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CURRENT CONCEPTS IN APOPTOSIS: THE PHYSIOLOGICAL SUICIDE PROGRAM REVISITED

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Abstract: Apoptosis, or programmed cell death (PCD), involves a complex network of biochemical pathways that normally ensure a homeostatic balance between cellular proliferation and turnover in nearly all tissues. Apoptosis is essential for the body, as its deregulation can lead to several diseases. It plays a major role in a variety of physiological events, including embryonic development, tissue renewal, hormone-induced tissue atrophy, removal of inflammatory cells, and the evolution of granulation tissue into scar tissue. It also has an essential role in wound repair. The various cellular and biochemical mechanisms involved in apoptosis are not fully understood. However, there are two major pathways, the extrinsic pathway (receptor-mediated apoptotic

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Abbreviations used: Apaf-1 – apoptosis protease activating factor-1; Bcl – B-cell lymphoma family; BH – Bcl-2 homology; tBid – truncated Bid; BIR – baculoviral IAP repeat; BRUCE – BIR repeat-containing ubiquitin-conjugating enzyme; CARD – caspase recruitment domain; CDR – cysteine-rich extracellular domain; DISC – death-inducing signaling complex; DD – death domain; DED – death effector domain; DR – death-inducing receptor; ER – endoplasmic reticulum; FADD – Fas-associated death domain protein; FLIP – FADD-like-ICE-inhibitory protein or FLICE inhibitory protein; G1 – GAP1; IAP – inhibitor of apoptosis; IL – interleukin; MOMP – mitochondrial outer membrane permeabilization; PCD – programmed cell death; PUMA – p53-up-regulated modulator of apoptosis; PS – phosphatidylserine; PTP – permeability transition pore; RIP – receptor interacting protein; TACE – TNF alpha-converting enzyme; TGF-β - transforming growth factor-β; TNF-α – tumor necrosis factor α; TNFR-1 –tumor necrosis factor receptor-1; TRAIL – TNF-related apoptosis-inducing ligand; TRADD – TNF-receptor associated protein with death domain; TUNEL – terminal deoxynucleotidyl transferase-mediated (TdT-mediated) dUTP-digoxigenin nick end labeling; VDAC – voltage-dependent anion channel

pathway) and the intrinsic pathway (mitochondria-mediated apoptotic pathway), which are both well established. The key component in both is the activation of the caspase cascade. Caspases belong to the family of proteases that ultimately, by cleaving a set of proteins, cause disassembly of the cell. Although the caspase-mediated proteolytic cascade represents a central point in the apoptotic response, its initiation is tightly regulated by a variety of other factors. Among them, Bcl-2 family proteins, TNF and p53 play pivotal roles in the regulation of caspase activation and in the regulation of apoptosis. This review summarizes the established concepts in apoptosis as a physiological cell suicide program, highlighting the recent and significant advances in its study.

Key words: Apoptosis, Programmed cell death, Pathways, Caspases, Bcl-2, p53, TNF, Apaf

APOPTOSIS

All multicellular organisms have endogenous mechanisms for selectively killing their own cells. This physiologically essential cell death helps them to control normal development. It is involved in the regulation of tissue homeostasis, which in turn tightly regulates cell numbers and tissue size, and in protection from rogue cells, and in the aging process [1]. The physiological cell death that occurs in multicellular organisms is called 'programmed cell death' (PCD). The first clue to the existence of PCD was provided by an Australian pathologist, John Kerr, and was initially called "shrinkage necrosis" [2, 3]. Later Kerr, Wyllie and Currie [4] termed the phenomenon "apoptosis", which is derived from an ancient Greek word (αποπτοστσ) coined in the fifth century BC, meaning "the falling or dropping off of petals from a flower or leaves from a tree in autumn" ('apo' means 'to separate from' and 'ptosis' means 'to fall from'). Apoptosis (referred to as Type I cell death) involves asynchronous, irreversible, genetically determined and complex network of biochemical pathways. Fine regulatory mechanisms control programmed cell death, which removes unwanted cells, maintaining homeostasis in metazoans [4-8]. Apoptosis is distinctly different from another form of cell death known as necrosis (Fig. 1). Necrosis (referred to as Type III cell death) is characterized as a pathological bioenergetic catastrophe resulting from ATP depletion, involving faster disintegration of cellular organelles, the rupture of the plasma membrane with inflammation and leakage of lysosomes, proinflammatory molecules and cell content in response to non-physiological inductions such as hypnotic shocks, hyperthermia, hypoxia, trauma, and the accumulation of toxic substances in the organism [5, 9-10]. Apoptosis is involved in aging and various diseases such as cancer, AIDS, Alzheimer's disease and Parkinson's disease. Apoptosis affects single cells; necrosis often affects sheets of cells within a tissue, due to the deleterious effects that necrotic cells have on surrounding cells.

Apoptosis is a biologically conserved phenomenon throughout evolution, observed from nematodes to mammals. This defensive mechanism of cell death

in development and homeostasis in metazoans was adopted and achieved over the course of evolution to multicellularity from single-celled organisms where cell death first evolved as a defense strategy. The process of apoptosis is the end point of an energy-dependent cascade of events initiated by death-inducing stimuli. It is grouped into four phases with overlapping components [11], as follows.

1. The early or initiation phase: a stimulus provokes or initiates the apoptotic response. This may be an external signal delivered through surface receptors or may originate inside the cell from the action of a drug, toxin, or radiation.
2. The signal transduction phase: the detection of a signal or metabolic state and the transduction of that signal to the cell death effector machinery.
3. The effector phase: proteases are activated as are their positive and negative feed-back regulators.
4. The post-mortem phase: the cell's chromatin and DNA are degraded. *In vivo* (but not necessarily *in vitro*) dying cells are recognized and engulfed by other cells.

This pathway of signal transmission is schematically represented as:
Death signal inducers → Receptors → Adaptors → Regulators (first-order proteases) → Regulators (second-order proteases) → Apoptosis.

AUTOPHAGY, ONCOSIS, PYROPTOSIS AND MITOTIC CATASTOPHE

Cells use different pathways for active self-destruction, as seen in different morphological studies. In recent years, several new definitions have been added to the dictionary of cell death: autophagy, oncosis, pyroptosis, mitotic catastrophe, and others (Fig. 1). Autophagy (Type II lysosomal cell death) is a phylogenetically old process that occurs during vertebrate development [12]. It features degradation of cellular components in autophagic vacuoles within the dying cell, primarily by cathepsins or proteosomal proteins [13]. The morphological characteristics of autophagy include vacuolization, degradation of cytoplasmic contents and slight chromatin condensation [14]. DNA fragmentation and caspase activation are very late events in this process. Oncosis (from "onkos", meaning swelling) is a counterpoint to apoptosis and is defined as a prelethal pathway leading to cell death accompanied by cellular and organelle swelling, blebbing, and increased membrane permeability [15]. This process is an active enzyme-catalyzed biochemical process that may result from toxic agents interfering with ATP generation or causing uncontrolled cellular energy consumption that ultimately leads to the depletion of cellular energy stores and failure of the ionic pumps in the plasma membrane [15]. Pyroptosis is characterized by cytoplasmic vacuolization that begins with progressive swelling of the mitochondria and endoplasmic reticulum (ER). It is mediated by mitogen-activated protein kinases, and can be triggered by the TNF receptor family |member TAJ/TROY or the insulin-like growth factor I receptor [16]. By contrast, mitotic catastrophe (mitotic failure) is cell death that occurs during abnormal

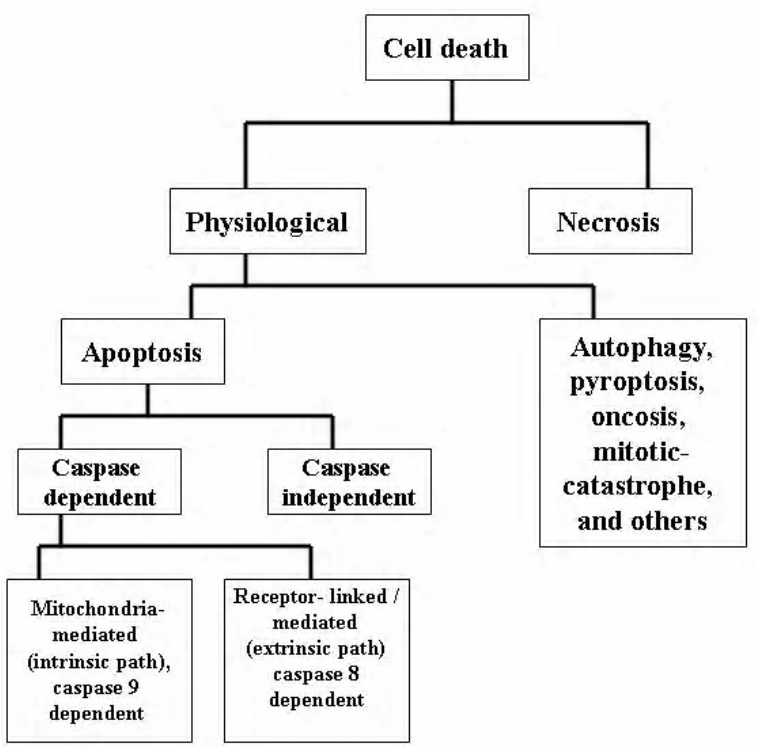

Fig. 1. A schematic representation of the types of cell death, based on morphological and biochemical studies.

mitosis that, unchecked, would lead to tetraploidy or endopolyploidy due to extensive DNA damage or deranged spindle formation coupled with the debilitation of different checkpoint mechanisms that would normally arrest progression into mitosis and hence suppress catastrophic events until repair could be achieved [17].

MORPHOLOGICAL FEATURES AND DETECTION OF APOPTOSIS

The discrete sequence of morphological changes in apoptotic cells can be observed with light and electron microscopy. In cell culture, the features of apoptosis include cell shrinkage, surface convolution, and the formation of protuberances with buddings, and the subsequent formation of apoptotic bodies. With hematoxylin and eosin staining, an apoptotic cell appears as a round or oval mass with an intensely eosinophilic cytoplasm with vacuolization and dense nuclear chromatin fragments. The formation of cytoplasmic blebs and the reduction of apoptotic bodies following phagocytosis by macrophages to unrecognizable residues are too rapid to be detected with light microscopy

[18-19]. However, electron microscopy reveals the more elaborate morphological features of apoptosis such as:

i. convolution of the nuclear and cellular outlines with cell shrinkage;
ii. nuclear chromatin compaction into sharply circumscribed masses;
iii. the formation of cytoplasmic blebs and membrane-bound apoptotic bodies; and
iv. phagocytosis of the apoptotic bodies by surrounding cells or by macrophages.

The biochemical complement of these morphological changes of apoptosis is rapid nuclear DNA cleavage, which occurs in two stages. Initially, cleavage occurs rapidly, mostly by topoisomerase II, into 200- to 300-Kb sized fragments. The cleavage of double-stranded internucleosomal DNA by DNAse I/DNAse II results in the formation of oligonucleosome-sized fragments. In necrosis, the cellular DNA also non-specifically degrades into different molecular base sizes.

The morphological evaluation of apoptosis remains the standard tool for the detection of apoptosis. Other techniques include:

i. DNA agarose gel electrophoresis for detecting the formation of a DNA ladder [20, 21];
ii. the flow cytometry assay [22, 23];
iii. terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) [24, 25];
iv. the annexin-V-FITC binding assay for identifying the early stage of apoptosis [26]; and
v. immunohistochemical detection of caspase cleavage (activated caspases) in tissue sections.

However, the simplest way to observe this phenomenon *in vitro* is to use a cell permanent DNA-staining fluorescent dye such as Hoechst 33342, which allows a striking visualization of chromatin condensation, a hallmark in apoptotic cells.

MOLECULAR REGULATORS OF APOPTOSIS

Each step of apoptosis requires the concerted effort of many proteins, and among the most influential are the caspases. The caspase activities are tightly regulated by the inhibitor of apoptosis proteins (IAP). Various other families of cellular proteins are also involved in the regulation of apoptosis at different stages, giving a tight control of its site specificity. These proteins are categorized as the Bcl-2 family, the TNF family and p53. The following is a descriptive and specific account of these regulators of apoptosis (see also Fig. 2).

Caspases

Caspases are a family of highly conserved cysteine-dependent aspartate-specific acid proteases that mediate the regulation and execution of apoptotic cell suicide [27-29]. The critical involvement of a caspase in apoptosis was first documented by Yuan *et al*. [30]. In mammals, 14 distinct caspases have been identified [31].

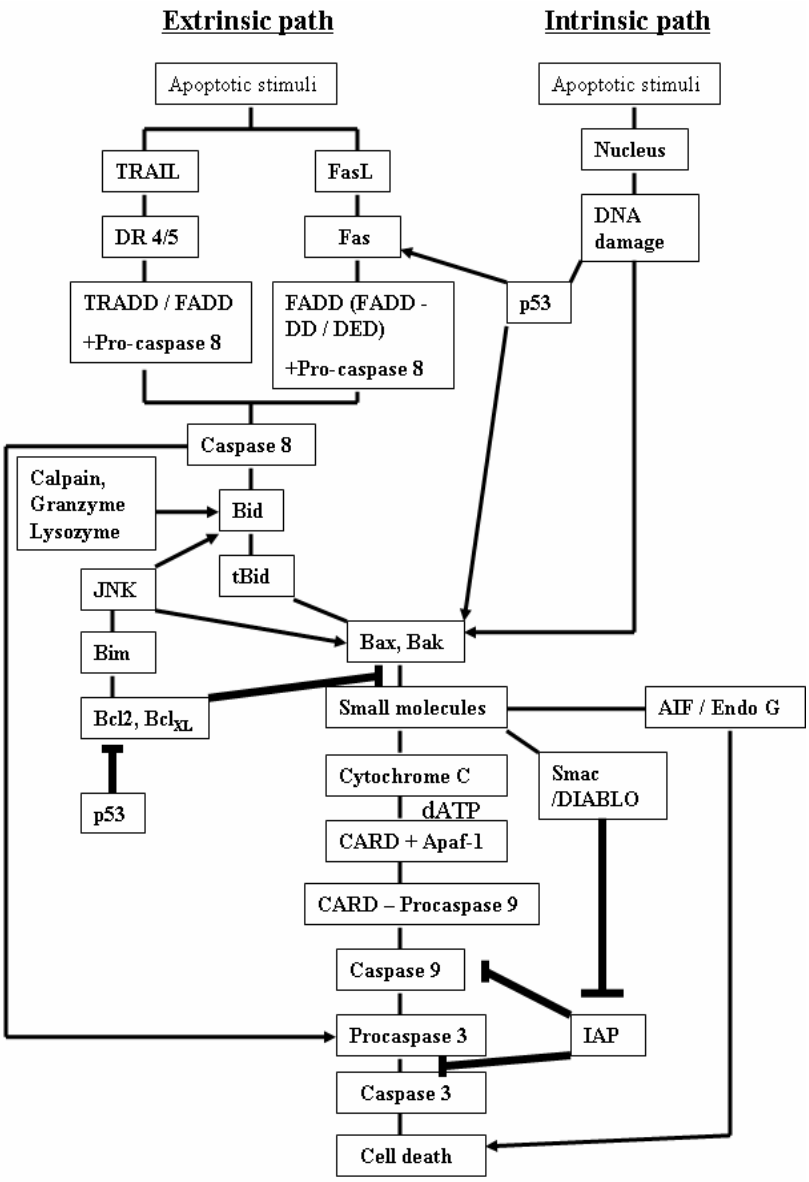

Fig. 2. A schematic representation of the extrinsic and intrinsic apoptotic signaling pathways of caspase activation showing the key events involved and cross-talk between the two pathways. The blunt-headed bold lines indicate the roles of the inhibitors regulating the apoptotic pathways.

All caspases exist within the cell as inactive latent pro-forms as precursor zymogen. These nascent caspases are synthesized as a single polypeptide chain with 3 domains in common: a 20-kDa central large internal domain (p20)

containing an active site called the death effector domain (DED); a 10-kDa small C-terminal domain (p10) called the caspase recruitment domain (CARD); and an NH2-terminus pro-domain called the death domain (DD). The DD is a member of the TNF receptor family and is involved in an early signaling event. DED and CARD are critical in the downstream portion of the pathway, recruiting caspases to the plasma membrane before activation. These inactive caspases become active in response to specific signals by selective proteolytic processing (two cleavages) at specific aspartic acid residues to produce subunits that form the active heterotetrameric protease to initiate apoptosis [32]. In mammalian cells, activation of the caspase zymogens occurs through three independent mechanisms: cleavage by upstream active caspases; cleavage by granzyme B, an aspartate-specific serine protease found in granules of cytosolic T-cells; and autoprocessing of zymogens with assistance from other caspase-interacting proteins that can occur in either a *cis*- or *trans*-acting manner [28, 33]. The active site of caspase is formed by the interface of the two subunits, *viz.*, one Arg, one His and one Cys of the large subunit and one Arg of the small subunit [34].

Depending on the structure of the pro-domain and their functions, caspases are typically categorized into 3 subclasses. The caspases with large prodomains (caspase-1, caspase-4 and caspase-5) play a role in cytokine maturation and inflammatory responses, and are called inflammatory caspases or group-I caspases [35]. The second group consists of caspases with a long prodomain (> 90 amino acids) containing either DEDs (caspase 8 and 10) or a CARD (caspase 2 and 9), and these are called initiator of apoptosis caspases or group-II caspases. The third group contains caspases with short prodomains (20-30 amino acids), called effectors, executioner caspases or group-III caspases (caspase 3, 6 and 7) [34, 36-38]. Caspases 12, 13 and 14 have not yet been categorized because of a lack of data, but they are expected to be involved in cytokine processing. These caspases have an amino acid sequence homology closer to caspase-1 than to the caspases involved in apoptosis. The signaling of caspases starts with the induction of the apoptotic signal via death receptors, resulting in the activation of an initiator caspase such as caspase 8 or caspase 10, whereas the mitochondrial signaling pathway initially involves procaspase 9. These caspases can then activate other caspases in a cascade. This cascade eventually leads to the activation of the effector caspases, principally caspase 3, 6 or 7, which in turn cleave a variety of substrates including the nuclease inhibitor, cytoskeleton and the key cellular proteins, leading to internucleosomal DNA degradation and the typical morphological changes observed in cells undergoing apoptosis [39-43].

By contrast, the inflammatory caspases (caspase 1, 4 and 5) are present in a complex called inflamasome with a CARD containing the protein NALP-1. In response to microbial pathogens, this complex activates the cytokines IL-1β and IL-18, which play a central role in the immune response [44].

Caspase inhibitors

Proteolysis is an irreversible process, and caspases have the potential to engage in amplifying cascades of proteolysis. In normal living cells, caspase activation and activity are carefully regulated on several levels by an endogenous family of cellular proteins called the inhibitor of apoptosis proteins (IAP) [45-47]. The IAP include 8 mammalian family members [48, 49] with highly conserved and differential expression patterns in various tissues. The IAP are characterized by a novel domain of ~70-80 amino acids termed the baculoviral IAP repeat (BIR) [32, 50]. Up to three tandem copies of the BIR domain can occur within the known IAP family proteins of viruses and animal species. In humans, six IAP relatives have been identified: NAIP, c-IAP1 (HIAP-2), c-IAP2 (HIAP-1), XIAP (hILP), survivin, and BRUCE [48]. The IAP do not bind or inhibit caspase 8, but they do bind to and inhibit its substrate caspase 3, thus arresting the cascade of proteolysis and providing protection from Fas/caspase 8-induced apoptosis [51-53]. In the mitochondrial pathway, caspase inactivation is done by XIAP, c-IAP1, and C-IAP2, which bind directly to the principal caspase, procaspase 9, thereby preventing its processing and activation induced by cytochrome *c*, both in intact cells and in cell extracts [52]. The overexpression of IAP family proteins inhibits apoptosis induced by Bax and other pro-apoptotic Bcl-2 family proteins [48].

The IAP are not the only natural inhibitors of caspases. The baculoviral p35 protein is a pan-caspase inhibitor, and it targets most caspases by forming an inhibitory complex that is characterized by a protected hoister link between the caspase and p35 [54, 55]. Another pan-caspase inhibitor, ser-pin Cram, derived from the cow pox virus, binds the active center of caspases by a covalent bond [56].

Bcl-2 family proteins

Although the proteolytic cascade of caspases has a central role in the apoptotic response, its initiation is tightly regulated by a variety of other factors. The B-cell/Lymphoma-2 family (Bcl-2) of proteins is known to play a pivotal role in the regulation of apoptosis as checkpoints (gatekeepers) between the cell surface and internal death signals, the formation of the apoptosome and the activation of the caspase cascade [57-59]. The Bcl-2 gene was first discovered in human B-cell lymphomas [60]. More than two dozen Bcl-2 family members have been discovered and categorized into two main subfamilies: the inhibitors or anti-apoptotic (Bcl-2, Mcl-1 and Bcl-XL) members, and the promoters or apoptotic (Bax, Bcl-2-associated X protein) members [8, 61]. All Bcl-2 family members are characterized by at least one of four BH1-BH4 domains, which correspond to α-helical segments [58]. In general, the anti-apoptotic members show sequence conservation in all four domains, while the pro-apoptotic molecules are characterized by a loss of the sequence conservation of the first α-helical segment, BH4. The BH3 domain is presumed to be a critical death domain in all the pro-apoptotic members [62]. An important feature of the members of the Bcl-2 family is their ability to form homo- and heterodimers, suggesting the

neutralization of competition between these proteins; they function either independently or together in the regulation of apoptosis. In the absence of death signals, Bcl-2 proteins are localized to distinct intracellular compartments [63]. Upon receiving death stimuli, the pro-apoptotic members can change their location within cells and undergo various pre- and post-translational modifications [59]. In response to death signals, the cytosolic pro-apoptotic proteins change conformations and integrate into the outer membrane of the mitochondria, and the anti-apoptotic family members are neutralized [64]. The anti-apoptotic members are initially integral membrane proteins localized to the mitochondria, endoplasmic reticulum and nuclear membranes [65, 66]. In the mitochondria, anti-apoptotic proteins form ion-channels and help to maintain mitochondrial integrity by allowing the export of H^+ ions from the inner mitochondrial space. The associations between various Bcl-2 family regulators are not static, but phosphorylation-dependent changes cause them to interact amongst themselves [67, 68].

p53

p53 is a stress-response 53-kDa nuclear protein which exists as a tetramer, accumulates in the cytoplasm during the G_1 (GAP1) phase and migrates to the nucleus at the start of the S (synthesis) phase. The activation of p53 is controlled by its biosynthesis and a large number of post-transcriptional modifications with sub-cellular relocalization. Importantly, other proteins and processes such as DNA damage in turn regulate each of the p53 kinases. p53 is well described as a transcription factor that can induce the expression of multiple different pro-apoptotic gene products, including inhibitors of cell cycle advancement, regulators that control p53 activity in negative feedback loops, mediators of oxidative stress and endoplasmic reticulum (ER) stress, components of the death receptor signaling pathway, and caspase activators and pro-apoptotic proteins of the Bcl-2 family by catalyzing mitochondrial outer membrane permeabilization (MOMP). However, p53 has another, transcription-independent pro-apoptotic effect, which involves direct interactions between p53 and MOMP inducers at the mitochondrial level [69]. DNA damage results in dramatic changes in p53. The activation of p53 as a transcription factor arrests the cell cycle, acting as the emergency brake of a cell. Once p53 is accumulated, it binds to DNA and mediates two major effects:

i. cell cycle arrest in the G1 phase, allowing time for cells to repair damaged DNA; and
ii. when DNA damage cannot be successfully repaired, activation of apoptosis-inducing genes, especially Bax, by up-regulating its transcription and down-regulating Bcl-2, thus favoring mitochondria-dependent apoptosis [70, 71].

In addition, p53 up-regulates the transcription of Fas to support Fas-mediated apoptosis. Further research on p53 is required to understand more of its role in the regulation of apoptosis. Overall, p53 controls the fate of cells by detecting genomically unstable cells and arresting the cell cycle by multiple mechanisms,

namely by stimulating the G1/S or G2/M checkpoint response, mitotic catastrophe, and apoptotic cell death as a guardian or molecular policeman [72].

Tumor necrosis factor (TNF) family

TNF is a soluble pleiotropic cytokine that mediates apoptosis, cell proliferation, immunomodulation, inflammation, allergy and autoimmune disease, among others. TNF is primarily produced as a type II transmembrane protein, arranged in stable homotrimers upon activation by the immune system (by macrophages, lymphoid cells or other cells). This membrane-integrated form, the soluble homotrimeric cytokine (sTNF), is released via proteolytic cleavage by the metalloprotease TNF alpha-converting enzyme (TACE). The members of the TNF ligand family exert their biological functions via interaction with their cognate membrane receptors. The TNF receptor (TNF-R) family contains one to six cysteine-rich repeats in their extracellular domain. There are two distinct TNF-receptors: type I (TNF-R1; CD120a; p55/60) expressed in all cell types; and type II (TNF-R2; CD120b; p75/80) expressed only on the cells of the immune system and endothelial cells. They bind membrane-integrated TNF (mem-TNF) and soluble TNF (sTNF), but also the secreted homotrimeric molecule lymphotoxin-alpha (LT-alpha) [73-76].

The signal transduction of cell death from TNF-R1 is via its cytoplasmic death domain (DD) by the activation of caspase 8 alone to activate caspase 3 or by the activation of the mitochondria-dependent amplification loop to cause apoptosis. TNF-R2 directly recruits TNF receptor-associated factors (TRAF), induces gene expression and intensively cross-talks with TNF-R1 [76].

APOPTOTIC PATHWAYS

In mammalian cells undergoing apoptosis, two distinct mechanisms or pathways are operated and are triggered by cell death stimuli from intra- or extra-cellular environments. The intracellular stimuli trigger the mitochondria-mediated signaling pathway which is generated by signals arising within the cell mainly by leakage of cytochrome *c* from the mitochondria, while the extracellular death stimuli induce the receptor-mediated pathway, which is triggered by the binding of death molecules to the cell surface receptors (death receptor-mediated events). In addition, there are other signaling pathways, such as the p53-dependent pathway (Fig. 2).

The mitochondria-mediated apoptotic pathway (the intrinsic pathway)

The mitochondrial electron transport chain component cytochrome *c* is a key variable for the activation of caspases; along with a protein known as apoptosis protease-activating factor-1 (Apaf-1), it initiates the intrinsic apoptotic pathway [41]. Apaf-1 is a 140-kDa cytosolic protein that resets in a latent state until bound by cytochrome *c*. The death signal (such as DNA damage) induces the pro-apoptotic BH3-only domain proteins (Bid, Bad, Noxa and p53-up-regulated modulator of apoptosis (PUMA)) to transfer the signals to the mitochondria

(Fig. 2). The BH3-only domain proteins facilitate the assembly of other pro-apoptotic proteins such as Bax and Bak, into the pores in the outer mitochondrial membrane [67, 77], and change the mitochondrial permeability to release or leak out various apoptosis-inducing factors, including cytochrome *c*, through the mitochondrial permeability transition pore (PTP) or voltage-dependent anion channels (VDAC) [78, 79]. The released cytochrome *c* and Apaf-1 bind to inactive caspase 9 in the presence of dATP or ATP and form a ~1-MDa oligomeric Apaf-1 complex (7 Apaf-1 + 7 cyt C + 7(d) ATP + 7-Procaspase 9) called the apoptosome or caspase 9-holoenzyme, or "wheel of death", which ultimately activates the effector caspase cascade (caspase 3 and 7), leading to cell death. The activation of the mitochondrial pathway is tightly regulated by the anti-apoptotic Bcl-2 family members via their inhibition of cytochrome *c* release [68, 80, 81]. Mitochondrial permeability is determined by the ratio of proapoptotic and anti-apoptotic members of the Bcl-2 family.

The death receptor-mediated apoptotic pathway (the extrinsic pathway)

The induction of this pathway is initiated by the binding of the death receptors (Fas (Apo-1 or CD95)), tumor necrosis factor receptor-1 (TNFR-1/p55/ $CD120_a$), and interferon (IFN) and TRAIL (TNF-related apoptosis-inducing ligand or Apo2-L) receptors to their ligands in the plasma membrane of the cell [82, 83]. The death receptors are transmembrane proteins with cysteine-rich extracellular domains (CDRs) that interact with ligands. Based on receptor types, there are two major signaling sub-types: the Fas-mediated signaling path and the TRAIL receptor-mediated signaling path (Fig. 2). Fas are glycosylated Type-I transmembrane receptors that either activate mitochondria-dependent or mitochondria-independent signaling paths in response to ligands [39, 84, 85]. The mitochondria-independent path is activated by the death-inducing signaling complex (DISC) prior to the loss of mitochondrial transmembrane potential. The mitochondria-dependent path is triggered by activated caspase 8 through the cleavage of the c-terminal fragment of the BH3-only member of the Bcl-2 family from protein Bid to truncated Bid (tBid), which translocates to the outer membrane of the mitochondria, allowing the loss of mitochondrial transmembrane potential and inducing cytochrome *c* release. Thus, Bid mediates the cross-talk from the extrinsic to intrinsic form of cell death [86, 87]. A similar mitochondria- independent event appears to be triggered when TRAIL binds to a different family of death-inducing receptors (DR-3, 4, 5 or 6) [88]. Both Fas and TRAIL bindings initiate ligation of the receptors and transmission of the apoptotic signals through the intracellular death domains (DD), death effector domains (DED) and caspase recruitment domains (CARD). The death domains are found in cytoplasmic proteins including the Fas-associated death domain protein (FADD/MORT1), TNF-receptor associated protein with a death domain (TRADD), and receptor-interacting protein (RIP), and in transmembrane proteins, including TNFR1, TRAIL-R1/DR4 and TRAIL-R2/DR5 [67]. The CARD mediates the activation of adaptor proteins and procaspases (procaspase 8 or 10) as

the death-inducing signaling complex (DISC), leading to the activation of a cascade of caspases including caspase 3, leading to cell death [89].
The signaling from death receptors through their adaptors to procaspase 8 is a well-regulated path, controlled by a polypeptide that contains a pro-domain similar to that of procaspase 8, but lacks a caspase active site called FLIP (FADD-like-ICE-inhibitory protein/FLICE inhibitory protein). FLIP (cFLIP) proteins are well-known inhibitors of death receptor-induced apoptosis [90]. There are three known c-FLIP isoforms: c-$FLIP_L$, c-FLIPs and c-$FLIP_R$ [91]. FLIP binds to FADD and competitively inhibits recruitment of procaspase 8 and procaspase 10, thereby interrupting signaling initiated by various death receptors [67, 40].

The p53-dependent pathway (DNA damage-mediated apoptosis)
The p53 pathway responds to the stresses that can disrupt the fidelity of DNA replication and cell division. A stress signal (either external or internal to cells) is transmitted to the p53 protein by post-translational modification (phosphorylation, acetylation, methylation, ubiquitination or sumolation). This results in the activation of the p53 protein as a transcription factor that initiates a programmed cell cycle arrest, cellular senescence or apoptosis. The transcriptional network of p53-responsive genes activates 20 different promoters, represses 26 different promoters and enhancers, and can interact with > 35 cellular and viral proteins. Thus, a number of positive and negative autoregulatory feedback loops act on the p53 response [92].

ROLE OF MITOCHONDRIA, ENDOPLASMIC RETICULUM (ER) AND GOLGI-COMPLEX IN APOPTOSIS

Each cellular organelle plays a key role in cellular life and possesses sensors that detect specific alterations, locally activates signal transduction pathways, and emits signals that ensure inter-organellar cross-talk. The mitochondria, the endoplasmic reticulum (ER), the *trans*-Golgi network and the lysosomes play critical roles in the regulation of apoptosis.
Mitochondria are not only the cell's main energy producers, but are also its arsenal. The mitochondria sequester a potent cocktail of pro-apoptotic proteins and constitute a major site for the integration of diverse proapoptotic signals (for the intrinsic pathway). The most prominent among them is cytochrome *c* (the humble electron carrier), which is one of the components (along with the adaptor protein Apaf-1) required for the activation of caspase 9 in the cytosol (93). This process is regulated by pro-apoptotic Bcl-2 family members by forming large voltage-dependent anion channels (VDAC) in the outer mitochondrial membrane and influencing mitochondrial homeostasis by changing the mitochondrial permeability transition pore (PTP), a large mega channel that participates in the regulation and functioning of Ca^{2+}, voltage and pH with several levels of conductance and ion selectivity. The mitochondrial mega channel is a point of integration of multiple pro-apoptotic pathways, as several

pro-apoptotic second messengers facilitate its opening, including Ca^{2+} ions, reactive oxygen species (ROS), p53-induced changes in cellular redox potentials, ceramide-derived ganglioside GD3, recombinant caspases and Bax. At high, irreversible levels of conductance, the mega channel causes the rapid loss of membrane potential and organellar swelling [94]. Thus, the mitochondria play a major role in regulating cell death, which occurs upon permeabilization of their membranes. Once mitochondrial membrane permeabilization (MMP) occurs, cells die either by apoptosis or necrosis.

The ER is involved in multiple cellular functions. The lumen of the ER is a unique oxidative environment for the critical formation of disulfide bonds and proper folding of proteins destined for secretion or display on the cell surface. It contains the highest concentration of Ca^{2+} within the cell due to the active transport of calcium ions by Ca^{2+} ATPases. A disturbance in the normal function of cell causes a stress response in the ER, leading to unfolding or misfolding of accumulated proteins in the ER and triggering cell death by activating proteases, kinases, transcription factors or Bcl-2 family proteins and modulators [95]. Various stimuli that cause the ER to dump Ca^{2+} precipitate cell death. The transport of excessive Ca^{2+} from the ER into the matrix of the mitochondria induces permeability of the mitochondrial membrane. In addition, local activation of calpains, a family of Ca^{2+}-dependent cysteine proteases and several caspases located in the ER is implicated in cell death. Finally, the Ca^{2+} of the ER alter the Ca^{2+}-dependent phospholipid scramblases, ubiquitously expressed plasma membrane proteins which alter membrane structure to promote apoptosis or necrosis, including transferring phosphotidylserine to the outer leaflet of the plasma membrane (a signal for clearance of the cells by phagocytosis) and transferring cardiolipin from the inner to the outer membrane of the mitochondria (a signal for targeted insertion of the proapoptotic Bid and Bax into the membranes) [for details, see reference 95]. Thus, the ER mediates as a probable instigator of pathological cell death and dysfunction by leading to downstream cell death mechanisms.

Similarly to the ER, the Golgi-complex (GC) has a potential role in the signaling pathway to alleviate stress, and if irreparable, it can trigger apoptosis. The highly dynamic steady-state structure of GC stacks is partly a consequence of the balance of anterograde and retrograde transport and stress signaling. Different stresses cause the GC to collapse into the ER due to retrograde traffic. Golgi-localized caspase 2 may be the best key to unlocking the role of GC in apoptotic signaling. A negative regulator of caspase 2, BRUCE (BIR repeat-containing ubiquitin-conjugating enzyme) is localized to the GC, preventing caspase activation at the GC. The death receptors, including tumor necrosis factor receptor-1 and Fas, are predominantly localized during the steady state at the GC. Since the ligands for these receptors are extracellular, some membrane trafficking must occur for maximal signaling [96].

RECOGNITION OF APOPTOTIC BODIES AND PHAGOCYTOSIS

The disassembly of the cell into membrane-limited cell fragments results in the formation of apoptotic bodies. These apoptotic bodies flip out their inner phospholipid layers and express phosphatidylserine (PS) in the outer layers of their plasma membranes. In some types of apoptosis, thrombospondin, an adhesive glycoprotein, is also expressed on the surfaces of apoptotic bodies. PS is also called an eat-me signal for phagocytosis and only little is known about how it is recognized as a ligand. Recent studies have demonstrated that phagocytes interact with PS on apoptotic cells through either the PS receptor or secreted bridging proteins called opsonins. The two secreted PS opsonins are identified as MFG-E8 and Gas6 and their receptors are $\alpha_v\beta_5$ and $\alpha_v\beta_3$ integrin and Mer tyrosine kinases (RTKs), respectively enabling the transduction of the single PS stimulus into multiple signaling outcomes [97].

Phagocytic clearance of apoptotic cells is characterized by the active production of anti-inflammatory cytokines, such as transforming growth factor-β (TGF-β) and interleukin 10 (IL-10), and the down-regulation of the pro-inflammatory cytokines tumor necrosis factor α (TNF-α) and IL-12, which promote an immunosuppressive environment in tissues [97]. These alterations permit the early recognition of apoptotic bodies by macrophages and adjacent cells and then their engulfing and digestion without the release of pro-inflammatory cellular components [98]. Thus, apoptosis results in the rapid and efficient removal of cells without those cells lysing and releasing their contents into the surrounding environment, thus avoiding an inflammatory response [4].

CONCLUSIONS

Apoptosis is an evolutionary, strictly conserved cell suicide mechanism. It operates in a variety of different ways for the sculpting of structure, developmental selection, check point control, damaged cell deletion and finally homeostasis of the cell population in metazoans. Further detailed studies of the cross-talk and transactivation of pathways in apoptosis will help in understanding these mechanisms, allowing the design of powerful therapeutic agents for conditions such as cancer and wound healing. This review attempted to briefly overview the recent concepts in apoptosis while revisiting the previously established dogmas.

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