Role of caspases and non-caspase proteases in cell death
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Abstract
Undoubtedly, caspases are the major driving force for apoptosis execution and mechanisms of their activation and inhibition have been largely unveiled. Recent progress has been made with regard to the exact intracellular ordering of caspases, monitoring their activities in vivo and unveiling their substrate degradomes. Moreover, non-caspase proteases seem to assist caspases in the completion of the death execution program. Here we will consider some very recent data dealing with these aspects. We will also provide novel insights into the mechanisms that dictate apoptotic variability within a cell population.

Introduction and context
Caspases coordinate, in a proteolytic cascade and with the help of other proteases, the rapid and efficient elimination of a cell by apoptosis. They are classified into monomeric initiator caspases (caspase-8 and -9) with long prodomains (death effector domain [DED], and caspase recruitment domain [CARD]), which are activated by recruitment to and dimerization on protein platforms, and already dimerized effector caspases (caspase-3, -6 and -7), which require for their activation the cleavage (e.g., by initiator caspases) of an inter-subunit linker that binds to the dimer interface [1].

In the extrinsic signaling pathway, the recruitment platform is the death-inducing signaling complex (DISC) consisting of the activated death receptor, the adapter FADD (Fas-associated protein with death domain) and initiator caspase-8 and -10. In the intrinsic mitochondrial pathway, the platform is the apoptosome, a complex of the adapter Apaf-1 (apoptotic protease activating factor 1), mitochondrial-released cytochrome c and initiator caspase-9 [1]. In both cases, the main role of caspase-8 and -9 is to cleave and activate effector caspase-3 and -7. Caspase-6 is an effector caspase with a limited substrate specificity whose exact role in apoptosis remains ill-defined [2]. Caspase-2 is recruited to another platform, the PIDDosome (PIDD is the p53-induced protein with death domain), which is involved in translating a danger signal, such as DNA damage, into either a repair or death response depending on the cellular or environmental context [3]. Similarly, caspases-1, -4 and -5 in humans and caspase-11 in mice are part of inflammasome platforms that assemble in response to external, pathogenic stimuli and guide the production and secretion of pro-inflammatory cytokines such as interleukin (IL)-1 and IL-18 [4]. Under certain circumstances, such as salmonella-infected macrophages, the antimicrobial, inflammatory response may result in a caspase-1-mediated form of programmed cell death, called pyroptosis [5]. Caspase-12 is an inhibitor of caspase-1 [4], and caspase-14 is probably the only truly non-apoptotic human caspase mediating keratinocyte differentiation [1].

In the caspase field, three major issues are still under scrutiny: why are three effector caspases needed? Are non-caspase proteases important to support or even replace caspase-mediated signaling? And why is effector
caspase activation and apoptosis not synchronized, even in a clonal cell population?

Major recent advances
Redundancy, substrate specificity and amplification loops in caspase signaling
Two recent papers reported on the caspase substrate repertoire in apoptotic cells [6,7]. In one case, the proteomes from control and apoptosis-stimulated systems were separated on one-dimensional SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) after gel elution [6]. In the other approach a gel-free technology was used by selectively biotinylating free protein amino termini that are generated in apoptotic cells upon caspase-mediated cleavages to enrich for LC-MS/MS [7]. Both groups identified hundreds of new substrates and support the concept that most of them are cleaved into domain-containing fragments that may either gain or lose function. The cleaved substrates are often stable, suggesting that dismantling the apoptotic cell is more like folding a tent after careful removal of pegs than disposing debris after an explosion. Walsh et al. [8] tested some of these substrates for caspase-3 or -7 specificity. They found that caspase-3 was more promiscuous than caspase-7 because 12 out of 20 substrates were preferentially cleaved by caspase-3 whereas only one was more susceptible to processing by caspase-7. Thus, caspase-3 and -7 may, in part, be functionally distinct, explaining why caspase-3 and caspase-7 null mice show distinct phenotypes on some genetic backgrounds [9]. In this regard, a recent study published for the first time a non-redundant role of caspase-7 in mediating lipopolysaccharide-induced lymphocyte apoptosis and mortality in mice [10]. This might be due to the fact that caspase-7, unlike caspase-3, can be processed and activated by caspase-1 [11]. Thus, under certain conditions caspase-7 may be crucial for inflammatory events triggered by the caspase-1 inflammasome.

Caspase-3 can also process other caspases, such as caspase-6 and -2. In turn, caspase-6 processes caspase-8 and -10 [2,12]. Using a specific inhibitor of caspase-3, and small interfering RNA-mediated ablation of specific caspases, Inoue et al. [13] recently validated this caspase ordering pathway not only for caspase-3 but also for caspase-7. This positions caspase-6 in an important amplification loop downstream of caspase-3 and -7. The role of caspase-2 in this loop still remains to be determined. Caspase-2 was previously suggested to function as an initiator caspase for DNA damage and heat-shock-induced apoptosis upstream of mitochondria. Meanwhile, this has been largely questioned [3]. It seems that caspase-2 may amplify apoptotic responses by being activated downstream of caspase-9/-3 [14] or, alternatively, to drive a DNA damage response that bypasses p53, B-cell lymphoma protein-2 (Bcl-2) and caspase-3 [15]. Moreover, a non-apoptotic role of caspase-2 was proposed in the context of DNA damage-associated cell cycle arrest through the recruitment and activation on a cytosolic and a nuclear PIDDosome platform [3,16] because caspase-2 deficient cells failed to show G2/M arrest, leading to more mitotic cells whose DNA was not repaired. In accordance with such a mechanism, Ho et al. [17] recently suggested a tumor-suppressive role of caspase-2 by controlling cell cycle checkpoints.

Monitoring caspases in vivo and ways to activate them therapeutically
Since the number of therapies associated with increased or decreased apoptosis continue to grow, it is crucial to directly visualize and quantify apoptosis in vivo. Edgington et al. [18] developed fluorescently-labeled activity-based probes (ABPs) against caspases that provided direct readouts of kinetics of apoptosis in thymi and tumors in live mice. In contrast to the already-used polymer-based quenched fluorescent substrates that are cleaved in multiple locations to produce fluorescent products, the ABPs are retained at the site of proteolysis by forming a covalent bond with the active-site cysteine of caspases [19]. Thus, the fluorescent ABPs show more rapid and selective uptake into tumors and brighter signals compared to substrate probes.

Human diseases characterized by high apoptosis resistance, such as cancer, may be treated in the future with small-molecule drugs that can activate effector caspase proenzymes. Wolan et al. [20] used high-throughput screening to identify a compound that promoted selective autoproteolytic activation of pro-caspase-3 at physiological concentrations by stabilizing the on-state conformation of the proenzyme. Moreover, Walters et al. [21] recently reported on a caspase-3 mutation that prevented the intersubunit linker from binding in the dimer interface, allowing the active site to form in the procaspase in the absence of proteolysis. This pseudo-activated caspase-3 was not inhibited by XIAP (X-linked inhibitor of apoptosis protein) and rapidly killed cells at low concentrations.

With a little help from your friends: non-caspase proteases assisting in cell death
In mammalian cells, cell death often continues when caspases are inhibited or deleted. This form of cell death seems to be mediated by other proteases, such as calpains, serine proteases or lysosomal proteases, which act in parallel with caspases to amplify apoptosis...
signaling or induce other forms of cell death such as necrosis [22].

Calpains mainly play a pro-death role when calcium signaling is involved. In 2005, Nicotera’s group showed that the plasma membrane Na+/Ca²⁺ exchanger is cleaved by calpain in the ischemic brain and in cerebellar granule neurons exposed to glutamate leading to a Ca²⁺-mediated neuronal demise [23]. Moreover, calpains were recently shown to redistribute to the nuclear envelope, where they degraded nuclear pore complexes and increased nuclear leakiness in response to a Ca²⁺ overload [24]. Furthermore, calpain-1 was found to be activated in spontaneous and Fas-mediated apoptosis of neutrophils by a caspase-induced cleavage of its inhibitor calpastatin. This resulted in the truncation of pro-apoptotic Bax (Bcl-2-associated X protein) [25] and the autophagy-related 5 (Atg5) protein [26], which both enhanced cytochrome c release and most likely deviated the cells from autophagy to apoptosis.

Masson and Tschopp [27] identified a family of serine proteases, the granzymes (Gzms), present in cytotoxic T cells and natural killer cells, which were capable of eliminating virally infected and malignant cells in cooperation with the FasL-Fas signaling system. Meanwhile, it has become clear that GzmB mediates caspase-dependent apoptosis by either cleaving Bid (BH3-interacting domain death agonist) and activating Bax/Bak-dependent cytochrome c release or directly processing caspase-3 [28]. By contrast, Metkar et al. [29] recently reported that GzmA contributes to anti-viral immunity, not via its pro-apoptotic action, but by triggering the processing of pro-IL-1β to mature IL-1β, thereby stimulating a pro-inflammatory response. Thus, Gzms resemble caspases in their capacity to influence both apoptosis and inflammation depending on the isoform. In this orphan human GzmM and GzmK may similarly act as pro-inflammatory proteases, explaining the observed ongoing anti-viral defence in gzmAxB/-/ mice [30,31]. Moreover, it is conceivable that other cell types may also exploit a serine protease-based mechanism to regulate apoptosis. A few candidate serine proteases have so far been proposed, such as HtrA2 (high-temperature-regulated A2), AP24 (24 kDa apoptotic protease) and serine proteases implicated in endoplasmic reticulum stress-induced apoptosis [22].

Cathepsins encompass three classes of lysosomal proteases, the serine proteases cathepsin A and cathepsin G, the aspartic proteases cathepsin D and cathepsin E and the 11 so far known human cysteine proteases, cathepsins B, C, F, H, K, L, O, S, V, W and X/Z [22]. They have been implicated in apoptosis regulation because of their cytoplasmic release due to increased lysosomal membrane permeabilization (LMP) [32]. Although they depend on acidic pH for optimal activity, they seem to retain some of their catalytic activity at physiological pH. Therefore, upon their release into the cytoplasm they can directly cleave or, as recently reported, pro- and anti-apoptotic members of the Bcl-2 family or XIAP [33]. Unfortunately, in most studies, LMP was artificially induced by lysosomotropic agents such as Leu-Leu-OMe (L-leucyl-L-leucine methyl ester), and it has therefore remained unclear if cathepsin release indeed plays an initiating role in apoptosis signaling. Now, Oberle et al. [34] report that cells deficient in Bax/Bak, apoptosomal components or caspase-3/-7 are resistant to LMP in response to various apoptotic stimuli, indicating that cathepsin release and action occurs downstream of mitochondria. Moreover, since some cathepsin knock-out cells (B, L but not D) exhibited delayed but not blocked apoptosis, this lysosomal signaling pathway seems to amplify rather than initiate apoptosis. This process could nevertheless be physiologically relevant because a lack of this amplification loop would lead to delayed cell death and/or inefficient removal of apoptotic bodies and may be the underlying cause of a lack of resolution of inflammation due to neutrophil accumulation in cathepsin D knock-outs [35], reduced keratinocyte apoptosis resulting in periodic hair loss in cathepsin L-deficient mice [36,37] and diminished liver damage upon tumor necrosis factor (TNF)-alpha challenge in mice deficient of cathepsin B [38]. More recently, Kirkegaard et al. [39] offered a possibility to stabilize lysosomes by delivering Hsp70 (heat-shock protein 70) via the endocytotic pathway. Through its interaction with an endolysosomal phospholipid, Hsp70 facilitates the activity of acid sphingomyelinase and thereby stabilizes the lysosomal membrane. This could be exploited to correct Niemann-Pick disease-associated pathology (in which acid sphingomyelinase is defective). Conversely, as tumor cells express high levels of lysosomal Hsp70, a reduction of this protein may sensitize these cells to LMP and cathepsin-mediated cell death.

Together with you I die: the molecular basis for synchronous dying

It has remained enigmatic why cells, even in clonal populations, undergo apoptosis at different rates despite simultaneous addition of an apoptotic stimulus. Although caspase-3 activation occurs within minutes after cytochrome c release, the duration of events preceding cytochrome c release can vary from 1 to 24 hours. A number of factors could contribute to this population variability, including differences in cell cycle stages, metabolic rates, the local environment of single cells or...
stochasticity in biochemical reactions triggered by apoptotic stimuli. Now, two recent papers reveal that the cell-cell variability of apoptosis times is due to the divergence of the molecular composition of the cells. Using a novel single fluorophore reporter to measure caspase-3/-7 activities inside cells, Bhola et al. [40] report that the onset of proteolytic activity is tightly synchronized amongst the most related sister cells and lost over successive generations. Similarly, Spencer et al. [41] found that variability in the apoptotic phenotype arises from cell-to-cell differences in protein levels of caspases, caspase inhibitors, Bcl-2 family members, and so on, that exist before TRAIL (TNF-related apoptosis-inducing ligand) exposure. A particular protein state of a single cell is then transmitted to the daughter cells, giving rise to transient non-genetic heritability in cell fate. Due to ongoing protein synthesis, the protein content of sister cells rapidly diverges so that soon these cells are no more similar to each other than pairs of cells chosen at random.

Future directions

Although we have learned much about the regulation of mammalian caspases, their ordering within signaling cascades and their specific roles in particular cell death regimens in vitro and in vivo, we do not know yet the full scope of action of the two effector caspases-7 and -6. Is caspase-7 mainly a downstream target of caspase-1 involved in inflammation? Is caspase-6 only an amplifier caspase or does it play a discrete role under particular stress responses? What about the large number of caspase substrates that have recently been identified by mass spectrometry [6,7]? Can they be validated in vivo (i.e., at physiologically relevant caspase concentrations of 1 nM in dying cells) and which of them, if any, exert a functional significance in mediating the execution of apoptosis? Another major task will be to determine if caspase-2 is only a checkpoint controller, or also an apoptotic caspase at specific cell cycle stages, for example, leading to mitotic catastrophe, a form of apoptosis that may depend on the molecular profile of the cell [42]. In this respect it will be exciting to continue the recent work on determining how the protein content of apoptosis signaling molecules sets the threshold for death induction in single cells, preferentially supported by systems biology approaches as shown by Spencer et al. [41]. This will have major implications for future anticancer therapies as resistance to chemotherapy is most likely due to such cell-to-cell variability within a given tumor cell population.

Another crucial aspect will certainly be the better detection of apoptotic cells in diseased tissues using specific fluorescent probes and the development of more drugs that activate the caspase-3 proenzyme. In particular, a drug mimicking the caspase-3 mutant described by Walters et al. [21] holds great promise to activate caspase-3 without proteolytic processing and eventually to kill even tumor cells expressing high XIAP levels.

Finally, we desperately need to understand the exact role of non-caspase proteases in cell death regulation. The implication of calpains, serine proteases, cathepsins and other proteases in this process seems to be a relatively recent evolutionary response to the complexity of biological processes in humans. Thus, these enzymes will be promising targets for future therapeutic intervention – for example, to save neurons or immune cells from degeneration – irrespective of whether these proteases amplify caspase-dependent signaling or trigger alternative caspase-independent processes. Our future work will include the identification and molecular characterization of more apoptotic and pro-inflammatory serine proteases and the discovery of the signaling pathway leading from caspases to LMP and cathepsin release from lysosomes.

Abbreviations

ABP, activity-based probe; Bak, B-cell lymphoma protein-2-antagonist/killer; Bax, B-cell lymphoma protein-2-associated X protein; Bcl-2, B-cell lymphoma protein-2; Fas, F7-associated surface protein; FasL, Fas ligand; Gzm, granzyme; Hsp, heat-shock protein; IL, interleukin; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; LMP, lysosomal membrane permeabilization; TNF, tumor necrosis factor; XIAP, X-linked inhibitor of apoptosis protein.

Competing interests

The authors declare that they have no competing interests.

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