

## News and Commentaries

# Cell death modalities: classification and pathophysiological implications

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Cell death can be classified according to the morphological appearance of the lethal process (that may be apoptotic, necrotic, autophagic or associated with mitosis), enzymological criteria (with and without the involvement of nucleases or distinct classes of proteases, like caspases or cathepsins), functional aspects (programmed or accidental, physiological or pathological) or immunological characteristics (immunogenic or non-immunogenic). Thanks to the advancing comprehension of cellular demise, it has become clear that the textbook equation 'programmed cell death = apoptosis = caspase activation = non-immunogenic cell death', although applicable to some instances of cell death, constitutes an incorrect generalization, at several levels. Thus, necrosis can be programmed both in its course and its occurrence. Apoptosis can be lethal without caspase activation, and caspase activation does not necessarily cause cell death. Finally, cell death with an apoptotic appearance can be immunogenic, in which case the immunogenicity is caspase-dependent. These examples illustrate the urgent need to strive towards a more detailed comprehension of cell death subroutines, with far-reaching implications for the pharmacological management of pathological cell loss and growth.

In conditions of homeostasis, in the adult organism, each event of cell duplication must be compensated by the elimination of another cell. Although in the human body cell deaths occur at the dazzling frequency of several millions per second, the subtle regulation of cell death – coupled to a perfect waste management – allows us to enjoy a peaceful existence for several years, until we are affected by disease. Pathological conditions are often, if not always, tied to deregulated (excessive or deficient) cell death (Figure 1). The loss of post-mitotic cells such as neurons and cardiomyocytes occurs acutely in stroke and infarction or progressively in degenerative diseases. Moreover, AIDS is caused by the loss of proliferating immune cells at a pace that cannot be compensated for by proliferation. Conversely, oncogenesis is characterized by the (at least) partial suppression of cell death programs, which in turn causes chemo- and radio-therapy resistance, thus ultimately sealing the patient's fate.

The physiological importance and pathological impact of cell death has spurred great interest, leading to the accumulation of more than 150 000 research papers over

the last 20 years. Nonetheless, apparently simple questions on the very definition of cell death (Table 1) and on the classification of cell death modalities in stereotyped patterns have not yet been solved. In this review, we will synthetically and critically enumerate the current classifications of cell death, laying special emphasis on the link between the morphological, biochemical and pathophysiological characteristics of different cell death modalities.

## Morphological Characterization of Cell Death

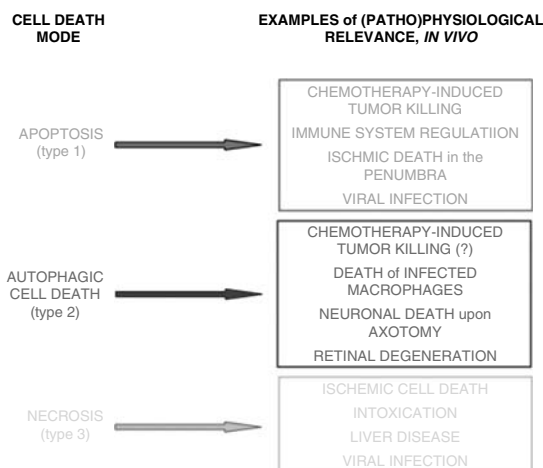
The Editors of *Cell Death and Differentiation* created in 2005 the Nomenclature Committee on Cell Death (NCCD) that was joined by a selected panel of experts.<sup>1</sup> After several months of discussion, the NCCD decided that the 'official' classification of cell death modalities had to rely on purely morphological criteria (Table 2), owing to the absence of a clear-cut equivalence between ultrastructural alterations and biochemical cell death characteristics.

The morphological changes that define the best-studied modality of cell death, apoptosis (type 1 cell death), include nuclear pyknosis (chromatin condensation) (Figure 2a) and karyorrhexis (nuclear fragmentation).<sup>1</sup> Apoptotic cells finally form small round bodies that are surrounded by membranes and contain intact cytoplasmic organelles or fragments of the nucleus. These apoptotic bodies result from progressive cellular condensation and budding, and eventually are engulfed by resident phagocytic cells (e.g. epithelial cells or fibroblasts). Biochemical analyses of DNA fragmentation or caspase activation should not be used to define apoptosis, because this type of cell death can occur without oligonucleosomal DNA fragmentation as well as without caspase activation.

While apoptosis involves the rapid demolition of all cellular structures and organelles, autophagy is a slow, spatially restricted phenomenon in which parts of the cytoplasm are sequestered within double-membraned vacuoles (Figure 2b) and finally digested by lysosomal hydrolases.<sup>2</sup> The functional relationship between apoptosis and autophagy is complex, and autophagy may either contribute to cell death<sup>3</sup> or constitute a cellular defense against acute stress, in particular that resulting from the deprivation of nutrients or obligate growth factors.<sup>4,5</sup> Massive autophagic vacuolization is ob-

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**Figure 1** Different cell death subroutines play an important role in human disease. Apoptosis, autophagy and necrosis have been associated with the course of several pathological conditions, either in a specific or (partially) overlapping fashion. Thus, cell death following an ischemic injury may manifest either with a necrotic or an apoptotic phenotype, according to the exact localization of the cell within the ischemic region. In other instances, as in the case of macrophages succumbing upon the intracellular infection by *Salmonella enterica*,<sup>43</sup> autophagic cell death, but not apoptosis nor necrosis, is specifically involved

served in some instances of cell death, which has been named 'autophagic cell death' (type 2 cell death). It is an ongoing conundrum, however, in which cases 'autophagic cell death' is truly mediated *through* autophagy (meaning that inhibition of autophagy would abolish cell death) and in which cases it simply occurs *with* autophagy (meaning that inhibition of autophagy would only affect the morphology of the process, but not the fate of cells).

Necrosis (type 3 cell death) is usually defined in a negative fashion, as a type of cell demise that involves rupture of the plasma membrane without the hallmarks of apoptosis (pyknosis, karyorrhexis, cell shrinkage and formation of apoptotic bodies) and without massive autophagic vacuolization. The principal features of necrosis include a gain in cell volume (oncosis) that finally culminates in rupture of the plasma membrane, and the unorganized dismantling of swollen organelles. Hence, necrosis lacks specific biochemical markers, apart from the presence of plasma membrane permeabilization, and can be detected only by electron microscopy (Figure 2c). Necrosis is considered to be harmful because it is often associated with pathological cell loss and because of the ability of necrotic cells to promote local inflammation that may support tumor growth.<sup>6</sup> Importantly, cell death that usually manifests with an apoptotic morphology can be shifted to a more necrotic phenotype when caspase

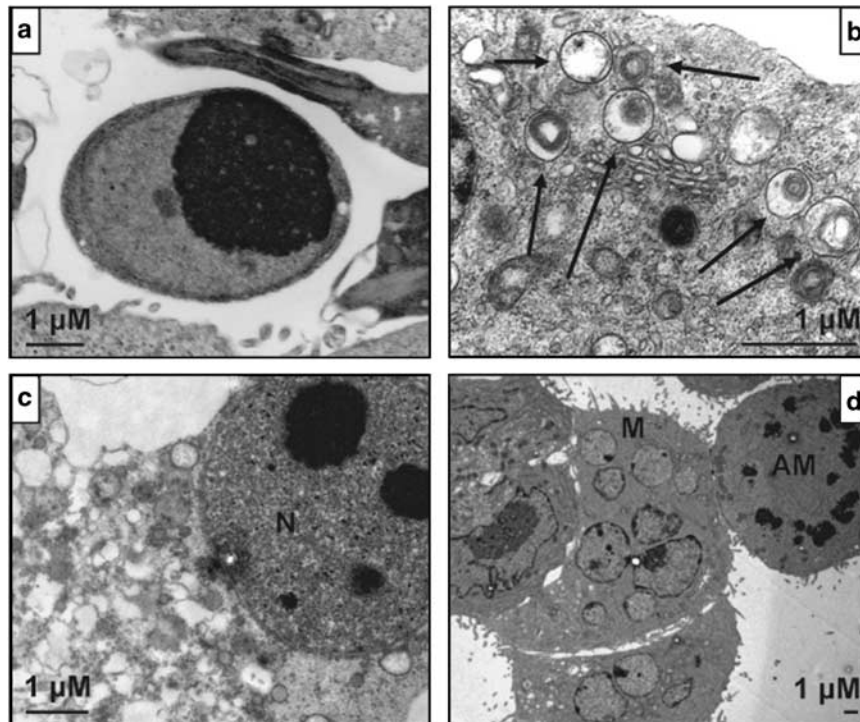
**Table 1** Possible definitions of cell death

| Definition                                                   | Notes                                                                                                                                                                                    | Technique                                                                                |
|--------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------|
| <b>Molecular-morphological criteria to define dead cells</b> |                                                                                                                                                                                          |                                                                                          |
| Loss of plasma membrane integrity                            | Plasma membrane has broken down, resulting in the loss of the cell's identity.                                                                                                           | Exclusion of vital dyes, <i>in vitro</i>                                                 |
| Cell fragmentation                                           | The cell (including its nucleus) has undergone complete fragmentation into discrete bodies (usually referred to as apoptotic bodies).                                                    | (IF) Microscopy<br>FACS quantification of hypodiploid events                             |
| Engulfment by adjacent cells                                 | The corpse or its fragments have been phagocytosed by neighboring cells.                                                                                                                 | (IF) Microscopy<br>FACS colocalization studies                                           |
| <b>Proposed points-of-no return to define dead cells</b>     |                                                                                                                                                                                          |                                                                                          |
| Massive activation of caspases                               | Caspases execute the classic apoptotic program, yet in several models caspase-independent death occurs. Moreover, caspases mediate numerous vital processes.                             | Immunoblotting<br>FACS quantification with fluorogenic substrates or specific antibodies |
| $\Delta\Psi_m$ dissipation                                   | Sustained $\Delta\Psi_m$ loss usually precedes MMP and cell death, however, transient dissipation is not always a lethal event.                                                          | FACS quantification with $\Delta\Psi_m$ -sensitive probes<br>Calcein-cobalt technique    |
| MMP                                                          | Complete MMP results in the liberation of pro-death proteins. Nonetheless, partial permeabilization may not lead to the fatal outcome.                                                   | IF colocalization studies<br>Immunoblotting upon fractionation                           |
| PS exposure                                                  | PS exposure on the outer leaflet of the plasma membrane usually is an early event of apoptosis, but may be reversible. PS exposure occurs also in T-cell activation, without cell death. | FACS quantification with Annexin-V                                                       |
| <b>Operative definition of cell death</b>                    |                                                                                                                                                                                          |                                                                                          |
| Loss of clonogenic survival                                  | This method does not distinguish cell death from long-lasting or irreversible cell cycle arrest. It is not applicable to post-mitotic cells.                                             | Clonogenic assays                                                                        |

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

**Table 2** Morphological aspects of different modes of cell death<sup>1</sup>

| Cell death mode             | Characteristic morphological aspects                                                                                                                                                                                                                                                                                         | Notes                                                                                                                                                                            |
|-----------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Apoptosis (Type 1)          | <ul style="list-style-type: none"> <li>● Rounding up of the cell</li> <li>● Reduction of cellular and nuclear volume (pyknosis)</li> <li>● Retraction of pseudopodes</li> <li>● Nuclear fragmentation (karyorrhexis)</li> <li>● Little modification of cytoplasmic organelles</li> <li>● Plasma membrane blebbing</li> </ul> | 'Apoptosis' is the original term introduced by Kerr <i>et al.</i> <sup>44</sup> to define a cell death with specific morphological features.                                     |
| Autophagy (Type 2)          | <ul style="list-style-type: none"> <li>● Lack of chromatin condensation</li> <li>● Massive vacuolization of the cytoplasm (double-membraned autophagic vacuoles)</li> </ul>                                                                                                                                                  | 'Autophagic cell death' defines cell death occurring <i>with</i> autophagy, though it may misleadingly suggest a form of death occurring <i>through</i> autophagy. <sup>45</sup> |
| Necrosis (oncosis) (Type 3) | <ul style="list-style-type: none"> <li>● Cytoplasmic swelling</li> <li>● Rupture of plasma membrane</li> <li>● Swelling of cytoplasmic organelles</li> <li>● Moderate chromatin condensation</li> </ul>                                                                                                                      | 'Necrosis' identifies, in a negative fashion, cell death lacking the features of apoptosis or autophagy, and usually appears as oncosis. <sup>46</sup>                           |
| Mitotic catastrophe         | <ul style="list-style-type: none"> <li>● Micronucleation</li> <li>● Multinucleation</li> </ul>                                                                                                                                                                                                                               | 'Mitotic catastrophe' refers to a cell death occurring during or shortly after a failed mitosis. <sup>11</sup>                                                                   |



**Figure 2** Morphological ultrastructural features of cell death by transmission electron microscopy. (a) Human non-small cell lung cancer (H1975) cell undergoing apoptosis induced by the treatment with an inhibitor of the epidermal growth factor receptor (EGFR). Note the cell's shrinkage and complete nuclear condensation (pyknosis). (b) Human epithelial cancer (HeLa) cell treated with the endoplasmic reticulum toxin thapsigargin. The cell is succumbing from autophagic cell death, as witnessed by the massive accumulation of double-membraned cytoplasmic vacuoles (arrows) containing organelles or parts of the cytosol. (c) Human colon carcinoma (HCT116) cell responding to a necrotic stimulus. Whereas the nucleus (N) is still intact and presents clearly defined nucleoli, the plasma membrane and the cytoplasm are extensively dismantled. (d) HCT116 cells treated with substances that alter the microtubule dynamics (e.g. taxanes) exhibit the hallmarks of mitotic catastrophe, including aberrant multipolar mitotic figures (AM) as well as multinucleation (M) phenomena. Scale bars represent 1  $\mu$ m

activation is prevented by pharmacological inhibitors or by the elimination of essential caspase activators such as Apaf-1.<sup>7,8</sup> Similarly, cell death that usually exhibits an autophagic phenotype can be shifted to a necrotic morphology by inhibiting the early steps of autophagy.<sup>3,9,10</sup> Attempts are underway to describe necrosis in biochemical terms,<sup>10</sup> yet still fall short of a redefinition of the process.

A special case of cell death is mitotic catastrophe, that is cell death occurring during or shortly after a failed mitosis. Usually, mitotic catastrophe involves micronucleation and multinucleation events that occur before cell death (Figure 2d). Mitotic catastrophe results from a combination of deficient cell cycle checkpoints (in particular the DNA structure and the spindle assembly checkpoints) and cellular damage (for a review see

Castedo *et al.*<sup>11</sup>). Failure to arrest the cell cycle before or at mitosis triggers an attempt of aberrant chromosome segregation, which culminates in the activation of self-destructive pathways and ultimately leads to the cellular demise.

### Enzymological Classification of Cell Death

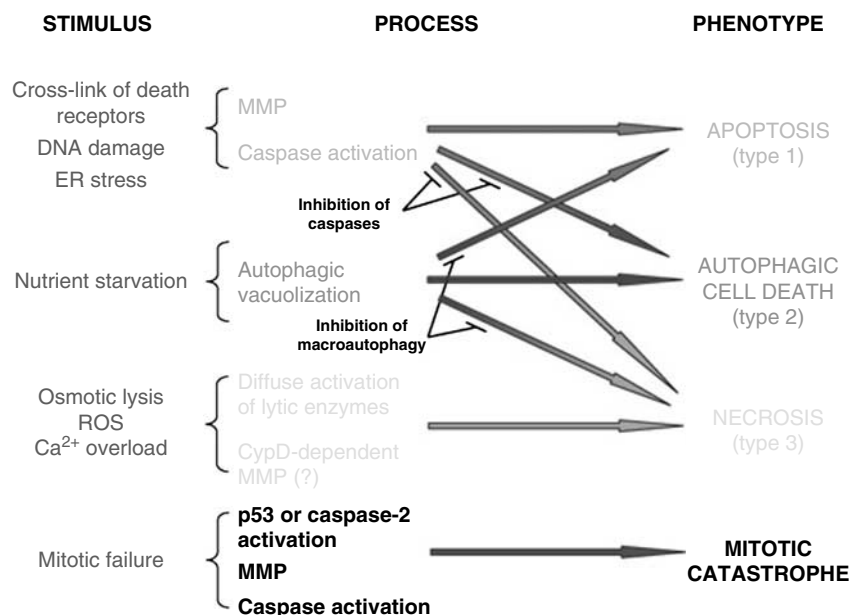
Admittedly, it is deceptive that at the dawn of the 21st century cell death modalities are still defined by morphological rather than by biochemical criteria. Beyond any doubt, catabolic enzymes and processes are activated in distinct cell death processes to different extents (Figure 3 and Table 3). For instance, the contribution of caspases is frequently observed in apoptosis, while calpains and cathepsins are often (but not exclusively) associated with necrosis. Mitochondrial membrane permeabilization (MMP) is involved in both apoptosis and necrosis,<sup>12–14</sup> and is commonly considered as the 'point-of-no-return' in the cascade of events leading to cell death (Table 1). Accordingly, MMP (involving either the inner or the outer mitochondrial membrane) is routinely assessed to identify cells irreversibly committed to death which have not yet acquired an apoptotic phenotype.<sup>15,16</sup> Finally, activation of the autophagic process according to a precise sequence of molecular events (formation of autophagic vacuoles followed by their fusion with lysosomes to form autophagolysosomes) occurs during type 2 cell death.

The metabolic context (i.e. replenishment of the intracellular energy stores, oxygen level) seems to play a crucial role in determining the subroutine of cell death, regardless of the initiating stimulus. It has been reported that while high glucose and ATP levels favor the execution of apoptosis, their depletion switches cell death to necrosis in several experi-

mental settings.<sup>17,18</sup> This may derive (at least partially) from the fact that ATP is required for caspase activation.<sup>19</sup> Nonetheless, metabolic determinants *per se* do not suffice to predict the pathway of cell death that will be executed following specific lethal signals.

One major difficulty in applying biochemical criteria to the classification of cell death modalities is the constant confusion between associations and mechanisms. While many if not most cases of apoptosis are accompanied by caspases, and caspase inhibition often hinders the morphological appearance of full-blown apoptosis (and hence 'inhibits apoptosis'), the inactivation of these proteases rarely prevents the death of mammalian cells succumbing to lethal signal transduction pathways. This is due to an inbuilt redundancy of the mechanisms underlying cell death. In this context, non-caspase death effectors (*e.g.* AIF, EndoG and HtrA2/Omi) as well as the deleterious consequences of failing mitochondrial metabolism lead to cell death even when effector caspases are inhibited.<sup>8,12,14,20</sup> The issue, however, remains controversial, since it has been demonstrated that the release of these factors occurs also via caspase-dependent pathways, at least in some experimental paradigms.<sup>21,22</sup> Thus, although apoptosis often occurs *with* caspase activation and the acquisition of some of the hallmarks of apoptosis are strongly caspase-dependent, apoptotic death does not (or rarely) occur entirely *through* caspase activation. A similar critique can be formulated for the so-called autophagic cell death, which occurs *with* but infrequently (if ever) *through* autophagy.

Instead of characterizing single enzymes or groups of enzymes in the apoptotic process, some attempts have been made to distinguish cell death modalities based on the involvement of distinct organelles. Thus, cell death that



**Figure 3** The biochemical processes underlying cell death. Specific proapoptotic stimuli are able to activate molecular pathways ultimately leading to one particular cell death mode. For instance, the ligand-binding-induced crosslink of death receptors results in a well-known cascade of intracellular events, which culminates in the activation of caspases and apoptotic cell death. Another example is given by the deprivation of nutrients, which induce massive autophagic vacuolization and type 2 cell death. However, the inhibition of intracellular processes like caspase activation or macroautophagy by pharmacological or genetic means may shift the phenotype of the lethal outcome from one type to another. CypD, cyclophilin D; ER, endoplasmic reticulum; MMP, mitochondrial membrane permeabilization; ROS, reactive oxygen species

**Table 3** Catabolic enzymes and processes involved in cell death

| Enzymes                 | Involvement                                 | Notes                                                                                                                                                                                                                      |
|-------------------------|---------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Nucleases</b>        |                                             |                                                                                                                                                                                                                            |
| EndoG                   | MMP – caspase-independent death             | Mitochondrial protein (resident in the IMS of healthy cells). During apoptosis, EndoG gains access to the nucleus following MMP. <sup>47,48</sup>                                                                          |
| DFF40 (CAD)             | Caspase-dependent apoptosis                 | Nuclear enzyme, maintained inactive by the interaction with DFF45 (ICAD). Activation occurs following DFF45 cleavage mediated by caspase-3 or -7. <sup>48</sup>                                                            |
| NM23-H1                 | Caspase-independent apoptosis               | Exonuclease of the ER-associated SET complex, activated by GrzA. <sup>49</sup>                                                                                                                                             |
| <b>Proteases</b>        |                                             |                                                                                                                                                                                                                            |
| Executioner caspases    | Extrinsic and intrinsic pathways            | Caspase-3, -6 and -7 account for the degradative phase of apoptosis triggered by different stimuli. <sup>50,51</sup>                                                                                                       |
|                         | Intrinsic pathway                           | Caspase-6 and -7 are involved in mitochondrial events of apoptosis. <sup>52</sup>                                                                                                                                          |
| Lysosomal cathepsins    | Lysosomal rupture                           | Lysosomal proteases released upon the rupture of the organelle. Cathepsins promote caspase-activation as well as the release of proapoptotic factors from mitochondria. <sup>2,53</sup>                                    |
| Granzymes               | Bid-dependent and -independent MMP          | Proteases released by cytotoxic lymphocytes. GrzA induces apoptosis by targeting the ER-associated SET complex. GrzB reportedly promotes Bid proteolytic activation. <sup>49,54</sup>                                      |
|                         | Degradation of antiapoptotic Bcl-2 proteins | Mcl-1 is degraded by GrzB, this promoting the release of proapoptotic BH3-only proteins (Bim). <sup>55</sup>                                                                                                               |
| Initiator caspases      | Extrinsic pathway                           | Caspase-8 and -10 are activated following the assembly of the DISC. <sup>56,57</sup>                                                                                                                                       |
|                         | ER stress                                   | Caspase-12 triggers p53-independent apoptosis in response to ER stress. <sup>58</sup>                                                                                                                                      |
|                         | Intrinsic pathway                           | Caspase-9 activation requires the cytosolic formation of the apoptosome, after Cyt c release from mitochondria. Caspase-2 is activated in the PIDDosome, as a result of DNA damage. <sup>56,59,60</sup>                    |
| Omi/HtrA2               | MMP – caspase-independent death             | Serine protease resident in the IMS of healthy cells. Upon MMP it translocates to the cytosol, where it promotes apoptosis via its catabolic activity and by antagonizing caspase inhibitors (e.g. XIAP). <sup>61,62</sup> |
| Calpains                | Ca <sup>2+</sup> -activated proteases       | Activate caspases, proapoptotic Bcl-2 family proteins (such as Bid), mitochondrial AIF release and lysosomal membrane permeabilization. <sup>63,64</sup>                                                                   |
| <b>Others</b>           |                                             |                                                                                                                                                                                                                            |
| Acidic sphingomyelinase | Extrinsic pathway                           | Activated upon the ligand-induced crosslink of death receptors, it promotes the accumulation of ganglioside GD3, which in turn has direct MMP-promoting effects. <sup>65</sup>                                             |
| Phospholipases          | Lysosomal rupture                           | Reportedly favors lysosomal destabilization. <sup>66</sup>                                                                                                                                                                 |
|                         | Intrinsic pathway                           | In some models of cell death, Ca <sup>2+</sup> -independent phospholipase A participates in MMP mediated by proapoptotic Bcl-2 family members. <sup>67</sup>                                                               |
|                         | Lysosomal rupture                           | Phospholipase C promotes the osmotic destabilization of lysosomes. <sup>66</sup>                                                                                                                                           |

Abbreviations: BH3, Bcl-2 homology domain 3; CAD, caspase-activated DNase; Cyt c, cytochrome c; DFF40, DNA fragmentation factor, catalytic subunit of 40 kDa; DFF45, DNA fragmentation factor, inhibitory subunit of 45 kDa; DISC, death inducing signaling complex; EndoG, endonuclease G; ER, endoplasmic reticulum; GrzA, granzyme A; GrzB, granzyme B; ICAD, inhibitor of CAD; IMS, mitochondrial intermembrane space; MMP, mitochondrial membrane permeabilization; Omi/HtrA2, Omi protease/High temperature requirement protein A2; XIAP, X-linked inhibitor of apoptosis protein

involves mitochondria (as occurring in type II cells succumbing upon the activation of the extrinsic pathway of apoptosis) has been distinguished from cell death that would not engage mitochondria (the case of type I cells, exhibiting 'direct' caspase activation after the ligation of death receptors).<sup>23</sup> However, this latter distinction has become superfluous in view of the discovery of a 'crosstalk' between the two pathways (for instance mediated by the proapoptotic BH3-only protein Bid).<sup>24–26</sup> Similarly, it has been attempted to label some instances of cell death as 'lysosomal', because they are associated with lysosomal membrane permeabilization (LMP) upstream and independent from MMP.<sup>2</sup> Future will tell whether such classifications will withstand experimental verification.

### Programmed Versus Accidental Cell Death

The term 'programmed cell death' was first coined to describe the developmental cell death of intersegmental muscle cell death in the developing silkworm, *Antheraea pernyi*.<sup>27</sup> While the cell death of insect muscle had an autophagic appearance, the term 'programmed cell death' has been used since in a generalized fashion, as a near-to-synonym of apoptosis in *Caenorhabditis elegans* and mammals. In contrast, necrosis has been considered as a merely 'accidental' consequence of non-physiological stress.

Nonetheless, necrosis can be programmed (*i.e.* genetically predetermined) as to both its course and its occurrence. A stereotyped cascade of biochemical events noted in different

models of necrosis<sup>10</sup> suggests the existence of a programmed course of events (how necrosis manifests) within the necrotic dying cell. Typical alterations that accompany (and perhaps define) necrosis are early signs of mitochondrial dysfunction (e.g. overproduction of ROS, swelling, ATP depletion and failure of the  $\text{Ca}^{2+}$  homeostasis), perinuclear clustering of organelles, activation of a limited set of proteases (in particular calpains and cathepsins), lysosomal rupture and finally plasma membrane breakdown.<sup>28–30</sup> The programmed occurrence of necrotic cell death (whether necrosis occurs) is indicated by the fact that necrosis can occur during mammalian development (e.g. the death of chondrocytes controlling the longitudinal growth of bones)<sup>31</sup> or in adult tissue homeostasis (e.g. intestinal epithelial cells).<sup>32</sup> Thus, necrosis can be triggered by the occupation of specific plasma membrane receptors by their physiological ligands,<sup>28–30</sup> a process that by no means can be considered as ‘accidental’. The inhibition of some enzymes and processes can prevent necrosis, meaning that these enzymes and processes play an active, decisive role in the lethal process. Finally, inhibition of caspases (which are often required for the morphological manifestation of apoptosis) can change the morphological appearance of cell death from type 1 to type 2 or 3 cell death.<sup>7,8</sup> Thus, the same upstream signal can produce different types of cell death as a function of the activation/inhibition of catabolic enzymes in the cell.

In conclusion, the semantic distinction between ‘programmed’ (implicitly physiological) and ‘accidental’ (implicitly pathological) cell death is a *non-sequitur*. Indeed, there are many examples in which it is merely the intensity of the signal or stress that determines whether the cellular demise occurs through a more apoptotic or a more necrotic subroutine. For instance, this applies (but is not limited) to cell death induced by cisplatin,<sup>33–35</sup> UV irradiation<sup>35</sup> and photodynamic therapy.<sup>36</sup>

### Immunogenic Versus Non-immunogenic Cell Death

Conventional text book knowledge indicates that apoptosis is a clean and silent modality of cell death in which corpses are rapidly engulfed and degraded by neighboring cells, while necrosis would lead to spilling of the intracellular content into the intercellular space, triggering an inflammatory response. In this sense, apoptosis would constitute a suicide without corpse. This notion was used to edify the myth of apoptosis as ‘physiological’ cell death and necrosis as ‘pathological’. Although it is likewise correct that physiological cell death (which often occurs through apoptosis) is immunologically silent (or even tolerogenic) – otherwise we would all be affected by autoimmune diseases – there are numerous indications that warn against the oversimplified equation that apoptosis equals non-inflammatory, non-immunogenic cell death.

When mice are injected with dying tumor cells (or dendritic cells incubated with dying tumor cells) and specific antitumor immune responses are monitored, it becomes clear that the distinction between immunogenic and non-immunogenic cell death does not follow the frontier between apoptosis and necrosis.<sup>37</sup> Subcutaneous injection of cell lysates or of cells killed by necrosis (by freeze-thawing cycles or hypotonic

shock) is non-immunogenic. Similarly, most stimuli inducing apoptosis (including mitochondrion-targeted, lysosomotropic and genotoxic agents) also result in poorly immunogenic cell death. However, a selected panel of agents (and in particular anthracyclins and ionizing irradiation) does induce a modality of cell death that is apoptotic in morphology and highly efficient in eliciting an immune response, in the absence of any adjuvant.<sup>38,39</sup> Furthermore, cells killed by flu virus have been reported to elicit an immune response, both *in vitro* and *in vivo*.<sup>40</sup>

Inhibition of caspases does not prevent anthracyclin- or irradiation-induced cell death, yet it abolishes the immunogenic property of anthracyclin-killed cells, while shifting the cell death subroutine from rapid apoptosis to more delayed necrosis. Similarly, destruction of the corpuscular nature of anthracyclin-elicited apoptotic bodies by freeze-thawing cycles or osmotic lysis abolishes the immune response. Apparently, immunogenic (as opposed to non-immunogenic) apoptosis is characterized by the translocation of calreticulin from inside the cell (presumably from the endoplasmic reticulum) to the cell surface, where it provides a potent ‘eat me’ signal for specialized antigen-presenting cells, and in particular for dendritic cells.<sup>39</sup>

These findings point to the possibility that the immunogenicity of cell death is dictated by specific signals of danger (such as calreticulin exposure) rather than by the cell death modality by itself. Unfortunately, at present, no information is available on the immunogenic properties of type 2 cell death, mitotic catastrophe and cellular senescence. Further exploration of this topic is urgently awaited.

### Conclusions

Scientific activity involves data collection, classification and explanation. While classification of cell death modalities was an easy task when few data were available, the vertiginous pace of accumulating knowledge has invalidated the first attempts of placing cell death scenarios into neat boxes, representing indeed gross oversimplifications. Thus, the simple equation

$$\begin{aligned} \text{programmed cell death} &= \text{apoptosis} = \\ \text{caspase activation} &= \text{non-immunogenic cell death} \end{aligned}$$

although correct in selected instances, is incorrect as a generalization at multiple levels, as discussed in this review.

In summary, the classification of cell death subroutines should never be based on morphological, biochemical or immunological criteria alone, but rather should take into account and integrate these data (and any other available) in order to delineate as precisely as possible each distinct experimental model. Starting from such premises, the challenge for the future is to link specific molecular processes and patterns to peculiar morphological, enzymological, pathophysiological and immunological characteristics of cell death, beyond the limits of casuistic classification. It is our hope that system biology approaches will help us in moving from linear (and oversimplified) cause–effect sequences to detailed and comprehensive analyses of cell death mechanisms in health and disease.

We are, indeed, in urgent need to understand more precisely cell death. The therapeutic suppression of cell death is still in its infancy, even in conceptual terms, irrespective of the advancing knowledge on the morphological appearance of pathological cell death occurring in our body. Cytoprotection by suppression of cell death should be the therapeutic goal of organ preservation as well as of the clinical management of major diseases, including stroke, infarction and neurodegeneration. However, so far very few strategies for cytoprotection have proven successful, even in animal models of acute cell loss. Similarly, we still anxiously await the development of chemotherapeutics that would elicit immunogenic cell death, in spite of the fact that there has been some success in developing cell death-inducing regimens for cancer chemotherapy.<sup>41,42</sup> Such an 'immunogenic chemotherapy' would allow for the immune system-mediated eradication of tumor (stem) cells that resist cell death induction, thereby increasing the efficacy of treatment and the probability of total remission. These examples illustrate how an exhaustive investigation of the mechanisms underlying cell death could have enormous impacts on human medicine.

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## Role of cardiolipin in cytochrome c release from mitochondria

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Mitochondria play a pivotal role in the regulation of apoptotic cell death as well as in several cellular metabolic processes,

including energy supply.<sup>1,2</sup> The latter is achieved by oxidative phosphorylation of ADP to ATP using the electrochemical

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