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Review

Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009

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Different types of cell death are often defined by morphological criteria, without a clear reference to precise biochemical mechanisms. The Nomenclature Committee on Cell Death (NCCD) proposes unified criteria for the definition of cell death and of its different morphologies, while formulating several caveats against the misuse of words and concepts that slow down progress in the area of cell death research. Authors, reviewers and editors of scientific periodicals are invited to abandon expressions like 'percentage apoptosis' and to replace them with more accurate descriptions of the biochemical and cellular parameters that are actually measured. Moreover, at the present stage, it should be accepted that caspase-independent mechanisms can cooperate with (or substitute for) caspases in the execution of lethal signaling pathways and that 'autophagic cell death' is a type of cell death occurring together with (but not necessarily by) autophagic vacuolization. This study details the 2009 recommendations of the NCCD on the use of cell death-related terminology including 'entosis', 'mitotic catastrophe', 'necrosis', 'necroptosis' and 'pyroptosis'.
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Cell death can be classified according to its morphological appearance (which may be apoptotic, necrotic, autophagic or associated with mitosis), enzymological criteria (with and without the involvement of nucleases or of distinct classes of proteases, such as caspases, calpains, cathepsins and transglutaminases), functional aspects (programmed or accidental, physiological or pathological) or immunological characteristics (immunogenic or non-immunogenic).[1]

The Nomenclature Committee on Cell Death (NCCD) has formulated a first round of recommendations in 2005, in *Cell Death and Differentiation*.[2] Since then, the field of cell death research has continued its expansion, significant progress has been made and new putative cell death modalities have been described. The NCCD provides a forum in which names describing distinct modalities of cell death are critically evaluated and recommendations on their definition and use are formulated, hoping that a non-rigid, yet uniform, nomenclature will facilitate the communication among scientists and ultimately accelerate the pace of discovery. This study contains the updated NCCD guidelines.

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Abbreviations: Ca^{2+}, calcium; $\Delta\Psi_m$, mitochondrial transmembrane potential; IL, interleukin; MMP, mitochondrial membrane permeabilization; NCCD, Nomenclature Committee on Cell Death; PCD, programmed cell death; PI, propidium iodide; Z-VAD-fmk, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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Recommendation to Authors, Reviewers and Editors of Scientific Journals

Authors still make frequent use of expressions like 'percentage apoptosis' without mentioning the method actually employed to assess ongoing cell death. In a totally inappropriate fashion, these terms are also employed to describe the results of cell-free assays based on purified cellular components. Such a vocabulary is confusing and imprecise and should definitively be abandoned. From 2009, *Cell Death and Differentiation* will actively enforce a policy in which terms like 'percent apoptosis', 'percent necrosis', 'percent cell death' and 'percent cell survival' must be replaced with more descriptive expressions including 'percent cells with condensed chromatin', 'percent cells with DNA fragmentation', 'percent cells with a low mitochondrial transmembrane potential ($\Delta\Psi_m$)', as well as 'percent propidium iodide (PI) positive', 'percent cleaved caspase-3 positive', 'percent terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive' and 'percent clone forming' cells. Similarly, the term 'percent autophagic cells' should be avoided and replaced with a language that precisely describes what has been measured (such as 'number of GFP-LC3 puncta/cell' or 'percent vacuolated cells'). This applies to the description of experimental results, be it in the text or in the abstract, as well as to the labeling of figures and figure legends. Moreover, NCCD encourages researchers to quantify cell death and/or other catabolic events (such as autophagy) with more than one assay, whenever possible, thereby reducing the probability of artifacts. The NCCD urges all life science journals and, more specifically, all journals in the areas of cell biology, cancer research and pharmacology to pursue a similar policy.

When is a Cell 'Dead'?

Dying cells are engaged in a process that is reversible until a first irreversible phase or 'point-of-no-return' is trespassed (Table 1). It has been proposed that this step could be represented by massive caspase activation,[6] loss of $\Delta\Psi_m$,[7] complete permeabilization of the mitochondrial outer membrane[8] or exposure of phosphatidylserine (PS) residues that emit 'eat me' signals for normal neighboring cells. However, there are dozens of examples in which caspases are activated in the context of non-lethal processes and differentiation pathways.[9,10] The $\Delta\Psi_m$ can be dissipated by protonophores without progression to immediate cell death.[11] PS exposure can be reversible, for instance in neutrophilic granulocytes.[12]

Table 1 Cell death methodology

Definition	Notes	Methods of detection[3–5]
Molecular or morphological criteria to define dead cells		
Loss of plasma membrane integrity	Plasma membrane has broken down, resulting in the loss of cell's identity	(IF) Microscopy and/or FACS to assess the exclusion of vital dyes, *in vitro*
Cell fragmentation	The cell (including its nucleus) has undergone complete fragmentation into discrete bodies (usually referred to as apoptotic bodies)	(IF) Microscopy FACS quantification of hypodiploid events (sub-G_1 peak)
Engulfment by adjacent cells	The corpse or its fragments have been phagocytosed by neighboring cells	(IF) Microscopy FACS colocalization studies
Proposed points-of-no return to define dying cells		
Massive activation of caspases	Caspases execute the classic apoptotic program, yet in several instances, caspase-independent death occurs. Moreover, caspases are involved in non-lethal processes including differentiation and activation of cells	Immunoblotting FACS quantification by means of fluorogenic substrates or specific antibodies
$\Delta\Psi_m$ dissipation	Protracted $\Delta\Psi_m$ loss usually precedes MMP and cell death; however, transient dissipation is not always a lethal event	FACS quantification with $\Delta\Psi_m$-sensitive probes Calcein-cobalt technique
MMP	Complete MMP results in the liberation of lethal catabolic enzymes or activators of such enzymes. Nonetheless, partial permeabilization may not necessarily lead to cell death	IF colocalization studies Immunoblotting after subcellular fractionation
PS exposure	PS exposure on the outer leaflet of the plasma membrane often is an early event of apoptosis, but may be reversible. PS exposure occurs also in T-cell activation, without cell death	FACS quantification of Annexin V binding
Operative definition of cell death, in particular in cancer research		
Loss of clonogenic survival	This method does not distinguish cell death from long-lasting or irreversible cell cycle arrest	Clonogenic assays

Abbreviations: $\Delta\Psi_m$, mitochondrial transmembrane permeabilization; FACS, fluorescence-activated cell sorter; IF, immunofluorescence; MMP, mitochondrial membrane permeabilization; PS, phosphatidylserine

Thus, the concept of a restriction point for cell death, as it was described by Pardee[13] for the cell cycle, has yet to be specifically defined.

In the absence of a clearly defined biochemical event that can be considered as the point-of-no-return, the NCCD proposes that a cell should be considered dead when any one of the following molecular or morphological criteria is met: (1) the cell has lost the integrity of its plasma membrane, as defined by the incorporation of vital dyes (e.g., PI) *in vitro*; (2) the cell, including its nucleus, has undergone complete fragmentation into discrete bodies (which are frequently referred to as 'apoptotic bodies'); and/or (3) its corpse (or its fragments) has been engulfed by an adjacent cell *in vivo*. Thus, *bona fide* 'dead cells' would be different from 'dying cells' that have not yet concluded their demise (which can occur through a variety of biochemically distinct pathways, see below). In particular, cells that are arrested in the cell cycle (as it occurs during senescence) should be considered as alive, and the expression 'replicative cell death' (which alludes to the loss of clonogenic potential), as it is frequently used by radiobiologists, should be abandoned.

Definition of 'Apoptosis'. The expression 'apoptosis' has been coined by Kerr *et al*.[14] to describe a specific morphological aspect of cell death (Table 2). Apoptosis is accompanied by rounding-up of the cell, retraction of pseudopodes, reduction of cellular volume (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis), classically little or no ultrastructural modifications of cytoplasmic organelles, plasma membrane blebbing (but maintenance of its integrity until the final stages of the process) and engulfment by resident phagocytes (*in vivo*). Hence, the term 'apoptosis' should be applied exclusively to cell death events that occur while manifesting several among these morphological features. It is worth noting that it is not correct to assume that 'programmed cell death' (PCD) and 'apoptosis' are synonyms because cell death, as it occurs during physiological development, can manifest non-apoptotic features.[19–21]

Specific biochemical analyses (such as DNA ladders) should not be employed as an exclusive means to define apoptosis, because this type of cell death can occur without oligonucleosomal DNA fragmentation. Similarly, the presence of proteolytically active caspases or of cleavage products of their substrates is not sufficient to define apoptosis. Frequently, the active suppression (by pharmacological and/or genetic means) of DNA fragmentation and/or caspase activation demonstrates that these changes are not required for the execution of the cell death program, although caspase activation may be necessary for the acquisition of the apoptotic morphology.[22–24] Moreover, the presence of active caspases and/or of specific products of their enzymatic activity can be linked to non-lethal biological processes.[9,10] The measurement of DNA fragmentation and/or of caspase activation, however, may be helpful in diagnosing apoptosis. Thus, it may be reasonable to use caspase activation not only to diagnose but also to better define (together with other features) the type of cell death.

It should be noted that the expression 'apoptosis' hides a major degree of biochemical and functional heterogeneity. There are several distinct subtypes of apoptosis that, although morphologically similar, can be triggered through different biochemical routes (for instance through the 'intrinsic' or the 'extrinsic' pathway, with or without the contribution of mitochondria, etc…).[25,26] Moreover, the apparent uniformity of apoptotic cell death may conceal heterogeneous functional aspects, for instance concerning the perception of apoptosis by the immune system.[27] Thus, although apoptosis mostly occurs in a non-immunogenic fashion, some lethal stimuli can lead to the exposure or secretion of proteins that elicit the engulfment of apoptotic material by dendritic cells, followed by efficient antigen presentation and stimulation of a specific immune response.[28]

Cell death is frequently considered to be 'caspase-dependent' when it is suppressed by broad-spectrum caspase inhibitors such as *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk). As a word of caution, however, it should be noted that Z-VAD-fmk does not act on all caspases with an equal efficiency, and it also inhibits calpains and cathepsins, especially at high concentrations ($>10\,\mu$M). Moreover, Z-VAD-fmk has been associated with several off-target effects that would result from the binding to cysteines on proteins other than cysteine proteases.[29] As an example, Z-VAD-fmk has been shown to interfere with the interaction between the adenine nucleotide translocase and cyclophylin D,[30] thereby favoring necrotic cell death.[31] For these reasons, the term 'Z-VAD-fmk-inhibitable' should be preferred to 'caspase-dependent'. A second difficulty arises from the fact that caspase inhibition often prevents the appearance of some morphological signs of apoptosis (such as chromatin condensation and DNA fragmentation), yet only retards cell death.[32] In many instances, caspase inhibition simply induces a shift from an apoptotic to a mixed cell death morphology, or even to full-blown pictures of necrosis or autophagic cell death, which, however, may manifest some delay.[33] Thus, 'caspase-independent cell death'[32] can occur despite the efficient inhibition of caspases and can exhibit some of the morphological signs of apoptosis (such as a partial chromatin condensation),[34] autophagy or necrosis.

Considerations on 'Autophagy' and 'Autophagic Cell Death'

Macroautophagy is characterized by the sequestration of cytoplasmic material within autophagosomes for bulk degradation by lysosomes. Autophagosomes, by definition, are two-membraned and contain degenerating cytoplasmic organelles or cytosol,[35,36] which allows them to be distinguished by transmission electron microscopy from other types of vesicles such as endosomes, lysosomes or apoptotic blebs.[3] The fusion between autophagosomes and lysosomes generates autolysosomes, in which both the autophagosome inner membrane and its luminal content are degraded by acidic lysosomal hydrolases. This catabolic process marks the completion of the autophagic pathway. When the fusion of autophagosomes with lysosomes is blocked, the former accumulate in spite of autophagy inhibition.[36,37] Hence, a massive increase in the number of autophagosomes is by no means a demonstration that the autophagic pathway is induced, and functional tests are required to investigate

Table 2 Distinct modalities of cell death

Cell death mode	Morphological features	Notes
Apoptosis	Rounding-up of the cell Retraction of pseudopodes Reduction of cellular and nuclear volume (pyknosis) Nuclear fragmentation (karyorrhexis) Minor modification of cytoplasmic organelles Plasma membrane blebbing Engulfment by resident phagocytes, *in vivo*	'Apoptosis' is the original term introduced by Kerr *et al*.[14] to define a type of cell death with specific morphological features. Apoptosis is NOT a synonym of programmed cell death or caspase activation.
Autophagy	Lack of chromatin condensation Massive vacuolization of the cytoplasm Accumulation of (double-membraned) autophagic vacuoles Little or no uptake by phagocytic cells, *in vivo*	'Autophagic cell death' defines cell death occurring with autophagy, though it may misleadingly suggest a form of death occurring by autophagy as this process often promotes cell survival.[15,16]
Cornification	Elimination of cytosolic organelles Modifications of plasma membrane Accumulation of lipids in F and L granules Extrusion of lipids in the extracellular space Desquamation (loss of corneocytes) by protease activation	'Cornified envelope' formation or 'keratinization' is specific of the skin to create a barrier function. Although apoptosis can be induced by injury in the basal epidermal layer (e.g., UV irradiation), cornification is exclusive of the upper layers (granular layer and stratum corneum).[17,18]
Necrosis	Cytoplasmic swelling (oncosis) Rupture of plasma membrane Swelling of cytoplasmic organelles Moderate chromatin condensation	'Necrosis' identifies, in a negative fashion, cell death lacking the features of apoptosis or autophagy.[4] Note that necrosis can occur in a regulated fashion, involving a precise sequence of signals.

autophagy. A very comprehensive description of the assays for monitoring autophagy in higher eukaryotes and a set of guidelines for their interpretation has been recently provided by Klionsky *et al*.[38] One technique commonly employed to detect autophagy relies on the redistribution of GFP-LC3 fusion proteins into vesicular structures (which can be autophagosomes or autolysosomes).[3,39] However, the exclusive use of GFP-LC3 as a marker of autophagy is not sufficient to diagnose an enhanced autophagic catabolism.[38]

'Autophagic cell death' is morphologically defined (especially by transmission electron microscopy) as a type of cell death that occurs in the absence of chromatin condensation but accompanied by massive autophagic vacuolization of the cytoplasm. In contrast to apoptotic cells (whose clearance is ensured by engulfment and lysosomal degradation), cells that die with an autophagic morphology have little or no association with phagocytes.[40,41] Although the expression 'autophagic cell death' is a linguistic invitation to believe that cell death is executed by autophagy, the term simply describes cell death with autophagy.[15,16,42] Thus far, involuting *Drosophila melanogaster* salivary glands provide the only *in vivo* evidence that the knockdown/knockout of genes required for autophagy truly reduces cell death.[43] This may be due to the limited number of studies that have investigated autophagic cell death *in vivo*, although there are no doubts that autophagy promotes cell survival, in multiple physiological and experimental settings.[44] Significantly, some reports indicate that cells presenting features of 'autophagic cell death' can still recover upon withdrawal of the death-inducing stimulus.[45] In most cases described to date in which autophagy is suppressed by genetic knockout/knockdown of essential autophagy (*atg*) genes, cell death is not inhibited but rather occurs at an accelerated pace,[15] pointing to the prominent role of autophagy as a pro-survival pathway. This said, it should be noted that most of these studies have been performed on immortalized cell lines *in vitro* and that autophagic cell death rarely affects individual cells *in vivo*.[19,41] Nevertheless, in specific cases, autophagy may participate in the destruction of cells, as a result of a protracted atrophy of the cytoplasm, beyond a not yet clearly defined point-of-no-return.[43,46] Thus, direct induction of autophagy by overexpression of the Atg1 kinase is sufficient to kill fat and salivary gland cells in *Drosophila*. Interestingly, although Atg1-driven autophagic cell death entails caspase-dependent mechanisms in fat cells,[46] the same does not hold true in salivary gland cells (which cannot be rescued from Atg1-induced death by p35 expression).[43]

Definition of 'Necrosis'

'Necrotic cell death' or 'necrosis' is morphologically characterized by a gain in cell volume (oncosis), swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents. For a long time, necrosis has been considered merely as an accidental uncontrolled form of cell death, but evidence is accumulating that the execution of necrotic cell death may be finely regulated by a set of signal transduction pathways and catabolic mechanisms.[4,47] For instance, death domain receptors (e.g., TNFR1, Fas/CD95 and TRAIL-R) and Toll-like receptors (e.g., TLR3 and TLR4) have been shown to elicit necrosis, in particular in the presence of caspase inhibitors. TNFR1-, Fas/CD95-, TRAILR- and TLR3-mediated cell death seemingly depends on the kinase RIP1,[48] as this has been demonstrated by its knockout/knockdown and chemical inhibition with necrostatin-1.[49–51] Although there is no generalized consensus on the use of this expression, some authors have proposed the term 'necroptosis' to indicate regulated (as opposed to accidental) necrosis. At a biochemical level, necroptosis may be defined

as a type of cell death that can be avoided by inhibiting RIP1 (either through genetic or pharmacological methods),[49,50] which may represent a convenient means to discriminate between programmed and fortuitous forms of necrosis.

Several mediators, organelles and cellular processes have been implicated in necrotic cell death, but it is still unclear how they interrelate with each other. The causative elements of necrosis are unclear, as well as its bystander effects. These phenomena include mitochondrial alterations (e.g., uncoupling, production of reactive oxygen species, i.e., ROS, nitroxidative stress by nitric oxide or similar compounds[52] and mitochondrial membrane permeabilization, i.e., MMP, often controlled by cyclophilin D), lysosomal changes (ROS production by Fenton reactions, lysosomal membrane permeabilization), nuclear changes (hyperactivation of PARP-1 and concomitant hydrolysis of NAD^+), lipid degradation (following the activation of phospholipases, lipoxygenases and sphingomyelinases), increases in the cytosolic concentration of calcium (Ca^{2+}) that result in mitochondrial overload and activation of non-caspase proteases (e.g., calpains and cathepsins).[4,53] In several (but not all) instances of necrotic cell death, a crucial role for the serine/threonine kinase RIP1 has been demonstrated.[54] Thus far, however, there is no consensus on the biochemical changes that may be used to unequivocally identify necrosis. In the absence of a common biochemical denominator, necrotic cell death is still largely identified in negative terms by the absence of apoptotic or autophagic markers, in particular when the cells undergo early plasma membrane permeabilization (as compared with its delayed occurrence, which is associated with late-stage apoptosis). For these reasons, caution should be used in classifying particular cell death routines as necrotic.

Definition of 'Cornification'

Cornification is a very specific form of PCD that occurs in the epidermis, morphologically and biochemically distinct from apoptosis. It leads to the formation of corneocytes, that is dead keratinocytes containing an amalgam of specific proteins (e.g., keratin, loricrin, SPR and involucrin) and lipids (e.g., fatty acids and ceramides), which are necessary for the function of the cornified skin layer (mechanical resistance, elasticity, water repellence and structural stability). Cornification is less often referred to as 'keratinization' or 'cornified envelope formation',[17,55] and it is generally considered as a terminal differentiation program similar to those leading to other anucleated tissues (such as the lens epithelium and mature red blood cells).[56,57] This is mainly due to the fact that these processes display the (often limited) activation of the molecular machinery for cell death, in particular of caspases.[9,10,57,58] In contrast with corneocytes, however, both mature red blood and lens epithelial cells retain the ability to undergo stress-induced death,[59,60] and hence only cornification should be regarded as a *bona fide* cell death program.

At the molecular level, cornification follows a specific mechanism of epithelial differentiation during which cells express all enzymes and substrates required for building up the epidermal barrier that allows for isolating the body from the external environment. This is obtained by the crosslinking enzymes (e.g., transglutaminase types 1, 3 and 5) acting on several substrates (e.g., loricrin, SPR, involucrin and SP100),[18] as well as through the synthesis of specific lipids that are released into the extracellular space (where they are covalently attached to cornified envelope proteins), and proteases, which are required for impermeability and desquamation, respectively.

Tentative Definitions of Atypical Cell Death Modalities

'Mitotic catastrophe'. Mitotic catastrophe is a cell death mode occurring either during or shortly after a dysregulated/failed mitosis and can be accompanied by morphological alterations including micronucleation (which often results from chromosomes and/or chromosome fragments that have not been distributed evenly between daughter nuclei) and multinucleation (the presence of two or more nuclei with similar or heterogeneous sizes, deriving from a deficient separation during cytokinesis). However, there is no broad consensus on the use of this term,[61–63] and mitotic catastrophe can lead either to an apoptotic morphology or to necrosis.[64] As a result, the NCDD recommends the use of expressions such as 'cell death preceded by multinucleation' or 'cell death occurring during metaphase', which are more precise and more informative.

'Anoikis'. Apoptosis induced by the loss of the attachment to the substrate or to other cells is called anoikis.[65] Besides its specific form of induction, the molecular mechanisms of anoikis-associated cell death match those activated during classical apoptosis.[66] The NCCD acknowledges the use of this term for historical reasons, as it is already quite diffuse in the literature. However, it will be necessary to determine whether under certain circumstances other modalities of cell death occur *in vivo* following detachment, that is, whether there are forms of anoikis refractory to caspase inhibitors and/or others that manifest necrotic features.

'Excitotoxicity'. This is a form of cell death occurring in neurons challenged with excitatory amino acids, such as glutamate, that leads to the opening of the *N*-methyl-D-aspartate Ca^{2+}-permeable channel, followed by cytosolic Ca^{2+} overload and activation of lethal signaling pathways.[67] Excitotoxicity seemingly overlaps with other types of death such as apoptosis and necrosis (depending on the intensity of the initiating stimulus), and involves MMP as a critical event. For these reasons, and for the presence of common regulators such as nitric oxide itself,[68] excitotoxicity cannot be considered as a separate cell death modality.

'Wallerian degeneration'. Additional less-characterized forms of cellular catabolism take place in the nervous system, such as Wallerian degeneration, in which part of a neuron or axon degenerates without affecting the main cell body.[69,70] This term does not describe a type of cell death *sensu stricto,* because neurons affected by Wallerian degeneration remain alive.[70]

'Paraptosis'. This term was originally introduced to describe a form of PCD morphologically and biochemically distinct from apoptosis.[71] In multiple cell types, paraptosis was triggered by the expression of the insulin-like growth factor receptor I, and it was associated with extensive cytoplasmic vacuolization and mitochondrial swelling, but without any other morphological hallmark of apoptosis.[71] The manifestations of paraptosis could not be prevented by caspase inhibitors, nor by the overexpression of antiapoptotic Bcl-2-like proteins,[71,72] and seemingly resulted from a signaling cascade involving specific members of the mitogen-activated protein kinase family.[72] At present, it is still unclear whether paraptosis represents a route of cell death that is truly distinct from all others.

'Pyroptosis'. Pyroptosis has first been described in macrophages infected with *Salmonella typhimurium*.[73] It involves the apical activation of caspase-1 (but not of caspase-3), a protease that is mostly known as interleukin-1β (IL-1β)-converting enzyme. Caspase-1 activation induced by *S. typhimurium* (and by other pathogens such as *Pseudomonas aeruginosa* and *Shigella flexneri*) occurs through Ipaf,[74–76] an Apaf-1-related NLR protein.[78] In contrast, pyroptosis induced by *Bacillus anthracis* lethal toxin does not require Ipaf and rather involves another NLR protein, that is Nalp1.[78] In addition, lipopolysaccharide-treated macrophages (either in the presence or in the absence of ATP) undergo pyroptosis mediated by the adaptor protein ASC, which together with caspase-1 forms a supramolecular cytoplasmic complex also known as 'pyroptosome'.[79] Thus, distinct routes to caspase-1 activation induce pyroptosis. As this form of cell death leads to the release of IL-1β (which is one of the major fever-inducing cytokines or pyrogens) and of IL-18, it may play a relevant role in both local and systemic inflammatory reactions.[80,81] As it stands, macrophages undergoing pyroptosis not only exhibit morphological features that are typical of apoptosis, but also display some traits associated with necrosis.[82]

'Pyronecrosis'. Nalp3 and ASC are involved in the necrotic cell death of macrophages infected by *S. flexneri* at high bacteria/macrophage ratios and associated with the release of HMGB-1, caspase-1 and IL-1β, which is called pyronecrosis.[83] Pyronecrosis and pyroptosis are distinguished based on the fact that the latter (but not the former) requires caspase-1. It remains to be determined whether RIP1 is implicated in pyronecrosis, as well as whether pyroptosis and pyronecrosis play any role outside of the innate immune system.[84,85]

'Entosis'. Entosis, originally described as a form of 'cellular cannibalism' in lymphoblasts from patients with Huntington's disease,[86] has been reported as a new cell death modality in which one cell engulfs one of its live neighbors, which then dies within the phagosome.[87] Intriguingly, the most efficient cells in performing entosis are MCF-7 breast cancer cells,[87,88] which lack both caspase-3 and beclin-1 and hence are (relatively) apoptosis- and autophagy-incompetent. This points to the possibility, which remains to be explored, that entosis is a default pathway that is unmasked (and hence can be observed) exclusively when other catabolic reactions are suppressed. Entosis is not inhibited by Bcl-2 or Z-VAD-fmk, and internalized cells appear virtually normal. Later they disappear, presumably through lysosomal degradation. In rare cases, however, internalized cells are able to divide within the engulfing cell or are released.[87] Hence, it is difficult to know whether the cell-in-cell morphology (entosis) truly represents a novel cell death modality.[89]

Postface

As it stands, three distinct routes of cellular catabolism can be defined according to morphological criteria, namely apoptosis (which is a form of cell death), autophagy (which causes the destruction of a part of the cytoplasm, but mostly avoids cell death) and necrosis (which is another form of cell death). Although frequently employed in the past, the use of Roman numerals (i.e., type I, type II and type III cell death, respectively) to indicate these catabolic processes should be abandoned. Moreover, several critiques can be formulated against the clear-cut distinction of different cell types in the triad of apoptosis, autophagic cell death and necrosis.

First, although this vocabulary was originally introduced based on observations of developing animals,[41,90] it has rapidly been adopted to describe the results of *in vitro* studies performed on immortalized cell lines, which reflect very poorly the physiology of cell death *in vivo*. In tissues, indeed, dying cells are usually engulfed well before signs of advanced apoptosis or necrosis become detectable. Thus, it may be acceptable – if the irreversibility of these phenomena is demonstrated – to assess caspase activation and/or DNA fragmentation to diagnose apoptotic cell death *in vivo*.

Second, there are numerous examples in which cell death displays mixed features, for instance with signs of both apoptosis and necrosis, a fact that lead to the introduction of terms like 'necroapoptosis' and 'aponecrosis' (whose use is discouraged by the NCCD to avoid further confusion).[53] Similarly, in the involuting *D. melanogaster* salivary gland, autophagic vacuolization is synchronized with signs of apoptosis,[91] and results from genetic studies indicate that caspases and autophagy act in an additive manner to ensure cell death in this setting.[43] Altogether, these data argue against a clear-cut and absolute distinction between different forms of cell death based on morphological criteria.

Third (and most important), it would be a *desideratum* to replace morphological aspects with biochemical/functional criteria (Table 3) to classify cell death modalities. Unfortunately, there is no clear equivalence between morphology and biochemistry, suggesting that the ancient morphological terms are doomed to disappear and to be replaced by truly biochemical definitions. In this context, 'loss-of-function' and 'gain-of function' genetic approaches (e.g., RNA interference, knockout models and plasmid-driven overexpression systems) represent invaluable tools to characterize cell death modes with more precision, but only if such interventions truly reduce/augment the rate of death, instead of changing its morphological appearance (as it is often the case). Present cell death classifications are reminiscent of the categorization

Table 3 Biochemical aspects of distinct modalities of cellular catabolism

Cell death mode	Biochemical features	Methods for detection[3–5]
Apoptosis	Activation of proapoptotic Bcl-2 family proteins (e.g., Bax, Bak, Bid)	IF microscopy localization studies Immunoblotting with conformation-specific antibodies
	Activation of caspases	Colorimetric/fluorogenic substrate-based assays in live cells Colorimetric/fluorogenic substrate-based assays of lysates in microtiter plates FACS/IF microscopy quantification with antibodies specifically recognizing the active form of caspases FACS/IF microscopy quantification with antibodies specific for cleaved caspase substrates FACS/IF microscopy quantification with fluorogenic substrates Immunoblotting assessment of caspase-activation state Immunoblotting assessment of the cleavage of caspase products
	$\Delta\Psi_m$ dissipation	Calcein-cobalt technique (FACS/IF microscopy) FACS/IF microscopy quantification with $\Delta\Psi_m$-sensitive probes Oxygen-consumption studies (polarography)
	MMP	Colorimetric techniques to assess the accessibility of exogenous substrates to IM-embedded enzymatic activities FACS-assisted detection of IMS proteins upon plasma membrane permeabilization FACS-assisted detection of physical parameters of purified mitochondria HPLC-assisted quantification of mitochondrial alterations in purified mitochondria IF microscopy colocalization studies of IMS proteins (e.g., Cyt *c*) with sessile mitochondrial proteins (e.g., VDAC1) IF (video) microscopy with Cyt *c*-GFP fusion protein Immunoblotting detection of IMS proteins (e.g., Cyt *c*) upon cellular fractionation
	Oligonucleosomal DNA fragmentation	DNA ladders FACS quantification of hypodiploid cells (sub-G_1 peak) TUNEL assays
	Plasma membrane rupture	Colorimetric/fluorogenic substrate-based assays of culture supernatants in microtiter plates to determine the release of cytosolic enzymatic activities (e.g., LDH) FACS quantification with vital dyes
	PS exposure	FACS quantification of Annexin V binding
	ROS overgeneration	FACS/IF microscopy quantification with ROS-sensitive probes
	ssDNA accumulation	FACS quantification with ssDNA-specific antibodies
Autophagy	Beclin-1 dissociation from Bcl-2/X_L	Co-immunoprecipitation studies
	Dependency on *atg* gene products	Genetic studies (e.g., knockout models, RNA interference, plasmid-driven overexpression systems)
	LC3-I to LC3-II conversion	IF microscopy with GFP-LC3 fusion protein Immunoblotting with LC3-specific antibodies
	$p62^{Lck}$ degradation	Immunoblotting with p62-specific antibodies
Cornification	Expression of TGs	Immunoblotting with antibodies specific for TG type 1, 3 and 5 qRT-PCR
	Expression of TG substrates	Immunoblotting with antibodies specific for TG substrates (e.g., loricrin, SPR, involucrin, keratins) qRT-PCR
	Crosslinking activity	HPLC detection of K-L isodipeptide bonds Monodansyl-cadaverine incorporation to detect TG activity in tissues Radiolabeled putrescine incorporation to detect TG activity in cell extracts
Necrosis	Activation of calpains	Colorimetric/fluorogenic substrate-based assays of cell lysates in microtiter plates
	Activation of cathepsins	Colorimetric/fluorogenic substrate-based assays in live cells Colorimetric/fluorogenic substrate-based assays of cell lysates in microtiter plates
	Drop of ATP levels	Luminometric assessments of ATP/ADP ratio
	HMGB-1 release	Immunoblotting of culture medium with HMGB-1-specific antibodies
	LMP	FACS quantification with lysomorphotropic probes
	Plasma membrane rupture	Colorimetric/fluorogenic substrate-based assays of culture supernatants in microtiter plates to determine the release of cytosolic enzymatic activities (e.g., LDH) FACS quantification with vital dyes
	RIP1 phosphorylation	Immunoblotting with phosphoneoepitope-specific antibodies
	RIP1 ubiquitination	Immunoprecipitation with anti-RIP1 antibodies followed by immunoblotting with anti-ubiquitin antibodies
	ROS overgeneration	FACS quantification with ROS-sensitive probes
	Specific PARP1 cleavage pattern	Immunoblotting with PARP1-specific antibodies

Abbreviations: $\Delta\Psi_m$, mitochondrial transmembrane permeabilization; Cyt *c*, cytochrome *c*; FACS, fluorescence-activated cell sorter; GFP, green fluorescent protein; HPLC, high-pressure liquid chromatography: IF, immunofluorescence; IM, mitochondrial inner membrane; IMS, mitochondrial intermembrane space; LDH, lactate dehydrogenase; LMP, lysosomal membrane permeabilization; MMP, mitochondrial membrane permeabilization; PS, phosphatidylserine; qRT-PCR, real-time quantitative reverse transcription PCR; ROS, reactive oxygen species; RNAi, RNA interference; TG, transglutaminase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; VDAC1, voltage-dependent anion channel 1

of tumors that has been elaborated by pathologists over the last one and a half centuries. As old morphological categorizations of tumors are being more and more supported (and will presumably be replaced) by molecular diagnostics (which allows for a more sophisticated stratification of cancer subtypes based on molecular criteria), the current catalog of cell death types is destined to lose its value as compared with biochemical/functional tests. In the end, such efforts of classification are only justified when they have a prognostic and/or predictive impact, allowing the matching of each individual cancer with the appropriate therapy. Similarly, a cell death nomenclature will be considered useful only if it predicts the possibilities to pharmacologically/genetically modulate (induce or inhibit) cell death and/or if it predicts the consequences of cell death *in vivo*, with regard to inflammation and recognition by the immune system.

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