

Secondary necrosis in multicellular animals: an outcome of apoptosis with pathogenic implications

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Abstract In metazoans apoptosis is a major physiological process of cell elimination during development and in tissue homeostasis and can be involved in pathological situations. *In vitro*, apoptosis proceeds through an execution phase during which cell dismantling is initiated, with or without fragmentation into apoptotic bodies, but with maintenance of a near-to-intact cytoplasmic membrane, followed by a transition to a necrotic cell elimination traditionally called “secondary necrosis”. Secondary necrosis involves activation of self-hydrolytic enzymes, and swelling of the cell or of the apoptotic bodies, generalized and irreparable damage to the cytoplasmic membrane, and culminates with cell disruption. *In vivo*, under normal conditions, the elimination of apoptosing cells or apoptotic bodies is by removal through engulfment by scavengers prompted by the exposure of engulfment signals during the execution phase of apoptosis; if this removal fails progression to secondary necrosis ensues as in the *in vitro* situation. *In vivo* secondary necrosis occurs when massive apoptosis overwhelms the available scavenging capacity, or when the scavenger mechanism is directly impaired, and may result in leakage of the cell contents with induction of tissue injury and inflammatory and autoimmune responses. Several disorders where secondary necrosis has been implicated as a pathogenic mechanism will be reviewed.

Keywords Apoptotic secondary necrosis · Apoptosis · Necrosis · Clearance · Autoimmunity · Infection

Introduction

“Life is uncertain, death is certain”. All cells are doomed to die. Cell death processes can be passive or active. Passive cell death is the direct, acute killing of a cell by an exterior aggression damaging enough to produce irreversible alterations; these occur without the participation of the cell and can be prevented only by the absence of the aggression. This is the case, among others, of cell death due to exposure to high concentrations of detergents or antiseptics, extreme heat or repeated freeze-thawing, that is, situations without biological relevance. In this mode of cell death there is immediate, extensive and irreparable damage to the cytoplasmic membrane and this damage affects the membrane from without.

In contrast, active cell death is a suicidal process that is carried out by the doomed cell using molecules and pathways that are endogenous to the cell. The term “programmed cell death”, coined by Lockshin and Williams to qualify developmental cell death [1], has gained wide use to characterize active cell deaths. However, the term “programmed” has been used in two distinct senses to characterize either (i) cell death processes that occur at a precise local and time according to a developmental program (for example in morphogenesis) or (ii) cell deaths that, once triggered (as part of a developmental program or occasionally as in some pathologies), follow cell-intrinsic biochemical programs controlled by the doomed cell. Therefore, we will use the term “active” instead of “programmed” when referring to cell death processes that, once triggered, unroll following a course of biochemical events processed and controlled by the dying cell.

Cell death is followed by cell elimination. In multicellular animals, cell elimination is essential for development and homeostasis, and is mainly carried out by apoptosis. The initial report by Kerr, Wyllie and Currie, introducing

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the concept of apoptosis [2] was based on *in vivo* observations in mammalian tissues and described that process as comprising a terminal cell elimination stage through the removal of the apoptosing cell by phagocytosis and degradation by a scavenger cell. However, it was soon recognized by Kerr and collaborators that *in vivo* [3, 4] and *in vitro* [5, 6], when removal by scavengers does not occur, the apoptotic process continues until a transition to necrosis ensues leading to cell elimination by cell disruption. This terminal disruption of apoptotic cells was initially called “secondary degeneration” [5], a designation later changed to “secondary necrosis” [7]. This designation achieved widespread acceptance and is now part of the glossary of cell death terms, although it is prone to some confusion. Indeed, necroses that can be also labeled as secondary can occur in diverse situations; for example, necrosis following ischemia due to vascular occlusion has been frequently called secondary necrosis, as in necrotizing fasciitis.

Secondary necrosis was viewed in the initial reports as a separate process occurring after completion of apoptosis [5, 6] and, thus, it has frequently been called post-apoptotic necrosis. An alternative view is to consider that necrotic outcome as part of the apoptotic program of cell elimination, in which case it has been called late apoptosis.

Secondary necrosis also terminates the apoptotic process in eukaryotic unicellular organisms like yeasts [8, 9]. A terminal necrotic elimination can occur at the end of other active cell death processes besides apoptosis [10, 11]. Therefore, the term apoptotic secondary necrosis is used in this review to refer the terminal cell disruption that may follow apoptotic cell death.

After being initially seen as a mere biological curiosity, apoptotic secondary necrosis was soon recognized as an event with pathogenic relevance when occurring in multicellular animals. The pro-inflammatory and cytotoxic consequences of lysis of cells under secondary necrosis due to the lack of removal by scavengers have initially been repeatedly predicted or assumed, but supporting evidence for the pathogenic potential of that outcome of apoptosis has been progressively gathered as will be here reviewed.

Apoptosis as the prelude of secondary necrosis

Secondary necrosis is a process that affects cells under advanced apoptosis; thus, the characteristics of secondary necrotic cells are influenced by alterations previously occurred during the execution of apoptosis.

After the initial concept that apoptosis was the mechanism behind active cell death, advances in the knowledge in this area revealed that active cell death may assume several genetically defined programs (reviewed in Ref. [12]). In typical situations, these diverse programs dictate

different phenotypes which can be used as a basis for the classification of active cell death modes [13, 14]. The presently known clear-cut forms of active cell death include, besides apoptosis, autophagic cell death [10, 15], primary necrosis (also known as programmed necrosis) [11, 16–18], mitotic catastrophe [19], and caspase 1-dependent pyroptosis [20]. These paradigmatic modes of cell death have been observed in multicellular organisms, but less clear-cut, intermediate or hybrid cell death modes have also been described as indicated by the use of terms as apoptosis-like or necrosis-like cell deaths [12], necrapoptosis [21] and necroptosis [22].

In multicellular organisms, apoptosis is a major physiological cell killing process used during embryonic morphogenesis and in adult life in tissue homeostasis and immune responses for the elimination of unnecessary, unwanted or dangerous cells [23, 24].

When a cell isolated from a metazoan and maintained *in vitro* responds to an apoptotic death stimulus it activates a sequence of molecular events [25] that proceeds through two phases which ultimately culminates in cell disruption [5, 25]. In the first phase, molecular alterations produce the classical apoptotic morphotype (see Table 1 and Figs. 1b, 2b and 4) that is used to identify this mode of cell death. During this phase the apoptosing cells may or may not fragment into apoptotic bodies, and, in contrast with necrosis, the membrane enveloping the apoptosing cells or the apoptotic bodies remains near-to-intact, that is, except for some structural alterations like externalization of phosphatidylserine and exposition of other “eat-me” signals for phagocytosis by scavengers, it is normal including in regards to selective permeability [7, 33, 34]. The terminal phase is secondary necrosis during which necrotic molecular alterations produce a new morphotype (see Table 1) which is more typical when there is no fragmentation into apoptotic bodies; this secondary necrotic morphotype is a mixture of alterations produced in the apoptotic phase (like nuclear fragmentation and intense chromatin condensation) and necrotic alterations (swelling of the cell and damage to the cytoplasmic membrane) [7]. Ultimately the cytoplasmic membrane ruptures and the cell (or the apoptotic bodies) is dismantled to cell debris (Fig. 1c, d). This experiment shows that the apoptotic death of an isolated cell, once triggered, is self-sufficient and leads to self-elimination by secondary necrosis.

When incorporated in the multicellular organism, that same cell will respond to an apoptotic death stimulus again by activating its endogenous apoptotic mechanism but the outcome may well be quite different from that occurring *in vitro* because now the cell is part of the social context of the multicellular organism [35]. Indeed, the apoptotic program triggers a mechanism for cell elimination that calls for the cooperation of a scavenger cell to engulf and digest by heterolysis the apoptosing cell before transition to

Table 1 Morphotypes in apoptosis, apoptotic secondary necrosis and primary necrosis

Apoptosis (see Figs. 1b, 2b and 4)	Cell rounding and shrinking Loss of microvilli Membrane blebbing Nuclear fragmentation and/or Intense chromatin condensation Mitochondrial “thread-grain” transition Production of apoptotic bodies Impermeability of the cytoplasmic membrane to PI
Apoptotic secondary necrosis (see Figs. 1c, 1d, 2c and 4)	Nuclear fragmentation Intense chromatin condensation <i>Cytoplasmic swelling</i> <i>Intense mitochondria swelling</i> <i>Damaged cytoplasmic membrane (revealed by PI-positive staining and release of LDH)</i>
Primary necrosis	No nuclear fragmentation Moderate chromatin condensation Cytoplasmic swelling Intense mitochondrial swelling Damaged cytoplasmic membrane (revealed by PI-positive staining and release of LDH)

Main morphological alterations that allow the identification of cells in the execution phase of apoptosis, in apoptotic secondary necrosis and in primary necrosis (reviewed in Refs. [7, 14, 26–29])

The apoptotic alterations of secondary necrosis are in bold; in italic are the necrotic alterations

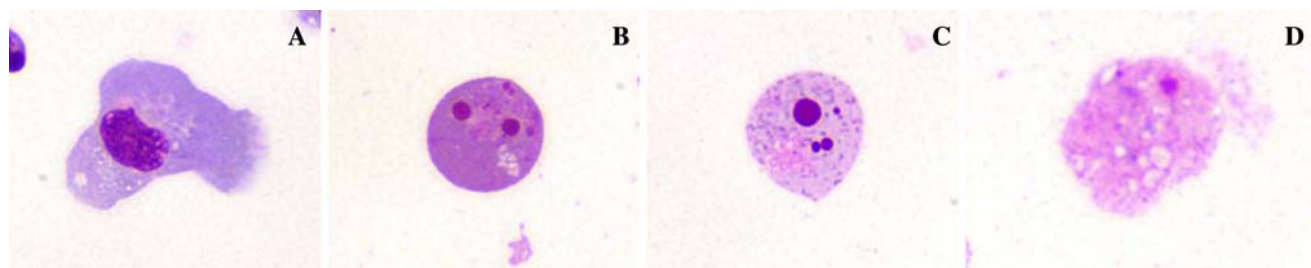


Fig. 1 Apoptotic cell death in vitro. Hemacolor staining of sea bass peritoneal phagocytes exposed in vitro to the apoptogenic exotoxin AIP56 of the gram-negative pathogen *Photobacterium damsela* ssp. *piscicida* [30]. (a) Normal phagocyte. (b) Phagocyte under apoptosis, showing cell rounding and shrinkage, nuclear fragmentation and

intense chromatin condensation. (c) Phagocyte under apoptotic secondary necrosis, showing nuclear apoptotic changes (fragmentation and intense chromatin condensation) and a lysed cytoplasm. (d) A more advanced secondary necrosis with chromatolysis and cell swelling. Technical details in Ref. [30]

secondary necrosis, that is, while the apoptosing cell still is enveloped by a near-to-intact membrane. This process of cell elimination, described in the initial report by Kerr, Wyllie and Currie as the second stage of apoptosis in mammals and as the physiological mechanism for elimination of apoptosing cells or apoptotic bodies [2] is activated in the in vitro setting but cannot operate because of the lack of the scavenger.

Biochemical events that are behind the apoptotic morphotype may include caspase activation, PARP-1 inactivation, mitochondrial permeability transition with loss of membrane potential ($\Delta\psi$), release of AIF, EndoG or cytochrome *c* from the mitochondrial intermembrane space, moderate increased production of ROS, moderate cytosolic Ca^{2+} overload and internucleosomal DNA degradation (reviewed in Refs. [25, 36, 37]). There is proteolytic degradation of many substrates mainly effected

by activated effector caspases [36] but caspase-independent effector mechanisms may also participate [38], and may have the participation of autophagy [39]. The degradative events occurring during the execution of apoptosis produce a partial cell dismantling which confers to apoptosis an autolytic character and is the basis of the apoptotic morphotype. This initial autolytic degradation is significantly increased when apoptosing cells are phagocytosed and heterolytically digested by scavengers or when apoptosis transits to secondary necrosis where further, extensive, autolytic degradation leads to cell disruption.

For the normal progress of the apoptotic pathways energy is required, and an initial increase in cytosolic ATP in apoptosing cells has been reported [40, 41]. ATP is required for cell shrinkage, bleb formation, caspase activation, enzymatic hydrolysis of macromolecules, chromatin condensation, DNA internucleosomal fragmentation,

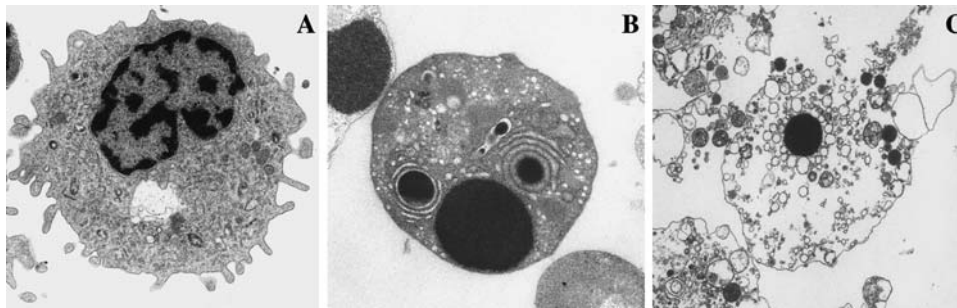


Fig. 2 Apoptotic cell death in vivo. Transmission electron microscopy of peritoneal sea bass phagocytes that were induced to enter apoptotic death by the exotoxin AIP56 secreted by intraperitoneally injected *Photobacterium damselae* ssp. *piscicida* [31, 32]. (a) A normal looking phagocyte. (b) A phagocyte under apoptosis, showing cell shrinkage, loss of microvilli, nuclear fragmentation and intense

chromatin condensation and a continuous cytoplasmic membrane. (c) A phagocyte under apoptotic secondary necrosis showing an apoptotic (hyper-condensed) nuclear fragment together with rupture of the cytoplasmic membrane and leakage of cell components. Technical details in Ref. [32]

nuclear fragmentation, exposure of engulfment signals and apoptotic body formation (reviewed in Refs. [25, 37, 41, 42]).

Apoptotic bodies may be formed during the degradation phase by the fragmentation of apoptosing cells as already described in the initial report of Kerr, Wyllie and Currie [2]. Although formation of apoptotic bodies is frequently considered as constant during apoptosis, in some otherwise typical apoptotic processes occurring in vitro or in vivo apoptotic bodies are not produced or are rare (see for example [6, 7, 26–28, 43, 44]), so that the elimination of apoptosing cells frequently affects non-fragmented cells. The non-fragmented, shrunken, apoptosing cell can be considered a single apoptotic body [45], as in the case of the so-called sunburn cells, apoptotic keratinocytes induced by UV irradiation of the skin [46]. Apoptotic bodies are produced while the apoptosing cell still has a near-to-normal cytoplasmic membrane and they are as well enveloped by a near-to-intact membrane [2, 47]. Like apoptosing cells, they express signals for engulfment and are phagocytosed by scavengers [2] or undergo secondary necrosis if not engulfed [7, 47].

In multicellular animals the timely removal of apoptosing cells through phagocytosis by scavengers is the clue for the physiological outcome of apoptosis because it prevents the occurrence of secondary necrosis [23, 48, 49]. In higher multicellular animals, phagocytosis is mainly accomplished by monocytes/macrophages and neutrophils, the professional phagocytes [50], which constitute up to 10–15% of cells in most mammalian tissues [23]. Typically, the professional scavenger of dying/dead cells is the macrophage [51, 52], which is located in all body territories and is highly phagocytic [53]. When macrophages are not available, for example due to a particular location of the dying/dead cells, neutrophils [54], or neighbor cells functioning as surrogate “amateur” phagocytes, may fulfill the role of removal of dying/dead cells; most cell types, including dendritic, epithelial, endothelial, and glomerular mesangial cells,

fibroblasts, myocytes, and tumor cells, are able to perform this task [7, 49, 51, 52, 55]. This situation is the rule in simple multicellular animals, like *Caenorhabditis elegans*, where professional phagocytes are not present and apoptosing cells are removed by neighbor cells [56].

Although less efficiently than macrophages [49, 57–59], dendritic cells may engulf apoptosing cells, mainly when extensive apoptosis overwhelms the macrophage availability [60–62] or in territories where dendritic cells outnumber macrophages [63]. It has been reported that dendritic cells are more prone than macrophages to develop a pro-inflammatory response following the uptake of apoptosing or secondary necrotic cells [61, 62]. This point will be further discussed in “Pathogenic consequences of apoptotic secondary necrosis” section.

Like molecules involved in cell death processes, molecules involved in the removal by scavengers of dying/dead cells are present, and are in part similar, in worms, flies and mammals [64], indicating that such a clearance represents a crucial mechanism that has been conserved through evolution. Encounter of apoptosing cells with phagocytes may require attraction of phagocytes; specific attraction signals released by apoptosing cells in a caspase 3-dependent manner have been described [65, 66]. The recognition and engulfment of dying/dead cells by phagocytes involves the interaction between a number of molecules (reviewed in Refs. [67–70]). “Eat me” signals are exposed on the surface of apoptosing cells and are recognized by receptors on the surface of scavengers. The interactions between the engulfment signals and the receptors are facilitated by bridging molecules present in serum such as $\beta 2$ glycoprotein, milk fat globule protein (MFG-E8), protein S, growth arrest-specific 6 (Gas6), thrombospondin, pentraxins and complement factors. This mechanism of attraction between apoptosing cells and scavengers is complemented by the loss by the apoptosing cells of expression of “don’t eat me” signals that normally prevent engulfment of living cells.

Much less is known about how scavengers recognize cells under active primary necrosis as compared to apoptosing cells, but it has been reported that some of the engulfment signals in necrosing cells are different from those exposed by apoptosing cells [68]. In this context, it is of note that many studies on this topic (and on the immunological effects of necrotic cells) used cells made necrotic by physical methods like heating, or exposure to detergents or fixatives. These methods produce passive necrotic cells with protein inactivation that consequently cannot be representative of cells dying *in vivo* due to active necrotic processes which induce proteolytic alterations and exposure of engulfment signals. It is therefore important that more studies be conducted on the process of recognition of necrosing cells by scavengers and on the pathogenic effects of necrosis using cells under active necrosis.

Elimination of apoptosing cells by a scavenger involves the degradation by the hydrolytic enzymes of the phagolysosomes of the scavenger (heterolysis). This heterolytic degradation superimposes on the autolysis ongoing in the apoptosing cell [2, 4]. Several results indicate that the role of macrophages in removal of apoptosing cells is not solely to eliminate cell corpses, but rather to degrade those cells to harmless and largely re-utilizable molecules; this dismantling can be extensive when the dying/dead cells are engulfed very early in the apoptotic process [71, 72]. Indeed, uptake of apoptosing cells can occur with different timings after death triggering, depending on the timing of exposition of surface

engulfment signals and on the availability of scavengers. Therefore, the extension of the executed apoptotic curriculum can vary, manifesting different morphologies. Apoptosing cells can be engulfed so early that the typical morphological changes or DNA fragmentation have not yet occurred [73–75] which would explain why under physiological conditions cells with apoptotic indicators are rarely seen in tissues [7, 76]. The swift removal of apoptosing cells is particularly advantageous in embryogenesis.

There are important differences in the processes of removal of cells under apoptosis or under active necrosis: (i) Apoptosing and necrosing cells are phagocytosed by macrophages through different mechanisms [77, 78]. (ii) Engulfment of cells under active necrosis is delayed and quantitatively and kinetically less efficient as compared to the uptake of apoptosing cells [77, 79]. (iii) Engulfment of apoptosing cells by scavengers normally occurs before the transition to secondary necrosis, that is when the dying/dead cells or the apoptotic bodies still have a near-to-normal cytoplasmic membrane [7, 33] (Fig. 3). In contrast, engulfment of necrosing cells usually occurs after cytoplasmic membrane damage and cell rupture so that cell fragments are engulfed [77, 78, 80] (Fig. 3).

Although the macrophage response to the phagocytosis of apoptosing cells may occasionally be pro-inflammatory (see discussion in Refs. [14, 52, 68], that phagocytosis typically fails to induce an inflammatory response [81] due to active inhibition of the release of pro-inflammatory

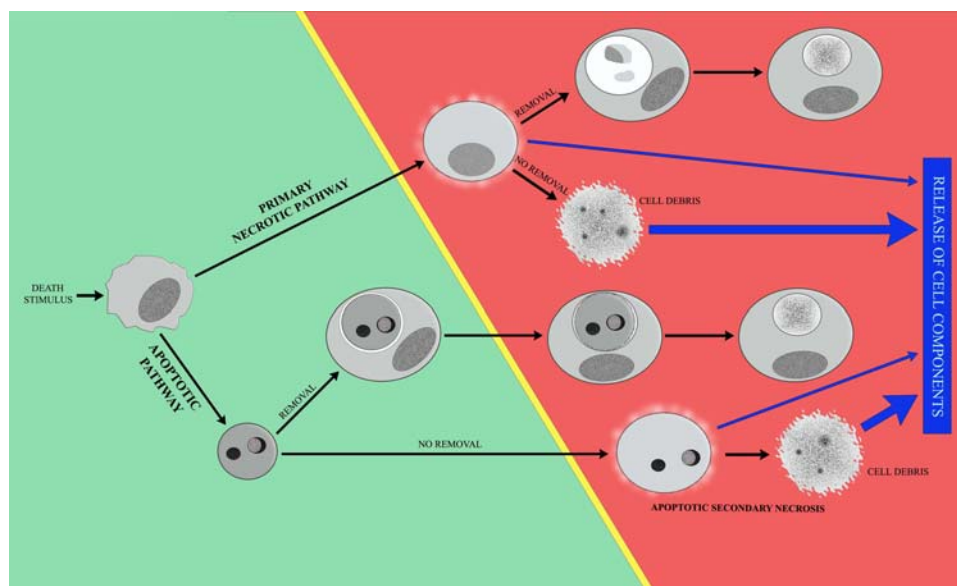


Fig. 3 Schematic representation of the two cell elimination processes that can operate in active primary necrosis and apoptosis (heterolysis after removal by scavengers or cell burst due to autolysis). The cytoplasmic membrane of the cells under death processes is normal in the green area and damaged in the red area. A central issue is that, in apoptosis, in contrast with primary necrosis, the timely removal by

scavengers of dying/dead cells is carried out before occurrence of damage to the cytoplasmic membrane. Leakage of cell contents is most extensive and involves large molecules when there is cell disruption (large blue arrows). See text in “Apoptotic secondary necrosis” section for details

mediators and to generation of anti-inflammatory factors such as transforming growth factor-beta (TGF-beta) and anti-inflammatory eicosanoids [82–84]. This contrasts with the effect of necrotic cell engulfment, which usually, but not always [77, 85], induces pro-inflammatory responses by the engulfing phagocytes [68, 69, 86, 87]. The prevailing view is that the delayed and deficient removal mechanism of cells under primary necrosis allows the persistence of leaky cells and necrotic debris which leads to activation of pro-inflammatory and immuno-stimulatory responses [11, 16] (see “Pathogenic consequences of apoptotic secondary necrosis” section). Necrotic cells may induce an inflammatory response not only by spilling their contents after lysis, but also by actively secreting inflammatory molecules, including cytokines (reviewed in Ref. [68]). However, there are reports of occurrence of extensive necrotic processes *in vivo* in the absence of important inflammatory responses [79, 80, 85, 88]. It is also of note that necrosis-associated inflammation, when not excessive, can be beneficial for the organism, for example during a protective immune response [17]. The removal by phagocytosis of cells under secondary necrosis will be discussed in “Pathogenic consequences of apoptotic secondary necrosis” section.

Central for the topic of this review is that, in multicellular animals, the physiological removal of apoptosing cells by scavengers may fail allowing the occurrence of secondary necrosis with potential pathogenic consequences. Failure of this process may be due to defects of the scavengers (insufficient number or functional incapacity) or of molecules involved in the recognition and engulfment of apoptosing cells, as will be reviewed later.

Apoptotic secondary necrosis

As already recognized in the initial reports on apoptosis [3, 4], when removal by scavengers fails *in vivo*, the apoptotic process fully unrolls and apoptosis transits to the self-elimination by secondary necrosis. A paradigmatic example is provided by apoptosis of cartilage chondrocytes; due to the avascular nature of articular cartilage and the lack of direct cell-cell contacts, apoptotic chondrocytes, induced for example in poly-arthritis [89], cannot be removed by scavenger cells and are eliminated by secondary necrosis [90]. Occurrence of *in vivo* secondary necrosis associated with pathology will be reviewed in “Pathogenic consequences of apoptotic secondary necrosis” section.

Contrary to the heterolytic degradation that dismantles apoptotic cells within scavengers, the hydrolytic processes of secondary necrosis involve autolytic enzymes. The available scant data regarding the molecular events in

secondary necrosis suggest that this is an active necrotic process with biochemical and structural features common to those of active primary necrosis.

Recent advances in the knowledge of signaling and biochemical events in active primary necrosis are reviewed in Refs. [11, 17, 18, 91, 92]. During the degradation phase of active necrosis several biochemical alterations have been described including mitochondrial dysfunction, high production of ROS, serious ATP depletion, intense ionic imbalance, and activation of non-caspase proteases; these alterations ultimately converge in extensive lysosome rupture, which assumes a decisive role in active necrosis [93]. ATP depletion, ROS production and Ca^{2+} fluxes involve complex feedback and feed-forward interactive loops, some of them self-amplifying, as comprehensively reviewed by Brookes [94]. The important Ca^{2+} overload [95–97] induces enhanced activation of hydrolysing enzymes, including calpains [98], and leads to exaggerated energy consumption and impairment of energy production [99]; ATP depletion and activated calpains were shown to induce lysosome rupture [93, 100, 101], and released lysosomal cathepsins contribute to cytoplasmic membrane damage [102, 103], a hallmark of necrosis [7]. Thus, contrary to what happens in passive necrosis, cytoplasmic membrane damage in active necrosis is produced from within. This damage is due to the cumulative activity of hydrolytic enzymes and of ROS, and to serious energy failure; these events result in progressive membrane permeabilization to molecules of increasing size until the rupture of the membrane. This rupture is a consequence of the continuous cell swelling (oncosis or necrotic volume increase [104]) due to the maintenance within the cell of large molecules, including proteins and nucleic acids (Gibbs–Donnan effect [105–108]). Thus, the progressive deterioration of the cytoplasmic membrane of necrosing cells leads to initial leakage of small ions like K^+ , then leakage of small macromolecules and finally leakage of large macromolecules [105].

The essential events occurring in active primary necrosis, culminating in cytoplasmic membrane rupture, are likely to operate in secondary necrosis. As discussed, the development of apoptosis requires energy. When apoptosis progresses without engulfment of apoptosing cells, the progression eventually leads to a late depletion of the intracellular ATP pool [109–111] likely due to early ATP-consuming apoptotic reactions together with a late complete loss of mitochondrial function [109]. This endogenously originated late serious energy depletion has been considered the trigger for the transition from the end of a fully developed execution phase of apoptosis to secondary necrosis [21, 101, 112, 113]. It is known that serious ATP depletion provoked during early apoptosis induces a switch to necrosis (see below).

On the other hand, occurrence of secondary necrosis in caspase 3-dependent apoptosis induced by ultra-sounds has been found to be inhibited by the antioxidant *N*-acetyl-L-cysteine, suggesting the involvement of ROS in the transition to secondary necrosis [114].

During apoptosis, caspase 3 cleaves proteins involved in intracellular Ca^{2+} regulation including the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, the inositol 1,4,5-trisphosphate receptors (IP3Rs), and the plasma membrane Ca^{2+} ATPases (PMCA) (reviewed in Ref. [115]). Caspase cleavage of the IP3R1 and of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger has been shown to result in cytosolic Ca^{2+} overload [115]. On the other hand, two groups have shown that the PMCA 4b isoform is cleaved by caspase 3 during apoptosis [116, 117], but there is discrepancy regarding the reported functional consequence of that cleavage. While the results from Nicotera's group suggest inactivation leading to a decreased rate of Ca^{2+} efflux from the cells and consequent cytosolic Ca^{2+} overload [116], the results from Enyedi's group suggest that the cleavage product remains functionally active and would not contribute to Ca^{2+} overload [117–119]. An intense rise in intracellular Ca^{2+} was observed prior to the transition of apoptosis to secondary necrosis in prostate and bladder cancer cell lines [120], human B cell line FMO [121] or chicken B-lymphocytes [115]. In two of these studies [115, 121], the dramatic Ca^{2+} cytosolic overload was considered to be involved in that transition.

As in active primary necrosis, serious energy depletion, high Ca^{2+} cytosolic overload and high ROS production will lead to activation of calpains and to lysosome rupture, two events which have been suggested to participate in the cytoplasmic membrane damage in secondary necrosis [101, 122]. That lysosomal enzymes participate in secondary necrosis is also suggested by the observation that, in apoptosis induced in vitro in the epithelial cell line LLC-PK1 by *S*-(1,2-dichlorovinyl)-L-cysteine, inhibition of cathepsin B resulted in inhibition of secondary necrosis [123]. In support of this interpretation, recent results with caspase 3-associated apoptosis induced in Jurkat cells by treatment with anti-Fas showed that the terminal cell lysis due to secondary necrosis was concomitant with lysosome rupture [124].

Two frequently used assays to evaluate the cytoplasmic membrane condition during cell death processes are vital staining with the membrane impermeant propidium iodide (PI) and quantification of release of the intracellular enzyme lactate dehydrogenase (LDH). Permeability to PI (molecular mass about 0.67 kD) indicates a relatively initial cytoplasmic membrane damage [106, 108], while leakage of LDH (molecular mass about 140 kD) indicates terminal membrane rupture [107]. When apoptosing cells enter the secondary necrosis process those two assays change from negative to positive [33, 125, 126], revealing the ensuing of

cytoplasmic membrane damage. Like in primary necrosis, in secondary necrosis PI-positivity precedes release of LDH and of other large molecules like activated caspase 3 (about 19 kD) (see Figs. 1D and 3C in Ref. [126]). As will be discussed in “Pathogenic consequences of apoptotic secondary necrosis” section, these observations are relevant in the context of the potential pathogenic role of secondary necrotic cells, since it is likely that only ruptured secondary necrotic cells will release large molecules like DNA, RNA and proteins including high mobility group box 1 (HMGB1) protein (about 30 kD) with inflammatory and immunogenic capacities.

The necrotic degradation in secondary necrosis affects cells that already were partially dismantled during the execution phase of the apoptotic process. The cell degradation during apoptosis involves proteolysis due to the activity of proteinases, mainly of effector caspases. During secondary necrosis there is additional proteolysis in a caspase-independent manner [127]. This late proteolysis, that contributes to the final dismantling of the apoptotic cell, may originate autoantigens that, when released by the lysing cell, send danger signals to the immune system and stimulate autoantibody responses [62, 128–130].

Serious energy failure provoked during early apoptosis by mechanisms external to the apoptotic process induces a switch to necrosis [131–133]. This switch occurs earlier than the spontaneous transition to secondary necrosis triggered by the endogenous failure of ATP associated with the complete progression of the apoptotic process. Indeed, the switch induced by ATP depletion from without interrupts the normal progression of apoptosis preventing the occurrence of biochemical events, like caspase activation, that produce the typical apoptotic morphotype. Consequently, cells under necrosis due to the switch apoptosis–necrosis do not exhibit the mixed secondary necrotic morphotype previously discussed (see Table 1 and Figs. 1c, 2c and 4) but rather that of active primary necrosis. This “switched necrosis” does not comply with the classical definition of apoptotic secondary necrosis discussed in the beginning of this review. However, it may lead to similar pathological consequences since the cells undergoing this switched necrosis autolyse and release cytotoxic or immunogenic molecules.

Apoptotic secondary necrosis was initially described in in vitro experiments [5, 6]. The frequent lack of consideration of the fact that induction of apoptosis in vitro (usually in the absence of scavengers) inevitably results in secondary necrosis can lead to misleading interpretations of induction of active primary necrosis, or of a switch from apoptosis to necrosis, when the actual event was the natural transition of an ongoing apoptosis to secondary necrosis. Confusion between the occurrence of active primary or secondary necrosis in vitro is particularly frequent when no

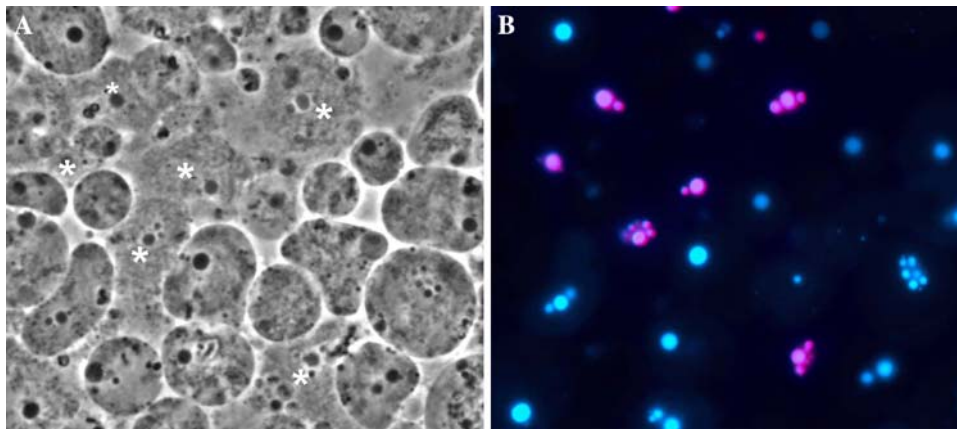


Fig. 4 A group of enterocytes of sea bass under detachment-induced apoptosis (anoikis). Cells incubated *ex vivo* and viewed by phase contrast (panel **a**) and by fluorescence after double vital Hoechst 33342 and PI stainings (merged in panel **b**). Apoptosis is identified by the presence of fragmented and/or hyper-condensed, PI-negative

nuclei in panel **b**. The cells labeled with asterisks in panel **a** are lysed as shown by the reduction in cytosol density; these cells have PI-positive fragmented and hyper-condensed nuclei (panel **b**), indicating that they are under apoptotic secondary necrosis. Reproduced from [134] with permission of the publisher

kinetic studies are carried out, preventing the observation of apoptosis preceding secondary necrosis. The same problem arises when studying apoptosis of unicellular eukaryotic cells like yeasts because also in this case apoptosis is always followed by secondary necrosis [8, 9].

Identification of apoptotic secondary necrosis

Detailed morphological descriptions of apoptotic secondary necrosis are available [26, 27, 134].

Since typically secondary necrosis is a necrotic event affecting late apoptosing cells, the secondary necrotic morphotype (see Table 1) is characterized by the co-existence in the same cell of some apoptotic alterations (like nuclear fragmentation and/or intense chromatin condensation) and some necrotic alterations (like a damaged cytoplasmic membrane). As already discussed, before they are cleared by scavengers or enter secondary necrosis, apoptotic cells exclude membrane impermeant stains like PI [33] (it is of note that the exclusion of membrane impermeant stains is only observed when these stains are used in low concentrations and for short periods [135] as in the protocol in reference [136]). PI positivity in secondary necrotic cells can be detected after combined vital staining with Hoechst 33342/PI (see Fig. 4 and Ref. [134]). Also typical of apoptotic secondary necrotic cells is the association of nuclear apoptotic alterations and cytoplasmic membrane rupture detected by transmission electron microscopy (Fig. 2c), depicting the terminal stage of secondary necrosis. Particularly informative is the time-lapse observation by phase contrast and fluorescence microscopy of Hoechst 33342/PI stained preparations [134]. This continuous scrutiny of the same

cell allows the observation of the apoptotic morphotype (with apoptotic nuclear alterations, cell shrinkage, blebbing, and production of apoptotic bodies (when it occurs) and PI-negativity) followed by entrance into the secondary necrotic process as indicated by the change to PI-positivity, followed by cell swelling with reduction in cytoplasm density (Fig. 4), chromatolysis, and finally cell burst.

Like LDH and other large molecules, activated caspases are released at the end of apoptotic secondary necrosis when membrane rupture occurs, while only procaspases are released in the case of cytolysis due to caspase-independent death processes [126]. Furthermore, the procaspases released by primary necrotic cells are not significantly processed extracellularly by proteolysis despite the presence of released proteases [126]. Therefore, when the cell death process has been characterized as caspase-dependent apoptosis, occurrence of relevant apoptotic secondary necrosis can be detected *in vivo* by the presence of increased levels of extracellular activated caspases which can be quantified in the blood [31, 137–139].

Non-pathogenic apoptotic secondary necrosis

Lack of removal by engulfment of apoptosing cells can occur *in vivo* without serious consequences. This is the case when those cells are shed into ducts or into territories topologically outside the organism (like the gut and airways lumen) where the chances of encountering or attracting scavengers are small but where release of cell contents is not critical.

For example, prostate apoptosing cells induced by castration detach and are shed in the lumina of the acini where

they undergo secondary necrosis [3]. During the first days of involution of lactating breast after weaning, abundant apoptosis of epithelial cells occurs; many of these apoptosing cells are shed into the alveolar lumen [140, 141] and undergo secondary necrosis [140].

In a mechanism of clearance, neutrophils and eosinophils in lung tissue can be removed through egression across the epithelial lining into the airway lumen where they may undergo apoptosis with engulfment by scavengers or progress to secondary necrosis [142, 143]. These granulocytes or their remnants are eventually eliminated with airway mucus and expectoration.

Pathogenic consequences of apoptotic secondary necrosis

Introduction

In physiological situations, the apoptotic mode of cell elimination is useful since it is triggered when appropriate, is silent, induces a safe mechanism for cell disposal by scavengers, and, although spending energy, is economical in terms of re-utilization of the products of this disposal [2]. Implicit in that perspective of apoptosis in multicellular organisms is that “the individual cellular fate is sacrificed for the benefit of a higher order of life—the organism” [144]. However, this perspective of apoptosis as a physiological, and sometimes programmed, process is restrictive since this mode of cell elimination can be subverted, leading to pathological situations that are harmful to the organism where it occurs. Apoptosis turns pathological when the killed cells are necessary and functional, for example when pathogens evade phagocytic defense mechanisms by inducing apoptotic destruction of macrophages, neutrophils or both, or in neurodegenerative disorders with apoptotic elimination of functional neurons. Or, within the frame of this review, pathological apoptosis may also result when the timely elimination of apoptosing cells by scavengers fails in vivo and secondary necrosis ensues.

The acquisition during evolution of the apoptotic cell death process [145], with the inherent mechanism for the elimination of cell corpses through removal by scavengers before damage to the cytoplasmic membrane, represented a crucial step forward for multicellular organisms because it avoids the potentially dangerous secondary necrosis. However, the normally efficient and non-pathogenic mechanism of assisted cell elimination associated with apoptosis can be overridden in vivo in pathological situations with the occurrence of secondary necrosis. The constraint associated with the process of removal by scavengers is that it is not self-sufficient: depending on a functional scavenger cell, that process fails if this cell is not

available despite exposition of engulfment signals, as is known to occur in several pathological situations to be reviewed below.

Failure of timely removal of apoptosing cells with occurrence of secondary necrosis may lead to two types of pathological consequences: (i) progress of secondary necrosis until cell disruption will result in leakage of cell contents which may be cytotoxic and induce tissue injury or (ii) secondary necrotic cells or their debris may be taken up by antigen presenting cells, mainly dendritic cells, and induce inflammation and autoimmunity. As discussed in “Apoptosis as the prelude of secondary necrosis” section, when macrophages fail to clear apoptosing cells, a second route can operate for the uptake using dendritic cells. Macrophages completely degrade ingested cells without antigen presentation, but when that uptake is by dendritic cells, these present antigens and activate the immune system, which may lead to autoimmunity [57, 58, 60, 146]. Phagosomes containing phagocytosed cells mature more rapidly in macrophage than in dendritic cells; in these cells, low levels of lysosomal proteases and a decreased ability to degrade internalized protein favors their ability to present antigen [69, 147].

Removal by scavengers of apoptotic secondary necrotic cells in vivo does not usually occur because those cells typically originate due to lack of functional scavengers or of molecules involved in the removal process. If engulfment does occur, for example because secondary necrosis resulted from delayed instead of absent clearance, or because dendritic cells replace the lacking macrophages as scavengers, the engulfment affects cells with damaged cytoplasmic membranes or even ruptured cells and involves mechanisms that would be similar to those operating with cells under active primary necrosis. It has been suggested that efficient removal of apoptosing cells versus deficient removal of primary or secondary necrotic cells depends on the presence of an intact or a damaged cytoplasmic membrane, respectively [77]. When injected into the peritoneal cavity of mice, cells under apoptotic secondary necrosis were found to be phagocytosed by macrophages [148]. Removal of secondary necrotic cells co-cultured with macrophages in vitro was found to be less efficient and slower than removal of apoptosing cells [77]. It is thus likely that, in the in vivo situations, if engulfment of cells under apoptotic secondary necrosis occurs, it would be a deficient process as in the case of primary necrotic cells. Moreover, as discussed in “Apoptosis as the prelude of secondary necrosis” section, phagocytosis of necrotic cells, in contrast to phagocytosis of apoptosing cells, usually results in pro-inflammatory and pro-immunogenic responses with potential to induce autoimmunity, as will be reviewed later.

Disruption of non-engulfed cells under apoptotic secondary necrosis may therefore ensue in vivo and it may

aggravate the consequences of the generalized, irreparable cytoplasmic membrane damage that occurs during the secondary necrotic process previous to cell rupture, because it causes the additional release of larger molecules with higher cytotoxic or immunogenic potential. Release of toxic molecules like elastase, myeloperoxidase and HMGB1 protein from cells under secondary necrosis has been demonstrated [149–154]. Although some reports suggest that phagocytosis by macrophages of secondary necrotic cells, like phagocytosis of early apoptosing cells, is not pro-inflammatory [59, 86, 87, 155], there are several publications showing that engulfment of secondary necrotic cells may be pro-inflammatory and immunogenic [60, 62, 129, 156–160]. The discrepant results may be explained by the use of secondary necrotic cells at early (non-ruptured cytoplasmic membrane) or terminal (ruptured cytoplasmic membrane) phases and/or by the use of different types of engulfing cells (macrophages, dendritic cells or other cell types).

As it will be reviewed in this section, the available data furnishes compelling evidence indicating that extensive or persistent *in vivo* secondary necrosis may have relevant pathogenic consequences.

Several mechanisms may lead to relevant *in vivo* secondary necrosis [76]. One regards situations of induction of excessive apoptosis that overwhelms the available scavenging capacity; this may occur in hyper-inflammation associated or not with infection. Other possibility is when the scavenging capacity is directly impaired by intrinsic defects in the phagocytic function of scavengers or by extrinsic defects in signaling molecules involved in the engulfment process; in this case accumulation of apoptotic cells and transition to secondary necrosis may occur even in situations of normal levels of apoptosis. Observation of high numbers of apoptosing cells in tissues implies a deficiency in their removal [7, 161, 162] with the likely occurrence of secondary necrosis. Examples of pathological situations generated by these different ways of induction of secondary necrosis will be now reviewed.

Deficiency in the clearance of apoptosing cells due to extensive apoptosis occurs particularly in solid organs [163]. Secondary necrosis of hepatocytes resulting in severe hepatitis has been described in rodents with extensive hepatocyte apoptosis induced by LPS [164], Fas antibody [138, 165], galactosamine [166] or carbon tetrachloride [137]. Apoptotic secondary necrosis was also reported in brains subjected to focal ischemia [167], in hepatic [112] and renal [168] ischemia-reperfusion, in acute myocardial infarction [169, 170], and in other heart pathologies [171].

Failure of removal of cells that entered an apoptotic process can also occur *in vivo* because of lack of exposure of engulfment signals when the ongoing apoptotic process

is interrupted by lack of activation of effector caspases before the caspase-dependent exposure of engulfment signals on the apoptosing cell [172, 173]. This interruption can be induced by impairment of energy metabolism [131–133], by defects due to genetic alterations or by generation of inhibitors in injured areas [173], and, as already discussed, leads to a switch to necrosis. These non-cleared, necrosing cells undergo lysis that may have pathogenic implications [174] as in the case of apoptotic secondary necrosis. The switch apoptosis–necrosis has been reported in several ischemic pathologies including in the brain [173–175] and heart [170, 176]. ATP dependence of apoptosis and (almost) independence of active necrosis [37, 40, 177] might explain the frequent appearance of necrotic and apoptotic cells in pathological areas *in vivo*, such as the center of solid tumors and ischemic regions in solid organs; indeed, limitation of blood flow in those areas leads to ATP exhaustion due to insufficient oxygenation and reduced glycolysis by lack of glucose [178] and may result in the switch apoptosis–necrosis.

In neurodegenerative disorders there is an increased rate of neuronal apoptosis [179]. As in other pathologies discussed above, this apoptosis may be blocked and switched to necrosis [173], again with possible pathogenic implications.

Secondary necrosis of monocytes/macrophages, eosinophils and neutrophils in acute and chronic inflammation

Tissue injury due to secondary necrosis of apoptosing cells may be particularly serious when it involves cells rich in cytotoxic molecules like monocytes/macrophages, eosinophils and mainly neutrophils, in situations of intense inflammation.

In the protective inflammatory response there is migration of neutrophils from the reserve pools to infectious and noninfectious inflammatory foci, where they accumulate in large numbers for *in situ* elimination of invading microorganisms or damaged tissues [180]. For these activities, neutrophils use phagocytosis and are stimulated to release effector molecules upon exposure to various cytokines and chemoattractants [181]. Neutrophils are very rich in inflammatory mediators and in proteases and oxidants which, if released in high amounts, can damage many types of cells, with the potential to produce tissue injury [182–184]. Released neutrophil contents also induce the secretion of pro-inflammatory cytokines and chemokines which may amplify leukocyte infiltration and associated tissue injury [185, 186]. Release of noxious neutrophil components may occur during neutrophil degranulation in inflammatory foci and after lysis due to apoptotic secondary necrosis.

Under normal conditions, neutrophil activities at inflammatory foci are tightly controlled. Mobilization of neutrophil cytoplasmic granules and secretory vesicles is regulated, minimizing the risk of externalization of excessive amounts of cytotoxic molecules [187]. Additionally, under physiological conditions, neutrophil cytotoxic proteases, including elastase, one of the most tissue destructive enzymes known [185], have their potentially dangerous activity tightly regulated by endogenous protease inhibitors [188]. Moreover, neutrophils have a short lifespan and are constitutively programmed to undergo apoptosis, which induces the exposure of engulfment signals and leads to their rapid recognition and removal by macrophages; this limits their pro-inflammatory and tissue injury potential and leads to resolution of inflammation with restitution of tissue homeostasis soon after the neutrophils have accomplished their task [76, 189, 190]. Pneumococcal pneumonia is a paradigm for complete resolution of inflammation without relevant subsequent lung injury indicating that, in some situations, extensive neutrophil accumulation can be adequately resolved [76]. Neutrophil apoptosis with timely removal of apoptosing neutrophils by lung macrophages was shown to be a mechanism for that resolution in a murine model of pneumococcal pneumonia [191]. However, this control of neutrophil activity and physiological resolution of inflammation by clearance of apoptotic neutrophils can fail and release of cytotoxic components by neutrophils accumulated at inflammatory foci can occur with the potential to produce collateral tissue damage; for instance, elimination of alveolar macrophages in the above model of pneumococcal pneumonia affected the clearance of accumulated neutrophils and resulted in extensive apoptotic secondary necrosis of neutrophils and in an exaggerated lung inflammation and increased lethality [191].

Several mechanisms can delay neutrophil apoptosis at inflammatory foci as has been observed in acute and chronic inflammatory diseases [192, 193]. This delay extends the time frame of neutrophil intervention but at the same time increases the chances of tissue injury. When accumulation of activated neutrophils at the inflammatory foci is massive, as is the case in several diseases, release of cytotoxic neutrophil molecules due to degranulation or lysis can be extensive [149, 150], overcoming the activity of the endogenous inhibitors [181, 188, 194]. Consequently, tissue injury ensues [195–197]. Removal by scavengers of accumulated apoptosing neutrophils can be deficient leading to neutrophil secondary necrosis. This occurs when neutrophil accumulation is massive and disproportionate compared to the availability of scavengers or when neutrophil apoptosis is accelerated by microbial products [198, 199]. Impairment of scavenger macrophages by bacterial products [31] or by functional defects (see later) also causes excessive neutrophil secondary necrosis.

The bypass of the balanced, physiological control of neutrophil pro-inflammatory activities due to extensive degranulation or secondary necrosis switches to pathogenic a beneficial defense mechanism. Neutrophil-mediated tissue injury can affect several organs like the liver [200, 201], the brain [202, 203], and the lung (see below).

A variety of severe infectious and noninfectious insults can result in the systemic inflammatory response syndrome (SIRS) [204]. Frequent complications of SIRS are the development of acute lung injury (ALI) and its more severe form acute respiratory distress syndrome (ARDS), and of multiple organ dysfunction syndrome (MODS) [205, 206]. Neutrophil accumulation has been associated with the above serious clinical situations [206], and released phagocyte proteinases have been implicated in the development of MODS [207].

Severe ALI with extensive accumulation of neutrophils can complicate human and experimental infection by the most virulent strains of influenza virus, including H1N1 [208] and H5N1 [209, 210]. However, no studies on the possible occurrence of neutrophil secondary necrosis in these infections have been conducted.

In patients with chronic lung inflammatory disorders like obstructive pulmonary disease (COPD) [211, 212], emphysema [213], chronic bronchitis [214], severe asthma [215, 216] or cystic fibrosis [217] there is accumulation of leukocytes including high numbers of neutrophils; there is evidence that these granulocytes release excessive amounts of proteinases including elastase [218, 219], a variety of inflammatory cytokines, and reactive oxygen species [212], which may cause airway tissue injury. Released proteinases overcome the activity of natural anti-proteases [194] and impair the anti-microbial activity of neutrophils [220]. Besides egression to the airway lumen [142, 143], an efficient mechanism for removal of neutrophils accumulated in the inflamed lung is through neutrophil apoptosis and removal by macrophages [76, 191]. Alveolar macrophages from patients with COPD are deficient in the capacity to engulf apoptotic cells [221], deficiency that is aggravated by smoking [222]. This clearance defect contributes to the accumulation of apoptotic cells in the lungs of COPD patients and in animal models of COPD [223], and the occurrence of pathogenic neutrophil apoptotic secondary necrosis associated with lung neutrophil accumulation has been considered in chronic lung inflammatory diseases including COPD and cystic fibrosis [186, 219, 220]. Secondary necrosis of neutrophils was also implicated in human [198, 199] and murine [224] lung infections by gram-negatives and in a mouse model of lung inflammation induced by nasal instillation of LPS [225, 226].

In accordance with a role of released neutrophil cytotoxic molecules in the above reviewed situations, the level of plasma neutrophil elastase or of products of elastase activity

were found to be significantly elevated in patients with SIRS and even more when this was complicated with ALI/ARDS [227, 228]. Active neutrophil elastase was also detected in increased levels in the blood of fish with massive neutrophil accumulation and neutrophil apoptotic secondary necrosis in advanced septicemic pasteurellosis [31].

Liu et al. [149] showed that neutrophils undergoing apoptotic secondary necrosis release active elastase with cytotoxic activity, indicating that this potent proteinase is not destroyed by the apoptotic and secondary necrotic proteolysis and directly demonstrating that neutrophil apoptotic secondary necrosis is prone to induce tissue damage. Moreover, topical or systemic administration of neutrophil elastase produced lung injury in rodents [185]. There is evidence in cystic fibrosis that neutrophil elastase, released by accumulated neutrophils possibly through apoptotic secondary necrosis, can impair apoptotic cell clearance [229] thus leading to a vicious circle that promotes further neutrophil secondary necrosis.

The effect of elastase inhibitors has been investigated in several situations with lung hyper inflammation, and some reports described beneficial results in patients with ALI/ARDS or in rodent models of ALI further supporting the pathogenic role of released elastase (see for example [230–232] and references herein).

Accumulation of eosinophils in airways occurs in patients with asthma or COPD (reviewed in Ref. [143]), and secondary necrosis of these leukocytes has been found in airways in patients with asthma [143] and in lung tissue of mouse models of the disease [233]. Eosinophil granules contain cytotoxic molecules [234] and apoptotic secondary necrosis of eosinophils was shown to induce inflammatory responses [156].

Although to a lesser degree as compared with neutrophils and eosinophils, monocytes/macrophages also contain tissue-damaging molecules including several proteases [235], which are released upon cell disruption due to secondary necrosis. Pathogenic macrophage secondary necrosis has been described in several situations including infection [31]. Besides inducing pro-inflammatory responses and tissue injury, macrophage secondary necrosis is a self-amplifying process with accumulation of apoptotic cells and further secondary necrosis because macrophages are the main scavenger cell [31].

Secondary necrosis in bacterial infection

Acute fish pasteurellosis is a lethal septicemic infection due to *Photobacterium damsela* ssp. *piscicida* (*Phdp*) and characterized by massive extracellular multiplication of the pathogen with extensive tissue necrosis [31]. One mechanism of the necrotic alterations typical of the advanced phase

of this septicemia is systemic secondary necrosis of accumulated neutrophils and macrophages induced by an apoptogenic exotoxin (AIP56) secreted by virulent *Phdp* [30, 31]. The AIP56-induced fish phagocyte apoptosis involves activation of caspase 8, 9 and 3, and mitochondrial dysfunction with loss of $\Delta\psi_m$ and cytochrome *c* translocation to the cytosol [30, 31, 236] (C. Costa-Ramos et al. unpublished results). That the AIP56-induced phagocyte apoptosis proceeds to secondary necrosis and cytolysis is directly demonstrated by the occurrence of lysing phagocytes with apoptotic nuclear alterations in advanced infection (Fig. 2c; see also Fig. 3b, c in Ref. [32]). The simultaneous apoptotic destruction of macrophages and neutrophils by AIP56 leads to reduction in the number of available phagocytes that accumulated during the initial phase of the infection and efficient evasion of the pathogen from the host phagocytic defense; moreover, that destruction seriously decreases the host capacity to clear apoptosing cells. This phagocyte destruction has additional pathogenic consequences for the host: AIP56 exotoxin secreted by *Phdp* in infected fish is systemically distributed and induces disseminated phagocyte apoptosis that proceeds to secondary necrosis with extensive lysis of the phagocytes and release of cytotoxic molecules including neutrophil elastase [31]. Two concurrent mechanisms operate in the induction of the secondary necrotic outcome of the apoptotic process induced in phagocytes by AIP56: first, the exotoxin-induced phagocyte apoptosis is extensive leading to the production of great numbers of apoptosing leukocytes and, most importantly, it affects the principal scavenger cell, the macrophage, thus impairing the capacity to clear apoptosing cells by a self-amplifying process. This is an example of cell elimination adding more pathogenic consequences to those associated with the loss of function accompanying cell death. The “clever” nature of this pathogenicity mechanism is that it uses the intrinsic apoptotic machinery of the host cells, which is put at work by the pathogen to its own advantage.

Extensive phagocyte secondary necrosis was also described in the skin of mice with experimental infection by *Mycobacterium ulcerans* [237]. This pathogen, agent of the human disease Buruli ulcer, secretes an apoptogenic exotoxin, mycolactone, which is a key virulence factor [238]. As in fish pasteurellosis, the secondary necrosis observed in *M. ulcerans* infection was considered as resulting from the mycolactone-induced apoptotic destruction of both neutrophils and macrophages and to contribute to the tissue necrosis typical of this mycobacteriosis [237].

Other acute and serious infections with necrotic lesions and destruction of host phagocytes have been reported. For example, septicemic plague is a rapidly fatal septicemia, with massive accumulations of extracellular *Yersinia pestis* and occurrence of apoptotic cell destruction with cell lysis and tissue necrosis [239]. The finding that in septicemic

plague, *Y. pestis* targets macrophages and neutrophils [240] suggests the possibility of occurrence of apoptotic secondary necrosis of phagocytes as in acute fish pasteurellosis, but such a possibility has not been assessed.

In a mouse model of lung infection by pyocyanin-producing *Pseudomonas aeruginosa* with extensive pulmonary accumulation of neutrophils and occurrence of neutrophil apoptotic secondary necrosis, a defect in the macrophage phagocytosis of apoptosing cells was recently described [224]; this defect was shown to be mediated by a non-apoptogenic effect of pyocyanin on macrophage phagocytic function.

In vivo studies on the role of apoptotic secondary necrosis of macrophages and neutrophils in the pathogenesis of infectious diseases have been neglected. New in vivo studies on bacteria-induced apoptotic phagocyte destruction may well reveal other situations where the pathogens use virulence mechanisms based on the induction of apoptotic secondary necrosis as in acute fish pasteurellosis and Buruli ulcer. In particular, infections caused by bacteria that, like *Phdp* and *M. ulcerans*, are known to produce apoptogenic molecules that target host phagocytes [241, 242], should be studied under this perspective.

Secondary necrosis in autoimmune diseases

Another possible consequence of in vivo occurrence of secondary necrosis due to increased rates of apoptosis [243, 244] and, mainly, failure of clearance of apoptotic cells is induction of autoimmune responses associated with disease. Several factors have been implicated in the pathogenesis of autoimmune disorders, including altered apoptosis [245]. Although autoimmunity may not follow impaired clearance of apoptosing cells [70, 246, 247], many studies show that such an impairment may result in immunogenicity and autoimmune disorders. There is a large body of evidence for the existence of a deficiency in clearance of apoptotic cells in autoimmune disorders including systemic lupus erythematosus (SLE), both in mouse models [61, 248] and in humans [58, 129, 244, 249–253]. Several genetically modified mice with defective apoptotic cell clearance have been developed and most of them show signs of autoimmune disease [52, 159, 254–256]. The defect in clearance of apoptosing cells in SLE and likely in other autoimmune disorders may have several mechanisms, including intrinsic impairment of engulfment capacity of phagocytes combined with extrinsic defects in bridging molecules involved in the recognition and engulfment of apoptosing cells by scavengers, like complement factors [61, 129, 253, 257, 258].

Apoptosis and secondary necrosis of neutrophils from blood of patients with SLE cultivated in vitro were found to

be increased as compared to neutrophils from controls; moreover, a poor ability of macrophages from patients with SLE to phagocytose apoptotic neutrophils was observed [251]. Although an alternative has been proposed [87], apoptotic secondary necrosis has been implicated in the pathogenesis of autoimmune disorders including SLE through the release of autoantigens and cytotoxic molecules which induce pro-inflammatory and autoimmune responses [48, 62, 129, 159, 244, 259].

One important molecule involved in cytolysis-associated pro-inflammatory situations is HMGB1 protein, a prototypical alarmin [260]. This non-histone nuclear protein with a role in transcriptional regulation, functions as a potent pro-inflammatory cytokine when released in the extracellular space [261]. This protein is released during primary necrosis [262] and, contrary to initial reports, was found to be also released during secondary necrosis of several cell types [11, 151–154] and has been considered to participate in the pathogenesis of autoimmune diseases [151, 152]. HMGB1 is a protein of about 30 kD, and its release during secondary necrosis follows a kinetics similar to that of LDH release [152, 154], suggesting that progress of secondary necrosis until cell lysis is required for its externalization (see in “Apoptotic secondary necrosis” section discussion on membrane damage in secondary necrosis).

Autoantigens cleaved during the execution phase of apoptosis [263] undergo an additional wave of caspase-independent proteolysis during secondary necrosis, with the production of lower molecular weight products [127]. If released during secondary necrosis, these products might induce inflammation and stimulate pathogenic autoimmune responses [127].

Nucleosomes produced during the apoptotic death process by internucleosomal DNA degradation can be released when secondary necrosis occurs [244]. Nucleosomes are present in increased amounts in the circulation of patients with SLE and are targets of anti-DNA and anti-histone autoantibodies (reviewed in Ref. [257]). Serum levels of nucleosomes were found to be increased in various autoimmune diseases besides SLE [139]; the fact that blood caspase levels were increased together with nucleosomes in those diseases, favors the interpretation that apoptotic secondary necrosis is involved in the release of nucleosomes. Autoantigens in SLE patients are composed of or are intimately associated with RNA and DNA sequences that are endogenous ligands, respectively, for toll-like receptor (TLR) 7 and/or TLR9 present in plasmacytoid dendritic cells and B lymphocytes, contributing to autoimmune disease [159, 264, 265]. TLR9 activation by DNA-containing immune complexes was found to require an interaction of the complexes with HMGB1 [265].

Conclusions

Active suicidal death programs are intrinsic to cells in unicellular and multicellular organisms. Activation of these programs not only kills cells but additionally promotes mechanisms for elimination of the dying/dead cells. In multicellular animals, cells under apoptotic death can be eliminated by two processes with contrasting physiopathological consequences. One is the safe, physiological clearance by phagocytosis of the dying/dead cell or of apoptotic bodies through the timely assistance of a partner cell (the scavenger); this intervention of the scavenger removes the apoptosing cells or the apoptotic bodies while they still have a near-to-intact cytoplasmic membrane thus preventing leakage of dangerous molecules. However, this physiologically convenient mechanism is not self-sufficient because it depends on the participation of other cell and can be overridden *in vivo* when the scavenging mechanism fails. In this situation cell elimination is by transition of full-blown apoptosis to secondary necrosis that leads to cytoplasmic membrane damage and cell disintegration. This has potential pathogenic consequences and available data provides evidence suggesting that secondary necrosis is implicated in the pathogenesis of several diseases including autoimmune and neurodegenerative disorders, ischemia, and infection. The pathogenic consequences of extensive or persistent apoptotic secondary necrosis result from (i) the leakage of cytotoxic molecules (mainly when involving neutrophils) that induce tissue injury and pro-inflammatory responses; (ii) the uptake by macrophages and dendritic cells of secondary necrotic cell debris and released autoantigens, which may be pro-inflammatory and immunogenic. Apoptotic secondary necrosis may therefore produce acute and chronic pathology, thus being a mechanism for apoptosis turning pathogenic. It is important to recognize that cell disruption due to a necrotic process can be an outcome of apoptosis *in vivo* so that pathological consequences of this outcome may be inhibitable by anti-apoptotic interventions, prompting the use of (and research on) therapeutic interventions based on anti-apoptotic drugs. Contrasting with the available extensive studies on apoptosis and on the clearance of apoptosing cells by scavengers, the study of apoptotic secondary necrosis has been largely neglected and is much needed to better understand the full potential of this outcome of apoptosis in promotion of pathology.

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