxis	FPR, FPRL1

Table excerpted from review article by Kono and Rock (10).
Abbreviations: DAMP, damage-associated molecular pattern; HMGB1, high mobility group box 1 protein; RAGE, receptor for advanced glycation end-product; BCR, B-cell receptor; FPRL1, formyl peptide receptor-like 1; FPR, formyl peptide receptor

autophagic flux in dying cells and increased numbers of cells with punctate LC3 (59). Cell death and autophagic features were inhibited by 3-methyladenine, an inhibitor of autophagy, and by RNAi inhibition of *beclin 1* or *atg5* (59). However, to our knowledge, no clear evidence of physiological autophagic cell death in mammals has been reported.

Autophagic cell death was first described by Schweichel et al. as a type of cell death that is accompanied by the presence of autophagosomes (64). Cell death is defined on the basis of the morphological features of dying cells, including massive accumulation of autophagosomes, which is often referred as autophagic flux (49,64,65). However, morphological features fail to explain the role of autophagy in cell death (49,66). Shen et al. proposed that autophagic cell death should be redefined as a type of non-apoptotic cell death or necrotic programmed cell death in which autophagy has a role in the mechanism of death and displays distinct characteristics, as follows (49): i) cell death without involvement of apoptotic signaling, such as caspase activation, ii) increased autophagic flux (not just an increase in autophagic markers, such as LC3 lipidation), and iii) suppression of autophagy by both pharmacological inhibitors or genetic approaches (e.g., knockdown of *atg* using RNAi or overexpression of dominant negative of *atg*) that rescue or prevent cell death.

Cell death and DAMPs

Cell death is a normal physiological process in the human body, although it sometimes occurs due to injury or disease. In physiological apoptosis, dead cells are phagocytosed and disposed of in the absence of inflammation by macrophage-lineage phagocytic cells (10). However, inflammation often occurs when a large number of necrotic cell deaths are present, for example, due to infarction or injury (10,11). Thus, when cells die by necrosis, DAMPs are released outside dead cells, bind to their receptor on the cell surface of immune cells, and trigger inflammation (10,11,67). Because DAMPs are released as a result of cell stress, they are also called alarmin molecules (10). Many examples of DAMPs, and their immunostimulatory function, have been reported, and they have been comprehensively reviewed by Kono and Rock (10). Table 1 shows DAMPs that reportedly have pro-inflammatory activities and their receptors. Interestingly, some DAMPs share receptors with pathogen-associated molecular patterns. For example, Toll-like receptor 4 (TLR4) recognizes both high-mobility group box-1 (HMGB1) and lipopolysaccharide (LPS). Therefore, consideration of necrotic cell death may be very important in understanding periodontal

diseases.

Possible relationship of cell death to periodontal disease

Recently we reviewed on roles of autophagy in the periodontal disease and proposed a possible role of autophagic cell death in initiation and progression of periodontal disease (68). Periodontal disease, which includes periodontitis and gingivitis, is a common chronic inflammation of tooth-supporting tissues and results from infection by periodontopathic bacteria that colonize the gingival groove as dental plaque. Periodontal tissue and the immune system cooperatively respond to the bacteria, thereby causing inflammation. Dental plaques contain numerous species of microorganisms and their metabolic products. The participation of anaerobic gram-negative bacteria, such as *Porphyromonas*, *Prevotella*, *Treponema*, and *Fusobacterium* species, in dental plaque is strongly implicated in the development of periodontal disease. These bacteria produce a variety of molecules that contribute to virulence (for example, LPS, proteases, fimbriae, and capsular polysaccharides), and these products stimulate host cells to release inflammatory effectors. However, the exact mechanisms by which these bacterial products trigger initiation of periodontal disease are unknown.

Mature subgingival dental plaques contain high concentrations of butyrate (2.6-14 mM), which is produced by anaerobic bacteria (69). We have shown that butyrate concentrations of 0.5 mM or higher induce death of epithelial cells (70). Although some dead cells exhibit features of apoptosis, such as phosphatidylserine redistribution, increased caspase-3 activity, and decreased anti-apoptotic *bcl-2* expression, only a small number were rescued by treatment with zVAD-fmk, a pan-caspase inhibitor (70). We also observed induction of a couple of autophagic features by butyrate, such as autophagic flux and increased-LC3 lipidation (70). Furthermore, cell death was reduced by the autophagy inhibitor 3-methyladenine, which suggests that butyrate induced autophagic cell death of gingival epithelial cells (70).

The nuclear protein HMGB1 is expressed ubiquitously and participates in DNA bending and transcriptional regulation (71-74). It also has an important role in extracellular signaling after it is released by cells (75-78) and is therefore categorized as a DAMP. Released HMGB1 reportedly functions as an activator during inflammation (78-80) and induces recruitment of inflammatory leukocytes (81) and cell migration (82,83). *In vivo* experiments also demonstrate that HMGB1-neutralizing antibody antagonizes LPS-induced lethality (84) and collagen-evoked arthritis (85). HMGB1 reportedly can be shuttled between the nucleus and cytoplasm, depending on its post-transcriptional modifications (74,86-88). Histone acetyltransferases (HATs) acetylate lysine residues of HMGB1 (89), and histone deacetylases (HDACs) eliminate the acetyl groups from the molecule (87). Acetylated-HMGB1 translocates from the nucleus to the cytoplasm (89), where it is released by cells that have lost cell membrane integrity. Butyrate also acts as an HDAC inhibitor (90). Thus, butyrate may increase acetylated HMGB1 and augment its presence in the cytoplasm. Consequently, HMGB1 may be located in the cytoplasm of epithelial cells lining the gingival crevice, which harbors mature dental plaque rich in butyrate. This hypothesis is consistent with a report that HMGB1 was present mainly in the cytoplasm of periodontal-pocket gingival epithelial cells (91). In such environments, acetylated HMGB1 in the cytoplasm can be easily released from the gingival cells by butyrate-induced autophagic cell death. The presence of high concentrations of HMGB1 in the gingival cervical fluid of patients with periodontal disease supports this putative mechanism (92).

Because extracellular HMGB1 works as a chemoattractant for polymorphonuclear leukocytes and stimulates inflammation and immune response (Table 1), HMGB1 might have a significant role in initiation and progression of periodontal inflammation. As mentioned above, we demonstrated that butyrate induced gingival-epithelial autophagic cell death (70) that displays necrotic features, including loss of cellular membrane integrity and cell swelling. Therefore, destruction of the cell membrane may cause HMGB1 to leak from cells. In fact, Ebe et al. reported that butyrate-induced death of Ca9-22 gingival epithelial cells was accompanied by necrotic features, and they concluded that dead cells may release HMGB1 (91). In sum, these studies suggest that autophagic cell death is closely related to butyrate-induced HMGB1 release.

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