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News and Commentary

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

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Different cell death types are 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 different cell death morphologies, while formulating several caveats against the misuse of words and concepts that slow down progress in the area of cell death research. Nomenclature must be open to improvements and amendments to entail new discoveries, and the NCCD will help to update and clarify these points. Authors, reviewers and Editors of scientific periodicals are invited to abandon expressions like 'percentage apoptosis' and to replace them by more precise descriptions of the parameters that are actually measured. Moreover, at the present stage, it should be accepted that 'apoptosis', as a form of cell death, can occur with or without, caspase activation and that 'autophagic cell death' represents a type of cell death with (but not necessarily through) autophagic vacuolization. This article details the 2005 recommendations of NCCD. Over time, molecular definitions are expected to emerge for those forms of cell death that remain descriptive.

Preface

It is obvious that clear definitions of objects that are only shadows in Plato's cage are difficult to be achieved. Cell death and the different subroutines leading to cell death do not escape this rule. Even worse, the notion of death is strongly influenced by religious and cultural beliefs, which may subliminally influence the scientific view of cell death. As an example, it appears counterintuitive that some cells exert essential functions when they are 'dead' (such as erythrocytes and keratinocytes), and this underscores the importance of clearly defining what is referred to as 'cell death' as well as the multiple processes leading to it. Knowing that the meaning of scientific words changes when knowledge advances and that words, especially when they express changing concepts,[1–4] can increase confusion, one may adopt one of two opposing views. A significant fraction of the community of cell death researchers refutes nomenclature as an intellectual cage and as forever 'premature', remembering that many investigators desperately searched for 'DNA ladders' during the 1980s and 1990s. Indeed, such an alteration was thought to constitute the obligate manifestation of apoptosis, and reviewers and Editors often insisted that authors should discriminate between apoptosis and necrosis, based on this criterion (which nowadays has become somewhat obsolete). Some in the research community refute the preponderant idea that words can be used in a subjective fashion. Scientists simply should not behave as a Court of Justice confronted with the issue of pornography: the witness said that although he could not provide a clear definition of pornography, he knew what it was when he saw it. As a result of the need for more precise classification and following earlier discussions,[5,6] the Editors of Cell Death and Differentiation have created the NCCD, which formulated the following recommendations and caveats.

The NCCD suggests that it is important to discriminate between dying as a process and death as an end point. Dying can, of course, occur by several mechanisms, each characterized by a number of criteria, although not all need necessarily be present to satisfy the definition. It must be remembered that dying in a cell population is not a synchronous but rather a stochastic process, and that at a given time, individual cells will be at different stages of the dying process. This makes it all the more important to precisely define the criteria used to assess a dying population.

It is not the intention of the NCCD to replace single terms in common usage with repeated cumbersome phrases like 'percentage of cells undergoing phosphatidylserine exposure'. Clearly, this would dramatically lengthen articles and presentations and may prove highly unpopular. Rather, the intention of the NCDD is to clearly define the available criteria used to evaluate a particular dying process. It is clear that as the field advances even more precise molecular definitions will likely emerge, and the nomenclature will need to be revised and updated. The NCCD suggests that more precise definitions will also serve a purpose to accelerate molecular understanding by demonstrating how much or how little we know about certain forms of cell death.

Recommendation to Authors, Reviewers and Editors of Scientific Journals

Percentage apoptosis

As discussed above, authors frequently use expressions like 'percentage apoptosis' without mentioning the method used to assess ongoing cell death. Such expressions are confusing and imprecise and should be abandoned. Cell Death and Differentiation will actively enforce a policy in which expressions like 'percent apoptosis', 'percent necrosis', 'percent cell death' and 'percent cell survival' must be replaced by more descriptive terms such as 'percent cells with condensed chromatin', 'percent propidium iodine-positive'; 'percent annexin V-binding', 'percent active caspase-3 positive', 'percent TUNEL positive' cells, 'percent cells with DNA fragmentation', 'percent cells with a low mitochondrial transmembrane potential', or 'percent clone forming' 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. The NCCD encourages investigators studying cell death to quantify this process using more than one assay whenever possible. This has been the general practice in the field by many, and it is hoped that over time specific criteria will emerge regarding what is necessary or sufficient to measure apoptosis or to predict death as an subsequent outcome based on any particular measurement.

Autophagy

Along the same lines, 'vesicular redistribution of LC3' or the 'presence of double-membraned microvesicles' (or whatever was actually observed) is better than 'autophagy'. Thus, rather than formalizing nomenclature, the use of functional terms should be encouraged as a general policy. The NCCD urges all life science journals and, more specifically, all journals in the areas of cell biology, cancer research and pharmacology to adopt a similar policy.

When is a Cell 'Dead'?

Point-of-no-return

Dying cells can engage in a process that is reversible until a first irreversible step or 'point-of-no-return' is trespassed. It has been proposed that this step could be massive caspase activation, loss of the mitochondrial transmembrane potential ($\Delta\Psi$m),[7] complete permeabilization of the outer mitochondrial membrane,[8] or exposure of phosphatidylserine residues that emit 'eat me' signals to neighboring normal cells. However, there are examples in which caspases are activated in nonlethal activation and differentiation pathways. Moreover, the $\Delta\Psi$m can be dissipated by protonophores without that this would lead to immediate cell death.[9] Finally, phosphatidylserine exposure can be reversible, for instance in neutrophilic granulocytes.[10] Thus, the concept of a restriction point for cell death as was described by Pardee for the cell cycle has yet to be specifically defined for apoptosis.

Dead cells

In the absence of a clear, generally accepted view of the 'point-of-no-return', the NCCD suggests that a cell should be considered dead when any of the following molecular or morphological criteria are met: (1) the cell has lost the integrity of the plasma membrane, as defined by vital dyes *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) have been engulfed by an adjacent cell *in vivo*. Thus, 'dead cells' would be different from *bona fide* 'dying cells' that are in the process of cell death, which can occur through a variety of different pathways (see below). Moreover, cells whose cell cycle is arrested (as it occurs in senescence) would be considered as alive and the expression 'replicative cell death' (which alludes to the loss of the clonogenic capacity) should be avoided.

Mechanism-based Definitions of Cell Death Types

It is evident that death may occur through different mechanisms leading to distinct morphologies. Consequently, different names have been coined. The NCCD strongly recommends a limit on new names except where specific molecular mechanisms, or at least pathognomonic morphological characteristics, have been defined.

Apoptosis

The word 'apoptosis' has been coined by Kerr *et al.*,[11] describing a particular morphological aspect of cell death, and it would be erroneous and confusing to replace this definition, which remains a merely morphological description. Apoptosis is a type of cell death that is accompanied by rounding-up of the cell, retraction of pseudopodes, reduction of cellular volume (pyknosis), condensation of the chromatin, fragmentation of the nucleus (karyorhexis), little or no ultrastructural modification of cytoplasmic organelles, plasma membrane blebbing, and maintenance of an intact plasma membrane until late stages of the process (Figure 1). Biochemical analyses such as DNA ladders should not be used to *define* apoptosis, because this type of cell death can occur without oligonucleosomal DNA fragmentation. Frequently, active suppression of DNA fragmentation or caspase activation

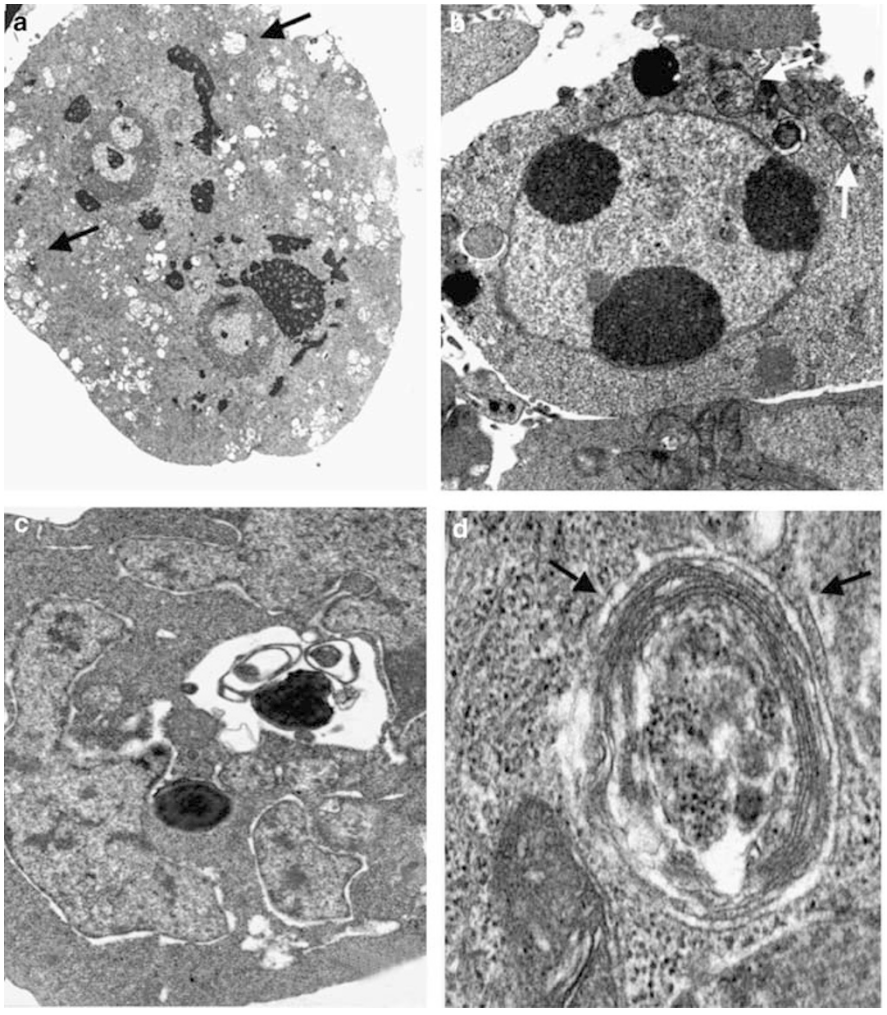

Figure 1 Morphological ultrastructural appearance of cell death by transmission electron microscopy. (**a**) Human epithelial cell undergoing necrosis after oxidative stress. Note the plasma membrane rupture, intracellular vesicle swelling and loss of mitochondrial ultrastructure (arrows). Magnification: $\times$ 4000. (**b**) Human epithelial cell undergoing apoptosis induced by radiation. Note the chromatin clumping and the integrity of mitochondria (arrow). Magnification $\times$ 4000. (**c**, **d**) Human epithelial cell undergoing autophagic vacuole formation after tamoxifen treatment. Note in (**c**) the normal morphology of the nucleus, the dilation of the perinuclear endoplasmic reticulum and the presence of some large vacuoles containing digested materials and in (**d**) the typical double membrane (arrows) characterizing the autophagic vacuoles. Magnifications: (**c**) $\times$ 8000 and (**d**) $\times$ 16000

demonstrates that these changes are not required for the execution of the cell death program, although caspase activation may be required for the acquisition of the apoptotic morphology. The measurement of DNA fragmentation or of caspase activation, however, may be helpful in *diagnosing* apoptosis. Another approach to consider here is to use the terms 'apoptosis associated with caspase activation' and 'apoptosis without evidence of caspase activation'. Thus, it may be reasonable to use caspase activation not only to diagnose but also to better define the type of cell death, that is, apoptosis associated with caspase activation. Indeed, at the biochemical level, apoptotic cell death is often accelerated by or even dependent on caspase activation.

Autophagy

'Autophagic cell death' is also morphologically defined, especially by transmission electron microscopy, as a type of cell death that occurs without chromatin condensation, accompanied by massive autophagic vacuolization of the cytoplasm. These vacuoles, by definition, are two-membraned and contain degenerating cytoplasmic organelles or cytosol.[12] Thus, autophagic vacuoles are distinguishable by electron microscopy from other types of vesicles such as endosomes, lysosomes or apoptotic blebs. Another *bona fide* marker of autophagic vacuolization is the redistribution of an LC3-GFP fusion protein into vesicular structures.[13] Although the term 'autophagic cell death' is a linguistic invitation to believe that cell death is occurring *through* autophagy, the term simply describes cell death *with* autophagy. Indeed, there is no *in vivo* evidence, thus far, that the knockdown or knockout of genes required for autophagy reduces cell death, and some reports even suggest that cells presenting features of 'autophagic cell death' can still recover upon withdrawal of the death-inducing stimulus.[14]

'Necrosis' and 'oncosis'

'Necrosis' is usually considered as a type of cell death with no signs of apoptosis or of autophagy, which is a negative definition.[15] The morphological appearance of necrosis is often that of oncosis. The expression 'oncosis' defines a cell death morphology with cytoplasmic swelling, mechanical rupture of the plasma membrane, dilation of cytoplasmic organelles (mitochondria, endoplasmic reticulum and Golgi apparatus), as well as moderate chromatin condensation. The NCCD recommends limiting the use of the expression 'oncosis', as it overlaps with necrosis, and with a partial apoptosis evolving into necrosis. Although the name 'oncosis' corresponds well to the morphological appearance of this type of cell death, 'necrosis' should be maintained for historical reasons. The NCCD recommends not using the term 'apoptonecrosis', which could generate further confusion (see below). Pathways leading to necrosis *in vivo* need to be elucidated so that in the future a more precise definition can be developed.

Mitotic catastrophe

'Mitotic catastrophe' is a cell death occurring during or shortly after a dysregulated or failed mitosis and can be accompanied by morphological alterations such as micronuclei (which often are chromosomes or chromosome fragments that have not been distributed evenly between the daugher nuclei) and multinucleation (the presence of two or more nuclei with similar or heterogeneous sizes, resulting from deficient separation during cytokinesis). However, there is no broad consensus on the use of this term,[16–18] and the NCDD recommends instead the use of terms such as 'cell death preceded by multinucleation' or 'cell death occurring during the metaphase', which are more precise and more informative. It is clear, however, that cell death during metaphase may have nothing to do with a cytokinesis or chromosome segregation defect but may occur because of exposure of cells to apoptotic stimuli during mitosis. The NCCD recommends including associated molecular events to describe cell death in any part of the cell cycle. For death during mitosis, it is clear there are potential overlaps with other terms such as loss of clonogenicity following radiation exposure due to chromosome damage.

There are some critiques that can be formulated against the clearcut distinction of different cell types in the triad of apoptosis, autophagic cell death, and necrosis. First, these terms have been developed to a large extent by observing cultured cells and can only partially reflect the *in vivo* physiology of cell death. In tissues, cells are usually engulfed well before signs of advanced apoptosis can be detected. Thus some criteria for apoptosis may not be evident *in vivo* whereas other may suffice (see above). Thus, *in vivo* it may be acceptable – if irreversibility of the phenomena is demonstrated – to detect caspase activation and DNA fragmentation to diagnose apoptotic cell death. In mammals, autophagic cell death has only been described in pathological situations, for instance in degenerating neurons in the central nervous system. Accumulating evidence is suggesting a molecular pathway associated with autophagy and a potential molecular definition (see above). There are also numerous examples in which cell death demonstrates mixed features, for instance with signs of both apoptosis and necrosis, a fact that coined expressions like 'necroapoptosis' and 'aponecrosis'.[19] Similarly, in the involuting Drosophila salivary gland, autophagic vacuolization precedes signs of apoptosis again arguing against a clearcut distinction between different forms of cell death.[20] Thus, the frontiers between distinct cell death types are indistinct, precluding a neat taxonomy. Moreover, it should be noted that expressions like 'apoptosis' suggest a biochemical uniformity although there might be in reality several distinct subtypes of apoptosis that, however morphologically similar, are functionally distinct cell death subroutines.

Anoikis

Apoptosis induced by loss of the attachment is to the substrate or to other cells is called anoikis. Besides its specific form of induction, the molecular mechanisms seem to be classic apoptosis. The NCCD suggests accepting this nomenclature for historical reasons, since it is already quite diffuse in the literature. However, it will be necessary to determine whether under certain circumstances other forms of death may occur *in vivo* following detachment, that is, whether there are forms of anoikis refractory to caspase inhibitors or others with features of autophagy.

Excitotoxicity

This is a form of death occurring in neurons when excitatory aminoacids, such as glutamate, leading to the opening of the *N*-methy-D-aspartate (NMDA) calcium channel, with subsequent increase of cytosolic calcium and death.[21] It is possible that this form of death may overlap with other types of death being unraveled and in the future it may be possible to determine whether this pathway involves ER stress and/or mitochondrial events.

Wallerian degeneration

Additional, less characterized forms of cell death occurs in the nervous system, such as Wallerian degeneration, where part of a neuron or axon degenerates without affecting the main cell body. As in the case of excitotoxicity more molecular characterization may better define this form of cell death as similar or distinct from other general processes or pathways.

Cornification

Cornification is a very specific form of programmed cell death occurring in the epidermis, different from apoptosis. It leads to the formation of the cornified envelope (or corneocyte), a dead keratinocyte containing an amalgam of specific proteins (e.g. loricrin, SPR, involucrin) and lipids (e.g. fatty acids, ceramides), necessary for the function of the cornified envelope (mechanical resistance, elasticity, water repellence, structural stability). Cornification is less often called 'keratinization' or 'cornified envelope formation'.[22]

Ill-defined Notions on Upstream Events and Terms Describing Cell Death Mechanisms

Programmed cell death

Frequently, apoptosis is referred to as synonymous to 'programmed cell death' (PCD). PCD is an expression that insinuates that cell death has been genetically programmed, as this is the case during development and aging. PCD is generally opposed to 'accidental cell death', that is necrosis induced by pathological stimuli. However, there are many circumstances that are difficult to consider as either 'programmed' or 'accidental', for instance when cytotoxic agents are added to cultured cells. So, these expressions are imprecise and should be replaced by expressions like 'developmental cell death', 'etoposide-induced cell death', 'cell death induced by osmotic shock', 'death induced by repeated freezing and thawing', etc. 'Death by neglect' can be conveniently translated by 'death induced by interleukin-3 withdrawal' or 'death induced by Akt inhibition'. Similarly, 'cell death following detachment' is more readily understood by those outside of the field, is less ambiguous than 'anoikis', and can be tested experimentally.

Caspase-independent apoptosis

Another classification of cell death types is based on the impact of inhibitors or genetic manipulations. Thus, cell death is frequently considered to be 'caspase-dependent' when it is inhibited 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 inhibit all caspases in an equally efficient fashion, yet does inhibit calpains and cathepsins. Thus, 'Z-VAD-fmk-inhibitable' is more precise than 'caspase-dependent', while using the same number of words. A second difficulty arises from the fact that caspase inhibition frequently inhibits signs of apoptosis (such as chromatin condensation and DNA fragmentation), yet only retards cell death.[23] In some instances, caspase inhibition simply induces a shift from an apoptotic morphology to a mixed morphology or even to full-blown pictures of necrotic or autophagic cell death, which, however, can manifest with some delay.[24] So under certain circumstances, an alternative to 'caspase-dependent death' may be 'Z-VAD-fmk-inhibitable chromatin condensation', if there is no information on the long-term behavior of the experimental system. 'Caspase independent cell death' (CICD)[23] can occur despite the effective inhibition of caspases and can manifest with the morphological signs of apoptosis, autophagy or necrosis. So while the idea that apoptosis equals caspase activation should be abandoned, caspase activation can be a major predictor of death and can be a defining feature of apoptosis.

Postface

Although it may be true that rigorous, 'legal' classifications of types of death are more important for the forensic department of the police than for the cell death research community, it is essential that scientists use terminology in a generally accepted and correct fashion. The NCCD has begun formulating clear recommendations that apoptosis, autophagic cell death and necrosis/oncosis are purely morphological entities and that conceptual short circuits such as programmed cell death = apoptosis = caspase-dependent cell death or accidental cell death = necrosis = caspase-independent cell death should be avoided. Moreover, the NCCD issues a firm recommendation against the use of poorly defined terms such as the percentage of apoptosis, necrosis, death or survival suggesting that they should be substituted by a precise description of the parameters that are measured. In the future, as progress unravels a clear distinction between different cell death subroutines, in molecular terms, the NCCD will formulate updated recommendations on cell death terminology.

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