Chapter 8

Cytoskeleton Rearrangements during the Execution Phase of Apoptosis

Jesús Porcuna Doncel, Patricia de la Cruz Ojeda, Manuel Oropesa-Ávila, Marina Villanueva Paz, Isabel De Lavera, Mario De La Mata, Mónica Álvarez Córdoba, Raquel Luzón Hidalgo, Juan Miguel Suarez Rivero, David Cotán and José Antonio Sánchez-Alcázar

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Abstract

Apoptosis is a regulated energy-dependent process for the elimination of unnecessary or damaged cells during embryonic development, tissue homeostasis and many pathological conditions. Apoptosis is characterized by specific morphological and biochemical features in which caspase activation has a pivotal role. During apoptosis, cells undergo characteristic morphological reorganizations in which the cytoskeleton participates actively. Traditionally, this cytoskeleton rearrangement has been assigned mainly to actinomyosin ring contraction, with microtubule and intermediate filaments both reported to be depolymerized at early stages of apoptosis. However, recent results have shown that microtubules are reformed during the execution phase of apoptosis forming an apoptotic microtubule network (AMN). Current hypothesis proposes that AMN is required to maintain plasma membrane integrity and cell morphology during the execution phase of apoptosis. AMN disruption provokes apoptotic cell collapse, secondary necrosis and the subsequent release of toxic molecules which can damage surrounding cells and promote inflammation. Therefore, AMN formation in physiological or pathological apoptosis is essential for tissue homeostasis.

Keywords: microtubules, actin, intermediate filaments, apoptosis, apoptotic microtubule network

1. Introduction

The term apoptosis refers to the process of programmed cell death characterized by a stereotypic sequence of cellular events including cell shrinkage, caspase activation and degradation

of cell content, blebbing and maintenance of plasma membrane integrity and condensation and fragmentation of DNA, followed by ordered removal by phagocytes [1]. Apoptosis was first described in 1972 as a vital biological phenomenon, with both physiological and pathological implications [2]. Apoptosis regulates cell number in tissues serving as a quality control mechanism in order to eliminate damaged and senescent cells, and it has been proven to be essential during development of multicellular organisms. In the proliferative environment of embryonic development, apoptosis helps to shape organs, drives morphogenesis and deletes structures that won't be required any longer; for instance, the formation of the four-chamber architecture of the heart is a consequence of this process [3].

Traditionally, the process of apoptosis occurs in three distinct phases: induction, execution and clearance. The first one comprises all the intrinsic or extrinsic environmental changes that lead to the activation of the apoptotic cascade. Meanwhile, the execution phase is distinguished by the activation of a caspase-dependent proteolytic cascade [4]. Caspases are aspartic acid-specific proteases responsible for cellular components degradation. Some of them, such as caspase-8 and -9 act as initiators of the apoptotic signalling pathway, while other caspases such as caspases-3, -6 and -7 operate as executor caspases which actively participate in the degradation of intracellular proteins [5]. Eventually, the dying cell is engulfed by professional phagocytes or by neighbouring cells. This process of apoptotic cell clearance is essential for tissue turnover and homeostasis [6].

The fate of apoptotic cells in multicellular animals is their prompt elimination by professional phagocytes. However, cells that perform apoptosis in vitro cultures progress to secondary necrosis, which implies the loss of membrane integrity and the release of cellular content into the culturing medium [7]. In vivo, apoptotic cells can also undergo secondary necrosis when they are not properly eliminated due to massive cell death or impaired phagocytosis [8].

Efficient apoptotic cell removal is driven by the interaction with phagocytes through the expression of “eat-me” signals, the release of “find-me” signals, engulfment of the dying cell and its eventual digestion in phagocyte phagolysosomes. This interaction prevents undesired immune reactions by contributing to the development of an immunomodulatory environment [9]. On the other hand, secondary necrosis is thought to be pro-inflammatory and immunogenic, as it causes the release of endogenous damage-associated molecular patterns (DAMPs) [8]. Among DAMPs are proteolytically processed autoantigens, nucleosomes, proteases, calcium-binding protein (calgranulin), high-mobility group box 1 (HMGB-1) and urate crystals.

2. Cytoskeleton rearrangements during the execution phase of apoptosis

Cell contraction, plasma membrane blebbing, chromatin condensation and DNA fragmentation are typical hallmarks of the execution phase of apoptosis, which lasts approximately 1 h [10]. In order to achieve such dramatic morphologic changes, apoptotic cells make profound cytoskeleton reorganizations. On the other hand, caspase-mediated cytoskeleton proteins digestion ensures the proper dismantlement of the dying cell [11].

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The eukaryotic cytoskeleton is mainly composed of actin filaments, microtubules and intermediate filaments. These three constituents act coordinately to increase tensile strength, allow cell motility, maintain plasma integrity, participate in cell division, contribute to cell morphology and provide a network for cellular transport [12]. It is widely accepted that actin cytoskeleton plays a central role in cell remodelling during the execution phase of apoptosis [13], while microtubules and intermediate filaments are disorganized at the onset of this phase [10]. However, recent work have demonstrated that microtubules are reorganized at later stages of apoptosis, giving rise to the formation of the apoptotic microtubule network (AMN) that

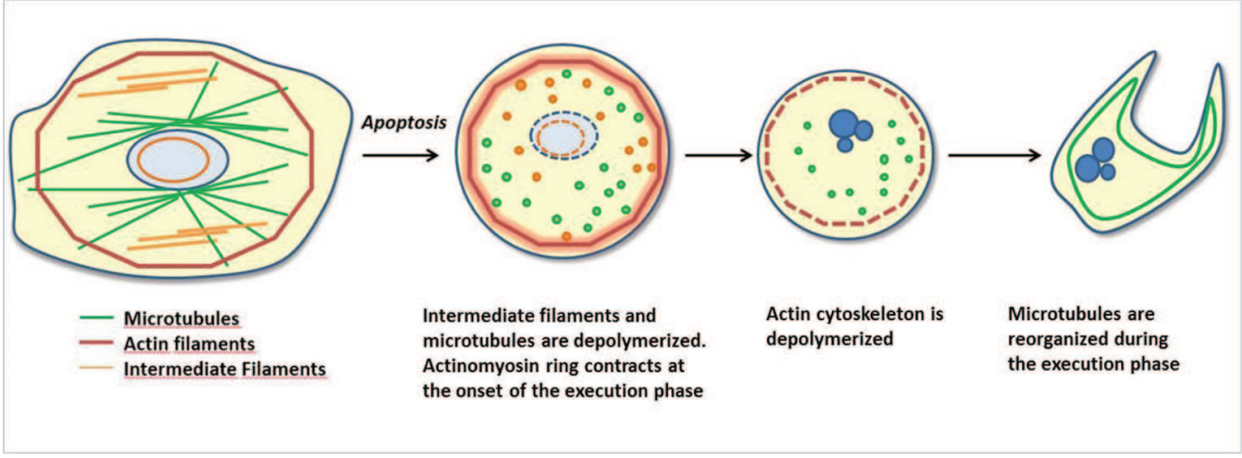

Figure 1. Cytoskeleton rearrangements during the execution phase of apoptosis.

contributes to the maintenance of the plasma membrane integrity [14, 15]. All these events are summarized in **Figure 1**.

Intermediate filaments are ubiquitous cytoskeletal components of 10 nm of diameter which provide mechanical strength and allow tissue growing among other functions. According to amino acid sequence identity, intermediate filaments can be classified into six types. Acidic keratins belong to type I, whereas basic keratins belong to type II intermediate filaments. They are typical components of hair and epithelium. Type III includes vimentin, desmin, glial fibrillary acidic protein and perinephrin. Nuclear lamins A and B, which support the cell nucleus, are represented by type V. Finally, type VI refers to nestin [16]. Intermediate filaments connect with other cytoskeletal components via cytolinker proteins of the plakin family, including desmoplakin, periplakin and plectin [17]. At the onset of the execution phase of apoptosis, type I keratins are targeted by caspases-3, -7 and -6 at their linker domain, whereas type II keratins are resistant to caspase-mediated proteolysis. Similarly, type III intermediate filaments, such as desmin or vimentin, are cleave by caspases. Once cleaved, intermediate filament subunits accumulate in the cytoplasm forming large aggregates. All these events contribute to cytoskeleton reorganizations [18]. Furthermore, caspase digestion of K18 (type I) has been proved to be indispensable for membrane integrity maintenance during apoptosis, as interference with keratin caspase cleavage shunts hepatocytes towards necrosis [19]. Keratins are not only caspase substrates but they also seem to regulate the induction phase of apoptosis.

Thus, it has been shown that deficiencies in keratins 8 and 18 favour tumour necrosis factor (TNF)/cycloheximide-induced cell death. Keratin 18 sequesters the adaptor molecule TNF receptor-associated death domain (TRADD) and thus prevents its interaction with the TNF receptor, regulating negatively apoptosis. In contrast, keratin type II K8 offers protection against Fas-mediated apoptosis.

With respect to the digestion of nuclear lamins, nuclear breakdown begins with the activation of caspases. Lamins appear to be specifically targeted by caspases 3 and 6 that become activated both via the intrinsic and extrinsic pathway of apoptosis [20]. However, little is known about the exact mechanism by which the cell nucleus dismantles. It has been established that active caspases are able to proteolyze lamins A and B, leading to lamina cleavage and chromatin condensation and fragmentation. These alterations contribute to the collapse of the nucleoskeleton [18].

Microtubules are depolymerized at the same time that intermediate filaments although the exact molecular mechanism involved is still unknown. Several hypotheses have been postulated to explain this process, which are not mutually exclusive [21]. Microtubules are polar protofilaments made up of α and β tubulin, which are involved in cell migration, growth, transport or mitosis [22]. Their dynamics is governed by microtubule-associated proteins (MAPs), Ran-GTP and proteins that bind to tubulin [22–24]. The cyclin-dependent kinase 1 Cdk1, associated with cyclin B is a key regulatory kinase which controls the entry in mitosis and regulates microtubule dynamics. In fact, it induces the depolymerization of interphase microtubules [25]. Cdk1 regulates some microtubule effectors such us MAP4, which reduces its ability to stabilize microtubules after phosphorylation [26]. In addition, Cdk1 is able to phosphorylate β-tubulin, thus inhibiting its incorporation to growing protofilaments. As Cdk1 and other Cdks activities have been observed during apoptosis, it has been suggested that they may act essential regulators in the apoptotic cytoskeleton reorganizations during apoptosis [27]. On the other hand, some authors have shown that microtubules depolymerization is associated with activation of the PP2A-like phosphatase, dephosphorylation of the microtubule regulator τ protein and deacetylation of tubulin [28]. This last mechanism can coexist with the hypothesis of Cdk1 regulation because PP2A downregulates the Cdk1 activator Cdc25 phosphatase [29].

In contrast to the apparent passivity of intermediate filaments and microtubules, the actin cytoskeleton is highly dynamic, and its remodelling turns out to be essential in the first stages of apoptosis. Actin filaments function in the generation and maintenance of cell morphology and polarity, endocytosis, intracellular trafficking, contractility, motility, cell division and apoptosis [30]. Actin is a 42-KDa globular protein (G-actin) which polymerizes to form actin filaments (F-actin). They adapt to the cell environment through actin-binding proteins (ABPs) which regulate actin cytoskeleton dynamics. A family of RhoGTPases are in charge of controlling ABPs in such a way that actin is organized into highly ordered structures such as stress fibres, lamellipodia and filopodia in non-apoptotic cells [13].

Once adherent cells have initiated apoptosis, they partially detach from the substrate by losing their focal adhesion sites. This is achieved by caspase-mediated cleavage of the focal adhesion kinase pp125, among other proteins [31]. Next, actin is reorganized into an actin-myosin II

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cortical ring with contractile force. Actinomyosin contraction is activated via the Rho/Rho-kinase (ROCK) signalling pathway which ends up with the phosphorylation of myosin light chain II (MLC-II) [32]. Rho-kinases are effectors of Rho-GTPase proteins, being RhoA and RhoC the most well characterized ROCK regulators [33]. Active GTP-bound Rho proteins activate ROCK by binding to their C-terminal portion of the coiled coil. Then, it induces actinomyosin contraction through two distinct mechanisms. First, it can increase the phosphorylation state of the MLCs by inhibiting the MLC phosphate or by directly phosphorylating MLC-II [32]. Alternatively, ROCK I but not ROCK II can be cleaved by caspase-3 at a conserved DETD1113/G sequence and its carboxy-terminal inhibitory domain is consequently removed [34]. The contractile force generated depends on other ROCK targets, such as the LIM kinase (a serine/threonine kinase containing LIM and PDZ domains), which phosphorylates and inactivates the actin stabilizer cofilin [35]. Cortical ring contraction results in the formation of membrane protrusions known as blebs. Their formation depends on a pressure gradient between the extracellular medium and the intracellular medium, taking place in areas of external negative pressure or in places where the plasma membrane is weakened as a consequence of caspase cleavage [11]. Thus, blebbing appears to be also dependent on the activation of ROCK by active caspases because it can be blocked by Y27632, a ROCK selective inhibitor. However, C3 toxin-induced inhibition of Rho is unable to block the formation of blebs [36].

Actinomyosin ring contraction is a fast process and coincides with the beginning of the execution phase. After that, cell content including organelles is packaged into apoptotic bodies and the actin cytoskeleton is dismantled by caspases [32]. Rho GTPases effectors contribute to this new cytoskeleton reorganization [11]. The Rho effector protein kinase C-related kinase (PRK1) is cleaved by caspase-3 generating a constitutively active kinase fragment that is able to induce actin structures disassembly [37, 38]. Similarly, the Rac effector p21-activated kinase (PAK2) may promote stress fibres dissolution after caspase cleavage [39]. Likewise, caspase-3 induces gelsolin fragmentation, contributing to the collapse of actin filaments in a calcium-independent manner [40].

At this time of the execution phase, apoptotic cells lack the main structured elements of cytoskeleton. It is then, when microtubules reorganize to give rise to the AMN [21]. Its organization, maintenance and properties will be reviewed in the next sections.

3. Influence of apoptotic cells on tissue remodelling

In tissues, apoptotic cells interact with their neighbouring partners and eventually must be eliminated. For example, in epithelia apoptotic cells are removed from the tissue by cell extrusion. Dying cell ejection is usually done apically, although it has been described that cells also extrude basally during Droshophila development. This coordinated process is necessary for tissue homeostasis and developmental morphogenesis [41]. In 2001, Rosenblatt et al. proposed a model to describe the sequence of events during epithelia extrusion [42]. They demonstrated that an actinomyosin ring is formed both in the apoptotic and in the neighbouring non-apoptotic cells. Apoptotic cells were proposed to send an undetermined early signal to the adjacent epithelial cells to induce the formation of an actinomyosin cable ring as well as the

activation of small RhoGTPases. Recently, spinsohine-1-phosphate receptor 2 pathway has been proposed as the mediator between apoptotic and non-apoptotic cells [43]. Contraction of the cable ring extrudes the now late apoptotic cell out of the epithelium. In other words, during apoptosis within epithelia, dying cells are able to not only rearrange their cytoskeleton but also induce actinomyosin reorganizations within adjacent cells. Importantly, apoptotic cell membranes do not permeabilize until cell extrusion is completed [44].

The mechanical force produced during apoptosis is used not only to extrude dying cells from tissues but also to change the morphology of neighbouring cells to fill the space originally occupied by the dying cell [45]. This finding suggests that apoptotic forces might be harnessed throughout cell death-related morphogenesis. Mechanical forces arising from the apoptotic process had been originally proposed as an "apoptotic force theory" [46] that would be important during animal development including elimination of interdigital webs, dorsal closure or leg folding [46, 47]. Therefore, apoptosis should not be seen as a passive carving process. Instead, it is a generator of mechanical forces and an active player during tissue remodelling that helps to the correct morphogenesis of embryos and control of tissue dynamics [47].

4. Apoptotic microtubule network

The reorganization of microtubules during the execution phase of apoptosis has been examined in a variety of cell lines such as H460, A431, HeLa cells, primary human fibroblasts and pig LLCPK-1α cells, under several apoptosis inducers such as camptothecin (CPT), anisomycin, staurosporine, serum withdrawal, UV irradiation and TNF-related apoptosis-inducing ligand (TRAIL). In addition, apoptotic microtubules have also been observed in cell fragments and apoptotic bodies [48, 49]. These findings suggest that AMN may play an important role during the apoptotic process.

Commonly, AMN is arranged beneath plasma membrane, adopting a cortical structure that gives a "cocoon-like" structure which confines most of the intracellular content of apoptotic cells. Furthermore, apoptotic microtubules may extend from the body of apoptotic cells as long and thin spikes, suggesting a key structural role in maintaining apoptotic cell morphology and surface extensions (**Figure 2**). Apoptotic microtubules organization beneath plasma membrane also suggests that AMN may function as a kind of support to preserve plasma membrane integrity and/or as a barrier for confining the degradation reactions inside apoptotic cells.

Apart from plasma membrane protection, another function of apoptotic microtubules has been associated with the process of apoptotic body formation by helping to sustain the peripheral localization of chromatin within surface blebs and by facilitating cell fragmentation [48].

To exclude the possibility that AMN could be an artefact of the process of fixation in the immunofluorescence protocol, apoptotic microtubules formation has been also monitored in vivo in pig epithelial (LLCPK-1α) expressing GFP-tubulin (Green fluorescent protein-tubulin) and A431 cells expressing YFP-tubulin (Yellow fluorescent protein-tubulin) by live imaging [48,

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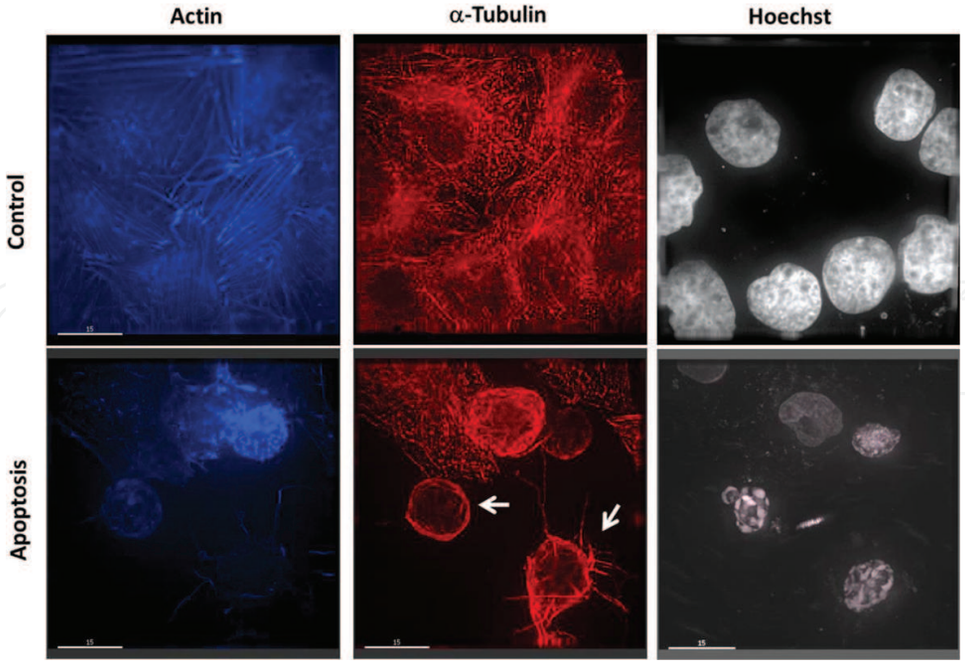

Figure 2. Immunofluorescence microscopy image of control and apoptotic cells in the execution phase. Apoptosis was induced in H460 cells by 10 μM camptothecin treatment for 48 hours. After fixation, control and apoptotic cells were stained with anti α-tubulin (red), and coumarin-phalloidin to visualize actin filaments (blue). Nuclei were revealed by Hoechst staining (blue). Arrows, apoptotic cells with AMN. Bar = 15 μM.

49]. In control interphase cells, microtubules are arranged in long fibres that fill the entire cytosol, growing from a central microtubule organizing centre (MTOC) corresponding to the likely position of the centrosome. In cells undergoing apoptosis, this radial network disappears and is replaced by a cortical arrangement of microtubules corresponding to the AMN observed by immunofluorescence of fixed cells. Initially, interphase microtubules are depolymerized while cells rounded up in the early stages of the execution phase of apoptosis. However, microtubules are soon reorganized beneath plasma membrane with a characteristic cortical localization.

Under physiological conditions, cytoskeleton proteins support plasma membrane integrity. Therefore, changes in the cytoskeletal components beneath plasma membrane can increase membrane permeability. During the execution phase of apoptosis, both the cell cortical actin network and intermediate filaments which support plasma membrane become depolymerized. Therefore, apoptotic microtubules are the only remaining cytoskeletal component supporting plasma membrane and cell shape during apoptosis. The organization of AMN beneath plasma membrane suggests that tubulin repolymerization in the execution phase of apoptosis may have a protective role, helping to maintain plasma membrane integrity and thus, delaying the transition to secondary necrosis. In fact, AMN is present in all genuinely apoptotic cells but is disrupted in cells undergoing secondary necrosis [49]. Furthermore, AMN disorganization by a short treatment with colchicine, an inhibitor of tubulin polymerization, increases cell permeability and the release of cell content into the culturing medium. In addition to a purely supporting role, AMN disorganization by colchicine treatment may also facilitate the access of

caspases to essential proteins localized in plasma membrane and cellular cortex such as calcium channels and fodrin (α II-spectrin) whose cleavage could induce ionic imbalance, cellular collapse and eventually secondary necrosis [50].

5. AMN formation

As mentioned above, formation of AMN is a biphasic process: first, during the early phase of apoptosis, interphase microtubules rapidly depolymerized, but soon after actin and intermediate filaments disassemble they are reorganized in extensive bundles of closely packed new tubulin polymers. The initial microtubule depolymerization phase correlate with the loss of centrosomal γ-tubulin, suggesting that the two events may be interconnected [48]. The mechanisms involved in centrosome disorganization remain unknown. One hypothesis is that pericentriolar proteins can be cleaved by active caspases, but to our knowledge, none of these proteins has been identified as caspase targets [51, 52]. Interestingly, it has been demonstrated that dynein, a microtubule motor protein, is essential for the centrosomal localization of pericentrin and γ-tubulin in living cells [53]. Cytoplasmic dynein function is abolished by caspase cleavage during the execution phase [54]. Therefore, an alternative hypothesis is that dynein hydrolysis reduces the content of pericentrin and γ-tubulin at the centrosome, thereby impairing its capacity to nucleate microtubules.

On the other hand, the mechanism of microtubules reassembly in the execution phase remains uncertain. Although the core centrioles remain essentially intact throughout apoptosis, they are unlikely to direct the formation of the novel apoptotic microtubule array, because they are not assembled with a radial pattern, and instead appear randomly throughout the peripheral cytoplasm. Furthermore, apoptotic microtubules assembly takes place in the absence of γ-tubulin ring complex, suggesting that AMN formation is produced by another unknown mechanism [48, 49]. Although they are tightly packed, apoptotic microtubules are dynamic—assessed by tracking the plus-end protein EB1 by time-lapse imaging [48]—indicating that their polymerization is regulated.

It has been postulated that active caspases may cleave the C-terminal regulatory regions of tubulins which increases their ability to polymerize and thus facilitate the formation of apoptotic microtubules [52, 55]. However, AMN reorganization during the execution phase of apoptosis has been also observed in the presence of caspase inhibitors [49].

In another approach, Jon Lane's group has described that active GTP-bound Ran is necessary for apoptotic microtubule polymerization, and that RanGTP release into the apoptotic cytoplasm triggers microtubule nucleation [56]. They showed that RanGTP-activated spindle assembly factor, TPX2 (targeting protein for Xklp2), escapes from the nucleus during the execution phase and associates with apoptotic microtubule bundles [57]. Furthermore, silencing of TPX2 expression by siRNA impairs apoptotic microtubule polymerization. They propose that apoptotic microtubule polymerization shares several common characteristics with mitotic and meiotic spindle assembly, with a particular dependence upon RanGTP and TPX2 [56]. These findings suggest that apoptotic cells utilize the RanGTPase pathway to promote the

reorganization of apoptotic microtubules. In another study, the examination of apoptotic microtubules components has showed that in addition to the expected tubulin subunits, they bind other microtubule-associated proteins (MAP) such as MAP-4. These findings may be interesting for elucidating the role of MAPs in AMN nucleation. Given previous evidences associating MAP4 with microtubule nucleation and stabilization [58, 59], this protein may participate in AMN formation and maintenance during apoptosis.

Although AMN lacks the morphological and functional accuracy of the mitotic/meiotic spindle apparatus, it nevertheless represents a model of regulated non-centrosomal microtubule polymerization, and further accentuates how apoptosis should be viewed as regulated process of cellular death.

6. Apoptotic microtubules delimit an active-caspase–free area in the cellular cortex

AMN indeed may work as physical barrier impeding active caspases to access into the cellular cortex where it can cleave critical proteins involved in plasma membrane integrity [60]. AMN disorganization in apoptotic cells by colchicine, a microtubule depolymerizing agent, allowed caspase-mediated cleavage of plasma membrane and cell cortex and proteins such as focal adhesion kinase (FAK), E-cadherin, α-spectrin, paxilin, Na^+/Ca^{2+} exchanger (NCX), plasma membrane Ca^{2+} ATPase-4 (PMCA-4), β4 integrin and Na^+/K^+ pump subunit β. This caspase-mediated proteolysis was associated with increase cell permeability, calcium and sodium overload and bioenergetics failure that eventually led to secondary necrosis [50]. The essential role of caspase-mediated cleavage of plasma membrane and cortical proteins in plasma membrane permeabilization was demonstrated because the concomitant addition of colchicine and Z-VAD, a pan-caspase inhibitor, blocked protein cleavage and significantly reduced plasma membrane permeability and secondary necrosis.

7. Apoptotic cells with AMN enhance phosphatidylserine exposure and interactions with macrophages

Clearance of apoptotic cells by phagocytes (or efferocytosis) can be divided into four distinct processes: aggregation of phagocytes near apoptotic cells, recognition of apoptotic cells by cell surface bridge molecules and receptors, engulfment of apoptotic cells, and degradation of apoptotic cells within phagocytes [61]. The elimination of apoptotic cells by macrophages reduces the probability of inflammation by ensuring that apoptotic cells are eliminated before the release of intracellular contents into de extracellular medium [62, 63]. Apoptotic cells are recognized by phagocytosis through the externalization of phosphatidylserine in the outer leaflet of the plasma membrane [64]. Phosphatidylserine translocation is an early event of apoptosis, occurring while the plasma membrane remains intact and cells exclude membrane-impermeant dyes [65]. Phosphatidylserine exposure has been reported to be a caspase and energy-dependent process [66, 67], but its mechanism is not completely understood. It has

been proposed that a combined effect of activation of a lipid scramblase and downregulation of a phospholipid translocase activity may contribute to phosphatidylserine exposure [68].

In agreement with a role of apoptotic microtubules for proper phosphatidylserine translocation, it has been shown that apoptotic cells with AMN show indeed high expression of phosphatidylserine on the cell surface and increased phagocytosis rate. However, both processes were markedly reduced when AMN was depolymerized by colchicine treatment [60]. Interestingly, phosphatidylserine externalization and phagocytosis of apoptotic cells were restored when AMN was depolymerized in the presence of Z-VAD, suggesting that caspase-dependent degradation of plasma membrane and cellular cortex proteins impairs proper phosphatidylserine externalization and apoptotic cell removal by macrophages. These findings

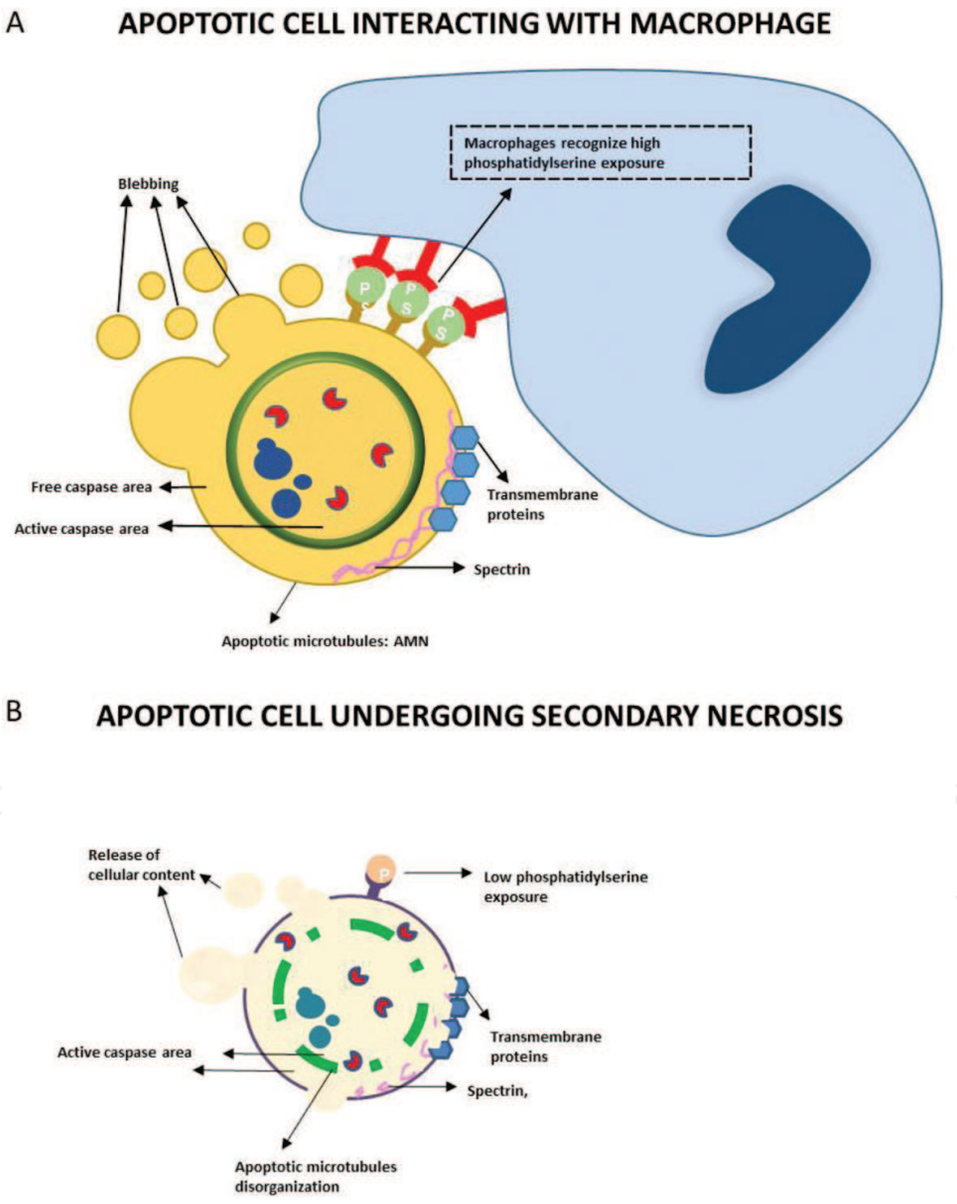

Figure 3. Scheme summarizing the main findings on AMN during the execution phase of apoptosis. (A) Apoptotic cell interacting with macrophage (B) secondary necrotic cell. PS = Phosphatidylserine.

corroborate previous observations showing that after AMN disorganization (by nocodazole treatment) the percentage of macrophages making contacts and engulfing apoptotic cells was significantly reduced compared to apoptotic cells with AMN [48]. The ability of apoptotic cells to stimulate their phagocytosis by macrophages before cell lysis is crucial to prevent the adverse effects (tissue damage and inflammation) associated with secondary necrosis [69] (**Figure 3**).

8. Apoptotic microtubules organization and maintenance depend on high cellular ATP levels and energized mitochondria

Microtubule polymerization is an energy-dependent process because β-tubulin hydrolyzes GTP during polymerization [70]. Therefore, it has been proposed that AMN formation depends on the bioenergetic status of apoptotic cells [71].

ATP levels must be kept high in apoptosis to allow all the energy-dependent processes occurring during the execution phase including AMN formation and maintenance. Thus, in vivo and in vitro experiments have shown that AMN was visualized predominantly in apoptotic cells with polarized/hyperpolarized mitochondria and, on the contrary, was dismantle in apoptotic cells with depolarized mitochondria. These observations suggest that AMN depends on energized mitochondria and high ATP levels [71]. Kinetics examination in pig LLCPK-1α cells expressing GFP-tubulin also showed that AMN was maintained during the execution phase of apoptosis until mitochondria depolarization marked the onset of secondary necrosis. Furthermore, mitochondria depolarization by treatments with uncouplers of mitochondrial oxidative phosphorylation (FCCP) or mitochondrial inhibitors (antimycin, rotenone and oligomycin) induced AMN disassembly associated with enhanced plasma membrane permeability. However, inhibition of glycolysis by 2-deoxyglucose treatment had no effect on mitochondrial polarization or either AMN organization or plasma membrane permeability. In contrast, stabilization of apoptotic microtubules by taxol prevented both mitochondrial depolarization and plasma membrane permeabilization. AMN stabilization also prevented the increased plasma membrane permeability when mitochondria were depolarized by rotenone or FCCP treatment. These results underline the essential role of AMN in plasma membrane integrity during apoptosis.

9. Zombie cells: Stabilization of apoptotic cells

Taken into account that apoptotic cells maintain the integrity of plasma membrane and cellular cortex proteins [60], an innovative method aimed to the temporal stabilization and preservation of apoptotic cells has been developed [72]. This method consists in the treatment of apoptotic cells with a cocktail of taxol, Zn^{2+} and coenzyme Q_{10} (CoQ). This experimental approach has been reported to prevent secondary necrosis for at least 96 h in cell cultures. The rationale for using this stabilizing cocktail is (a) taxol, a microtubule stabilizing agent, prevents AMN depolymerization and the access of active caspases into cellular cortex [73, 74];

(b) Zn^{2+}, a caspase inhibitor, prevents caspase-dependent cleavage of cellular cortex and plasma membrane proteins [75–78]; and (c) CoQ, an antioxidant, that protects against oxidative membrane damage which is increased in apoptotic cells [79].

Stabilized apoptotic cells can be considered as dying cells in which the cellular cortex and plasma membrane are intact or alive. Metaphorically, they can be considered as "living dead" or "zombie cells". Stabilized apoptotic cells retain many of the hallmarks characteristic of apoptotic cells such as cellular cortex and plasma membrane integrity, low intracellular calcium levels, plasma membrane potential, high phosphatidylserine exposure and the ability of being engulfed by phagocytes.

Recently, interest in apoptosis research has increased remarkably for a number of reasons including the technological development of cell cultures and the expansion of new therapeutic strategies. Furthermore, apoptotic cell quantification plays an important role in biomedicine because it is widely used to evaluate the cytotoxic effects of drugs [80]. However, apoptosis determination is often affected by the process of cell manipulation (harvesting, cell centrifugation, cell pipetting…), especially in adherent cell cultures, required for flow cytometry assays. Very often, these manipulations disrupt plasma membrane permeability and leads apoptotic cells to secondary necrosis [81]. As a consequence, reliable apoptosis quantifications are particularly difficult in adherent cell cultures. Stabilization of apoptotic cells before cell harvesting may allow a more accurate and reliable quantification of the actual number of apoptotic cells or the correct determination of biochemical parameters such as mitochondrial membrane potential, intracellular calcium concentration, pH or caspase activity in genuine apoptotic cells.

Currently, apoptotic cells are used for various forms of therapy, especially with the objective of promoting immunological tolerance in recipient individuals [82]. Therefore, stabilization of apoptotic cells before their administration to patients may ensure that apoptotic cells will retain their characteristic features until they are removed by macrophages. The administration of stabilized apoptotic cells can also be of interest for the delivery of proteins (for protein replacement therapy) or drugs to recipient macrophages [83].

There are forms of cell death which by their nature impair the correct formation of AMN (e.g.mitochondrial toxics and cold exposure) [71] and, as a result, apoptotic cells are not able to maintain plasma membrane integrity. Therefore, apoptotic cell stabilization may provide a new approach for preventing the adverse effects of early secondary necrosis.

10. Conclusion

Microtubule cytoskeleton is reformed during the execution phase of apoptosis forming an AMN. AMN is required to maintain plasma membrane integrity and cell morphology during the execution phase of apoptosis. AMN disruption leads cells to secondary necrosis and the release of toxic molecules which can damage neighbour cells. Therefore, AMN formation, preservation or stabilization in apoptosis is essential for tissue homeostasis preventing cell damage and inflammation.

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The authors submitted a patent application describing the stabilization of apoptotic cells.

Author details

Jesús Porcuna Doncel, Patricia de la Cruz Ojeda, Manuel Oropesa-Ávila, Marina Villanueva Paz, Isabel De Lavera, Mario De La Mata, Mónica Álvarez Córdoba, Raquel Luzón Hidalgo, Juan Miguel Suarez Rivero, David Cotán and José Antonio Sánchez-Alcázar*

*Address all correspondence to: jasanalc@upo.es

Andalusian Centre for Developmental Biology, CSIC, University Pablo de Olavide, Seville, Spain

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