Cell Death: A One-Way Journey to the Graveyard

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Abstract: Tissue homeostasis is ensured by the correct balance between cell proliferation and death, the latter mainly occurring through a multi-step program, named apoptosis, which ultimately leads to the breakdown of cellular DNA and proteins. Apoptosis is activated under physiological developmental conditions, during metamorphosis and atrophy of tissues and organs, sexual differentiation and cell turnover, and can also be triggered by various external stimuli, including DNA damage, growth factor deprivation and metabolic stress. The main features of apoptosis will be described in detail. Although apoptosis is recognised as the main type of programmed cell death, cells may die by alternative mechanisms, e.g. autophagy and necrosis. Their properties will be discussed in this review.

APOPTOSIS: A HISTORICAL OVERVIEW

Individual cells may die through a finely orchestrated sequence of biochemical and morphological changes which define the so-called Programmed Cell Death (PCD). This notion was first developed when Lockshin and Williams described the controlled cell death occurring during insect development [1-4], and further extended to different organisms [5-8]. After the discovery of PCD in insects, the best-studied animal model was the nematode Caenorhabditis elegans, which is characterised by the selective elimination of 131 out of 1090 cells during embryo development [9]. Genetic studies in this organism allowed the identification of the four essential gene products governing PCD: CED-3 (Cell Death Abnormal), a cystein-protease involved in protein degradation; CED-4, the upstream activator of CED-3; CED-9, able to bind CED-4, thus abolishing its death function; EGL-1, a CED-9-interacting protein that prevents CED-9/CED-4 interaction, thereby promoting PCD [9-11].

Apoptosis is the most well known form of PCD, defined for the first time by Kerr, Currie and Wyllie as an event reminiscent of the falling of leaves from trees [12]. The greek word apoptosis was used by Hippocrates to describe the removal of bone cells after a fracture and by Galen to define the falling of scabs [13], and was found in a Spanish dictionary of medicine written in 1878 [14].

DISTINCTIVE FEATURES OF APOPTOTIC CELLS

Morphological Changes

Apoptotic cells can be easily recognised by morphological hallmarks, such as nuclear shrinkage, chromatin condensation and aggregation, changes in the surface of plasma membrane, appearance of blebs and consequent formation of the so-called apoptotic bodies. Apoptosis can trigger cell death without rising of any inflammatory response or autoimmune reaction, because apoptotic cells display “eat-me signals”, including phosphatidylyserine [15], annexin I [16], a large variety of sugars, such as mannose and galactose [17], and ICAM-3 [18] (reviewed in [19]), which favour their recognition by phagocytes [20-22]. However, as recently reviewed [23], the non-immunogenic nature of apoptosis is presently debated, given that the apoptotic response elicited by anti-cancer therapies could exert an immunostimulatory side effect [24].

Degradation of Proteins

Apoptotic cells undergo controlled and organised DNA and protein digestion, carried out by specific endonucleases and proteolytic enzymes, respectively [25]. Among the proteases that promote the orderly destruction of structural and regulatory proteins [25], the best known are caspases. Caspases are cystolic proteases so-named because of their mechanism of action: they contain a cystein residue in their catalytic site and cleave their substrates after an aspartic acid [26]. To date, 14 distinct mammalian caspases have been identified. Their primary structure consists of an N-terminal pro-domain, a large subunit, and a small subunit linked by a region flanked by two aspartate residues [27]. As illustrated in Fig. (1), mammalian caspases with a long pro-domain are named initiator caspases; those with a short pro-domain are called effector caspases [28-30]. Caspases are present within the cell as inactivezymogens and are converted into active enzymes only in response to apoptotic stimuli. The conversion of initiator caspase-8 [31] and -9 [32] into active proteases occurs through their dimerisation, while the transformation of effector pro-caspases to mature enzymes requires one cleavage to remove the N-terminal pro-domain and one to separate the large from the small subunit. These cleavages are followed by heterodimerisation of the subunits (Fig. 1). Once activated, caspases can in turn activate other caspases. Many targets of caspases are known, some of them involved in the maintenance of cell structure, such as actin, fodrin, tubulin and lamin; others, with an enzymatic activity, control DNA metabolism [33]. A recent survey revealed that at least 400 substrates of caspases exist [34]. The cleavage of structural proteins impairs their function in cytoskeleton dynamics. Analogously, most enzymes, once proteolysed, are generally inactive. The nuclear protein PARP-1 (poly(ADP-ribose) polymerase-1), which is involved in the recognition of DNA
single and double strand breaks, is the most utilised apoptotic marker. Its proteolysis by caspases converts the 113 kDa protein into two inactive fragments of 89 and 24 kDa easily detectable by immunological assays [35,36]. Although caspases held the predominant role in proteolysis, there is growing evidence that other proteolytic enzymes are involved in the execution of apoptosis. A family of serine proteases proved to degrade proteins during apoptosis [37]. Among them, the HtrA2/Omi (high temperature requirement protein A) protease plays a crucial role in mitochondrial homeostasis and is released from mitochondria into the cytosol during apoptosis, thus being able to antagonize anti-apoptotic factors [38]. Cathepsins are proteases normally localised intralysosomally; after a specific signal that targets lysosomes, cathepsins are released into the cytoplasm, where they trigger apoptosis [39,40]. Another class of proteolytic enzymes involved in apoptosis are calpains (i.e. calcium-dependent proteases), which are present in the cytosol as zymogens and activated when Ca^{2+} is released from the endoplasmic reticulum into the cytosol [41,42].

Degradation of DNA

During the last steps of apoptosis, DNA is firstly degraded into molecules ranging from 50 to 300 kb; then, the cleavage at internucleosomal DNA regions generates oligonucleosomal-sized fragments (180 bp and multiples), giving rise to a typical ladder when analysed by agarose gel electrophoresis [43]. Many DNase activities involved in apoptotic DNA degradation have been described, including Ca^{2+}-/Mg^{2+}-dependent endonucleases, Mg^{2+}-dependent DNases, acid DNases, DFF (DNA fragmenting factor), CAD (caspase-activated DNase) and L-DNase II [24]. It has also been reported that the Apoptosis Inducing Factor (AIF) is involved in the formation of high molecular weight DNA fragments, even if AIF does not have an intrinsic endonuclease property but could induce DNA fragmentation through the interaction with other proteins [44,45]. The activation of AIF requires its translocation from the mitochondria to the nucleus; this event is promoted by poly(ADP-ribosylation) [46]. Other nucleases (e.g. DFF40/CAD) are activated through the proteolysis of their specific inhibitors by caspases, thus demonstrating the existence of an interplay between protein and DNA degradation [47,48]. A particular acid DNase, that is L-DNase II, is involved in caspase-independent apoptosis [48].

APOTOTIC CELL DEATH PATHWAYS

Apoptosis is a genetically-regulated and energy-dependent process that can be induced by a large number of pro-apoptotic stimuli through two distinct pathways: the extrinsic, activated

![Fig. 1](image-url) Caspase family. (A) Initiator caspases show, from C- to N-terminal, a small subunit, a large subunit and a pro-domain containing either a CARD or a DED motif. (B) Effector caspases show a C-terminal small subunit, a large subunit and a short pro-domain. (C) Active caspase conformation.
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The extrinsic apoptotic pathway is activated by soluble molecules belonging to the Tumor Necrosis Factor-α (TNF-α) superfamily, such as TNF-α, FasL (Fas Ligand, also known as CD95L), TRAIL (TNF-related apoptosis inducing ligand) and TNFSF10 (TNF ligand superfamily member 10). These molecules can bind to their respective receptors TNFR-1, Fas, TRAIL-R1,2 [49]. A common feature of death receptors is an 80 amino acids domain, named death domain (DD), in their cytoplasmic tail [50], which is crucial for the interaction with adaptor molecules that can transduce the death signal. Fas and TRAIL-R1,2 receptors can bind directly to the adaptor molecule FADD (Fas-associated molecule with death domain) [51,52], while the interaction between FADD and TNFR1 is mediated by TRADD (TNFR1 associated death domain protein) [53]. The interplay between death receptors and adaptor factors is regulated by homotypic interactions with the DD present in both classes of molecules [54].

FADD recruitment to death receptors is essential to transduce the death stimulus to the initiator caspase-8 or -10 to promote their activation through the formation of the supermolecular complex DISC (death-inducing signalling complex) [54], where FADD is essential, being able to interact with death receptors (thanks to its DD domain) and with initiator caspases through its DED domain (death effector domain). According to the first proposed “induced-proximity model” [55], once recruited to DISC, initiator caspases are activated through their FADD-induced dimerisation, thus being able to cleave each other promoting their complete activation. Based on this first model, two further models have been postulated, the “proximity-induced dimerisation” and the “induced conformation”. According to the former model, caspase activation is achieved by dimerisation favoured by their close proximity promoted by FADD, whose role would

by soluble molecules that can bind to plasma membrane receptors, and the intrinsic, sensible to physical and chemical injuries. The main features of these pathways are schematised in Fig. (2).

Fig. (2). Simplified view of the apoptotic pathways. The extrinsic pathway is triggered by TNF-like molecules that activate their receptors causing DISC formation and caspase-8/10 activation. The intrinsic pathway is induced by internal stimuli sensed by Bid, thus causing Bax/Bak activation, mitochondria permeabilisation, apoptosome formation and caspase-9 activation. Each pathway independently converges on caspase-3 that can promote DNA and cellular protein dismantling.
be to increase local concentration of inactive caspases [56], thus caspases would be activated only by dimerisation. The latter model implies that caspase binding to DISC allows conformational changes within the active site responsible for the activation [57]. After DISC formation, the initiator caspase-8 and -10 became active and can, in turn, activate initiator and effector caspases. Since apoptosis requires a tight regulation to be induced only when it is necessary, signal transduction mediated by DISC could be down-regulated or blocked by the so-called Decoy receptors, such as DcR1 and DcR2, also known as TRAIL-R3 and TRAIL-R4, respectively [58]. Both these inhibitors show high homology with the extracellular domain of death receptors, but they completely (DcR1) or partially (DcR2) lack the DD intracellular domain [58], thus being unable to transduce the apoptotic stimulus because they cannot trigger DISC formation.

The intrinsic apoptotic pathway is activated by a variety of chemical and physical injuries generating stimuli that converge on mitochondria [59]. The key event leading to the activation of this pathway is the mitochondrial outer membrane permeabilisation (MOMP) due to the activity of two pro-apoptotic proteins belonging to the Bcl-2 family, i.e. Bax and Bak [60]. On the basis of their function in apoptosis regulation, Bcl-2 family members can be divided into two classes: anti- and pro-apoptotic proteins. Bcl-2, Bcl-X₁, Bcl-W, MCL1 and A1, all characterised by four BH domains, contrast apoptosis [61]. On the contrary, pro-apoptotic members include BH3-only proteins, such as Bad, Bik, Bid, Bim, Noxa and Puma, which show only one BH domain, and proteins (Bax, Bak and Box) that contain three BH domains (reviewed in [62]). To date, two models have been proposed to explain Bax/Bak complex activation. According to one of them, given that anti-apoptotic members normally bind to and inhibit the Bax/Bak complex, the physical binding of pro-apoptotic members other than Bax and Bak to the anti-apoptotic ones could release Bax/Bak complex and promote its pro-apoptotic function [63]. An alternative model postulates a direct activation of Bax/Bak through their interaction with BH3-only proteins [64]. Once activated, Bax and Bak can insert themselves into the outer mitochondrial membrane [65,66] and form a homo- or hetero-dimer, the so-called MAC (mitochondrial apoptosis-induced channel), a pore large enough to release different pro-apoptotic factors into the cytosol [67-71]. Moreover, Bax and Bak can indirectly trigger MPTP (mitochondrial permeability transition pore) opening thanks to the ability to release Ca²⁺ from the endoplasmic reticulum [72]. After its release, Ca²⁺ can induce MPTP opening followed by transmembrane potential (Δψm) dissipation [72]. As a consequence of mitochondria permeabilisation, some factors are released into the cytosol, including cytochrome c, AIF, endonuclease G, Smac/DIABLO (second mitochondria-derived activator of caspase) and HtrA2/Omi [73]; this event triggers cell death either in an intrinsic cell death or independent manner.

Intrinsic cell death goes through the activation of the initiator caspase-9, which requires the assembly of an activation platform called apoptosome, where the base is represented by the cytosolic protein Apaf-1 (apoptotic protein activating factor-1). This protein exists in different spliced isoforms [30]; all of them contain an N-terminal CARD (caspase recruitment domain), a central CED-4 like domain and a 13-14 repetition of a WD40 domain which can bind cytochrome c [74]. In its inactive form, Apaf-1 is present in the cytosol bound to an ATP molecule in a conformation that inhibits its activation; cytochrome c binding induces a conformational change in Apaf-1 that activates its intrinsic ATPase function [75]. Once activated, Apaf-1 recruits other Apaf-1 molecules to assemble the apoptosome that has, in its three-dimensional structure, a wheel-shaped form with 7-fold symmetry. In this conformation, CARD and CED-4 homology domains constitute the hub of the wheel, which binds to caspase-9 monomers, while the seven spokes are made of WD40 domains [74]. Many steps are required to drive correct apoptosome assembly, including Apaf-1 proteolysis by caspases, possibly serving as a negative feedback to counteract unwanted apoptosis activation [76]. Other factors that can prevent apoptosis formation are K⁺ [77] and Ca²⁺ [78]; these ions can inhibit apoptosis function in a cytoplasmic concentration-dependent manner. Furthermore, apoptosome assembly could be avoided because of the action of chaperones Hsp70 and Hsp90, which prevent caspase-9 binding to apoptosome and/or cytochrome c-mediated oligomerisation of Apaf-1 molecules [79-81].

Many factors block either the activation or the function of zymogens and activated caspases; such proteins are called IAPs (inhibitors of apoptotic proteins) [82]. Human genome encodes for 8 different IAP proteins: X-linked IAP (the best characterised IAP, also known as XIAP), cIAP1, cIAP2, ML-IAP, NAIP, ILP2, Apollon/Bruce and Survivin [83-85]. These proteins contain 1-3 tandem repeated copies of the so-called BIR domain (baculovirus IAP repeat); some of them show a C-terminal domain with an E3 ubiquitin ligase activity possibly involved in promoting caspase destruction by the proteasome [83,84].

IAPs are not the only proteins involved in caspase regulation. Indeed, various pro-apoptotic proteins interact with them to establish if apoptosis can go forward or needs to be blocked. Such factors are Smac/DIABLO and HtrA2/Omi, that can bind to [86] or cleave some IAPs [87,88], respectively. These two classes of apoptotic proteins interact to regulate apoptosis execution and their interplay is controlled by their different localisation during cell life. Indeed, in viable cells Smac/DIABLO and HtrA2/Omi are confined to mitochondria while IAPs are in the cytosol, thus caspases are blocked in an inactive state. When a death stimulus triggers mitochondrial permeabilisation, the release of pro-apoptotic factors such as Smac/DIABLO and HtrA2/Omi counteracts the inhibitory action of IAPs, thus making apoptosis possible.

IS APOPTOSIS THE ONLY PCD?

In recent years, it has been found that many forms of cell death other than apoptosis exist [20,89-91]. Among them, anoikis, autophagy, and necrosis are below described.

**Anoikis**

In normal cells, loss of adhesion is counteracted by a death mechanism termed anoikis, which plays an essential role to avoid the undesired metastatic spread of cancer cells through their detachment from the primary tumor followed by their migration to the lymphatic and circulatory systems [92]. The features of this process are reminiscent of the apoptotic ones,
being characterised by the activation of the extrinsic and/or intrinsic pathways, and the consequent involvement of caspase-8 and/or -9 [92]. Anoikis is accompanied by cytoskeleton perturbations and alterations of the integrin complex [93]. The mediators responsible for anoikis have not been well characterised. Among the putative crucial factors, reactive oxygen species (ROS) produced through the involvement of the small GTPase Rac-1 upon integrin engagement have been reported to be able to transduce a pro-survival signal that ensures that cells escape from anoikis [94]. Given that anoikis impairment copes with the ability of cancer cells to survive in a non-adherent status, the understanding of the molecular bases of this form of cell death will greatly help in designing strategies able to bypass this crucial point.

**Autophagy**

Autophagy is a genetically controlled mechanism present in different species, from yeast to humans. In all organisms, this special pathway takes care of cellular components recycling [95] that can be required under extreme conditions such as hypoxia, nutrient starvation and oxidative stress [96]. Although autophagy is a basic process mainly involved in degradation and recycling of macromolecules and organelles, its extensive activation can lead to the so-called type II cell death (to distinguish it from apoptosis, also defined as type I cell death). However, due to our limited knowledge of this process, it is very difficult to define the boundary between survival and lethal autophagy. From a morphological point of view, autophagy is characterised by the appearance within the cytosol of large vacuole-like structures with a double-layered membrane. These structures then fuse each other to form the so called autophagosome that in turn fuses with lysosomes to allow the digestion of their content [97]. The possible impact of mitochondria disruption (the so-called mitophagy) on the dynamics of autophagosome formation has been recently discussed with respect to the role of ROS production [98,99]. From a molecular point of view, autophagy involves the activation of various genes. Even if a well-established pathway has not been so far described, some important factors involved in autophagy regulation have been discovered. Various regulators of this process belong to the ATG family (autophagy-related genes), originally discovered in yeast and conserved among different species [100]. In mammals, one autophagic marker is Beclin-1 protein, an orthologue of the Atg-6 yeast protein. This protein seems to be a key regulator of the autophagic death machinery and, interestingly, proved to be involved in the cross-talk with apoptosis, given that it could interact with Bcl-2 [101-103]. In fact, Maiuri et al. [104] recently reported a physical association of Beclin-1 with Bcl-2 and Bcl-X, whose inhibition allows the increase of cell number with cytosolic aggregation of the autophagic marker LC3 (a protein involved in the autophagosome maturation). In addition, Bad interferes with Bcl-2/Beclin-1 interaction by binding to Bcl-2, thus stimulating autophagy [104]. Recent studies demonstrate that autophagy is regulated also by p53. Although it is not clear which is the precise mechanism by which p53 can regulate this pathway, probably it acts as an autophagy suppressor [105]; however, these emerging data need to be supported by other evidences. Taken together, these results show that a connection between autophagy and apoptosis exists, although the exact mechanisms leading to their interaction remain to be elucidated.

**Necrosis**

Necrosis, the first type of cell death discovered, is often correlated with pathological conditions, like inflammation and virus infection. From a morphological point of view, necrotic cells are characterised by the loss of control of intracellular ion concentration, leading to cellular volume increase and swelling, mitochondria swelling and perinuclear organelle clustering [106]. As for the biochemical features, proteases and DNases seem to be activated without a well defined program [106]. Given that necrosis occurs without energy requirement, it was often described as a passive form of cell death, but emerging data support the idea that necrosis would be programmed [91,107,108]. For this reason, necrosis is now established as type III PCD, although a well-defined molecular pathway leading to necrosis is still lacking. However, encouraging data support the existence of some markers able to discriminate necrosis from apoptosis [109]. One paradigm of programmed necrosis implies that increased cytosolic Ca2+ concentration could allow calpain activation and a further enhanced Cr3+ concentration by cleavage of some transmembrane channels that regulate intracellular concentration of this ion, leading to osmotic imbalance and, finally, cell lysis [110]. Moreover, necrosis could involve PARP-1 over-activation in response to DNA damage. Prolonged activation of PARP-1, which forms ADP-ribose from NAD, would cause intracellular NAD depletion that, in turn, impairs ATP synthesis, therefore leading to energetic imbalance and cell death by necrosis [107,111,112]. Additional evidence that necrosis would be a programmed cell death come from different reports showing that the inactivation of both apoptosis and autophagy triggers necrosis [106,113,114]; however, further experiments would be performed to increase our knowledge on necrosis.

**CONCLUSION**

Given that tissue homeostasis is maintained through a balance between proliferation and death, it is obvious that Programmed Cell Death is “as intrinsic for cells as mitosis” [115], and plays a crucial role in governing physiological and pathological conditions. In fact, a deregulated cell death could contribute to the ethiology of a number of disorders, including neurodegenerative diseases, where massive death of neurons occurs [116], and cancer, which is promoted by a defective removal of cells [89]. The growing body of evidence supporting the existence (and intercorrelation) of various forms of PCD renders this field extremely complex. Among the various forms of PCD, the interest towards apoptosis is still growing, due to the discovery that it plays an active role in cancer development. Indeed, tumor development is often characterised by an impaired apoptosis [74]; this behaviour stimulated the search for drugs able to antagonize anti-apoptotic factors and to restore the apoptotic potential of cancer cells [117,118].

**AKNOWLEDGEMENTS**

The research at the author’s laboratory is supported by Regione Lombardia (Project Metadistretti 4238). We thank
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