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The Apoptotic Microtubule Network During the Execution Phase of Apoptosis

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Abstract

Apoptosis is a regulated energy-dependent process of cell death characterized by specific morphological and biochemical features in which caspase activation has a central role. During apoptosis, cells undergo characteristic morphological rearrangements in which the cytoskeleton participates actively. From a historical point of view, this reorganization 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 the microtubule cytoskeleton is reformed during the execution phase of apoptosis, forming an apoptotic microtubule network (AMN). AMN is closely associated with the plasma membrane, forming a cortical ring or cellular “cocoon.” Apoptotic microtubules’ reorganization has been reported in many cell types and under many apoptotic inducers. Recently, it has been proposed that AMN is essential for preserving plasma membrane permeability and cell morphology during the execution phase of apoptosis. Apoptotic microtubules’ depolymerization leads cells to secondary necrosis and the release of toxic intracellular contents that can harm surrounding cells and initiate inflammation. Therefore, microtubules’ reorganization in physiological apoptosis during development and in the adult organism or in pathological apoptosis induced by anticancer treatments or chronic inflammation is essential for tissue homeostasis, preventing cell damage and inflammation.

Keywords: apoptosis, microtubules, cytoskeleton

1. Introduction

1.1. Apoptosis

Apoptosis is an intracellular signaling pathway conserved across evolution dependent on a caspase-mediated proteolytic cascade that leads to programmed cell death through a series of cellular changes distinct of cell necrosis. Apoptosis plays a critical role in tissue remodeling during development, tissue homeostasis, cleaning of senescent cells, and removal of the cells with severe DNA damage. Given that cell necrosis causes the release of toxic molecules and causes inflammation, an important function of apoptosis is to isolate specific cells and prepare them for disposal by phagocytosis. This program of cell death is carried out by organelle-directed regulators including the Bcl-2 proteins and ultimately executed by proteases of the caspase family.

The apoptotic process can be divided into three functionally distinct phases : (a) induction – cellular environmental changes that result in the activation of intracellular apoptotic mechanisms (entry into the execution phase); (b) execution – processes that result in the degradation of intracellular components by a family of proteases called caspases which cleave a variety of important structural and regulatory proteins at conserved aspartic acid residues to alter their function irreversibly; and (c) cleaning – those events associated with the removal of apoptotic cells and cellular debris by “professional” phagocytes such as macrophages, immature dendritic cells, and neutrophils.

2. Apoptosis and secondary necrosis

In contrast to apoptotic cells *in vivo*, cells that perform apoptosis *in vitro* cell cultures are not removed by phagocytes and suffer a late process of secondary necrosis, which is defined as the loss of membrane integrity and release of cell contents into the extracellular space. Secondary necrosis *in vivo* will take place in the presence of massive apoptosis that overloads the ability of available macrophages as well as deficiencies in the number of macrophages and/or saturation of the immune system as seen in chronic inflammation. Secondary necrosis is the natural result of apoptosis in unicellular eukaryotes but can also occur in multicellular organisms and is involved in the genesis of many human pathologies.

Elimination of apoptotic cells by phagocytosis instead of progressing to necrosis secondary has major advantages to multicellular organisms. These advantages include increased speed for cell elimination, increased degradation of cellular component and a most cell-energy-efficient reuse of the components of engulfed apoptotic cells, allowing proper recycling of molecules. Furthermore, and most significantly, apoptotic cell elimination by phagocytosis when the apoptotic cell is still wrapped by a plasma membrane integrated allows that cell disintegration including membrane permeabilization takes place in the secure compartment of the macrophage phagolysosome. This mechanism prevents secondary necrosis, which is potentially pathological through the release of partially degraded cellular components such

as "damage-associated molecular patterns" (DAMPs) that are pro-inflammatory and immunogenic. Among DAMPs are proteases, nucleosomes, proteolytically processed autoantigens, calcium-binding protein (calgranulin), high mobility group box 1 (HMGB-1), and urate crystals. The cells in secondary necrosis may also be phagocytosed by macrophages *in vivo* following different mechanisms that have been reviewed recently. The removal process of apoptotic cells and cells undergoing secondary necrosis are not identical and generally they have several consequences in terms of the inflammatory and immunogenic responses. Apoptotic clearance *in vivo* includes the sensing of corpses via "find me" signals, the recognition of corpses via "eat me" signals and their cognate receptors, the signaling pathways that regulate cytoskeletal rearrangement necessary for engulfment and the responses of the phagocytes.

While typically the removal of apoptotic cells is anti-inflammatory and "immunologically silent", phagocytosis of cells in secondary necrosis is pro-inflammatory and immunogenic and thus represents another mechanism by which secondary necrosis generates pathogenic consequences. Secondary necrosis, therefore, can produce acute and chronic diseases and it has been recently implicated in a growing number of clinical situations that occur with acute and chronic inflammation including many autoimmune alterations, ischemia, atherosclerosis, chronic obstructive pulmonary disease (COPD), pulmonary inflammation associated with oxidative stress in smokers, cystic fibrosis, asthma, bronchioestasis, and infections. On the other hand, secondary necrosis that affects tumors has recently gained prominence because of its recognition as a process with beneficial implications in anticancer therapies by promoting the activation of the immune system response and consequently the disposal of tumor cells. Chemotherapy and radiotherapy generally act by the induction of apoptosis in tumor cells. It has been demonstrated that after treatment with ionizing radiation or chemotherapeutic agents, cancer cells became highly immunogenic when administrated into immunocompetent mice. Several observations suggest that such immunogenicity is associated with progression of apoptotic cells to secondary necrosis and release of DAMPs molecules.

3. Reorganization of the cytoskeleton during apoptosis

The execution phase of apoptosis lasts approximately one hour and is characterized by typical morphological features: cell shrinkage, plasma membrane "blebbing," chromatin condensation, and DNA and cells fragmentation. To perform these dramatic morphological changes that accompany the execution phase of apoptosis, apoptotic cells make a series of profound changes (breaks and rearrangements) in the cell cytoskeleton.

The cytoskeleton is made up of three main types of filamentous proteins, actin filaments, intermediate filaments, and microtubules, that assemble into higher-order polymers in healthy cells and coordinately act to increase tensile strength, allow cell motility, maintain cell morphology, participate in cell division, and provide platforms for positioning and transport of cellular components.

Previous experiments have shown that actinomyosin cytoskeleton plays an essential role in cellular remodeling during the early events of the execution phase of apoptosis while micro-

tubules and intermediate filaments are disorganized. However, some researchers have demonstrated the reorganization of microtubules in apoptotic cells at late stages. This rearrangement occurs during the execution phase of apoptosis, playing a key role in maintaining the integrity of the plasma membrane and dispersion of cellular and nuclear fragments.

Basically, cytoskeletal rearrangements during apoptosis can be summarized as in Figure 1. Initially, actinomyosin ring contraction is activated via phosphorylation of myosin light chain (MLC) II. MLC phosphorylation is under the control of the Myosin Light Chain kinase (MLCK), ROCK (Rho-associated coiled-coil-forming protein) kinases and is regulated by MLC phosphatase (MLCP). It has been discovered that the ROCK kinases actively phosphorylate a large cohort of actin-binding proteins and intermediate filament proteins to modulate their functions. ROCK kinases activates MLC by direct phosphorylation and by inhibiting the activity of MLCP. This movement of the actinomyosin contractile ring is facilitated by the early disruption of microtubules and intermediate filaments, which allows full contraction of the ring and the formation of protrusions of plasma membrane ("blebs"). Subsequently, actin cytoskeleton is depolymerized and, coinciding with the absence of an organized structure of the various elements of the cell cytoskeleton, apoptotic microtubules are repolymerized close to the cytosolic side of the plasma membrane.

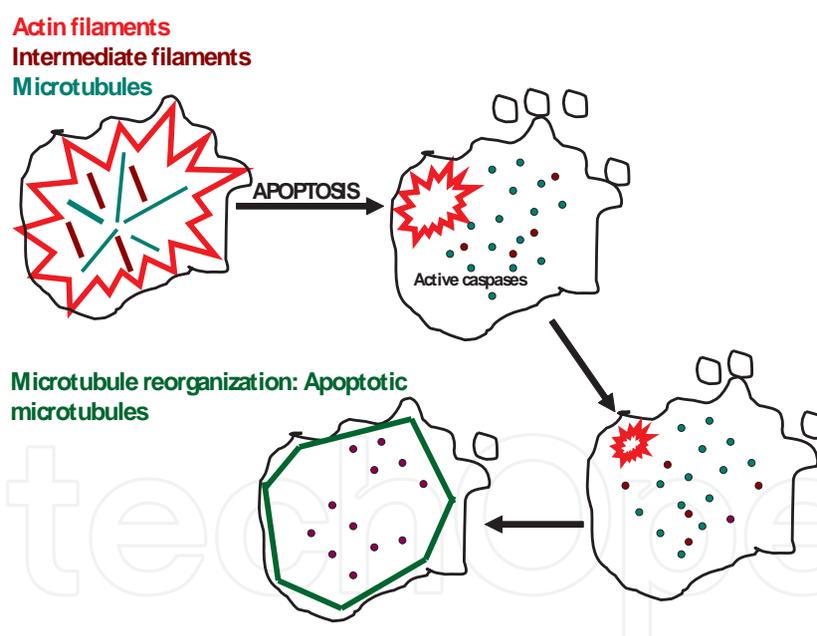


Figure 1. Reorganization of the cytoskeleton during apoptosis.

During the execution phase of apoptosis, ROCK I is cleaved by caspase-3 at a conserved DETD1113/G sequence and its carboxy-terminal inhibitory domain is removed, resulting in deregulated and constitutive kinase activity that is necessary and sufficient for actinomyosin ring contraction.

MLC phosphorylation is also regulated by the calcium-dependent MLCK calmodulin. Thus, an increase in calcium may also activate actinomyosin ring contraction. In the ring contraction

activated by ROCK kinases not only MLCK is involved but also LIM kinase through phosphorylation and inactivation of cofilin, which stabilizes actin polymers.

After actinomyosin ring contraction, actin filaments are depolymerized by the action of active caspases. The Rho effector protein kinase C-related kinase (PRK) 1 is cut by active caspases leading to a constitutively active kinase fragment and after that, PRK1 can induce depolymerization of actin filaments. Likewise, the kinase p21-activated kinase (PAK) 2, a Rac effector, can be activated by caspases and active PAK has been demonstrated to induce stress fibers disassembly. Furthermore, caspases can cleave gelsolin which depolymerizes the actin cytoskeleton in a Ca^{2+} -independent manner.

Intermediate filaments that help maintain the integrity of tissues and cells are disrupted at the onset of apoptosis by the action of caspases. The intermediate filament cleavage causes fragmentation and aggregation and the breaking of the nuclear lamins facilitates nuclear disintegration. Cleavage occurs at a conserved location within the rod domain, causing loss of filament integrity and disorganization of the nuclear and cytoplasmic intermediate filaments networks. Caspase cleavage of intermediate filaments is important for the timely execution of apoptosis as evidenced by the delay incurred when caspase-insensitive forms of lamins and desmin are overexpressed. Equally interesting, the caspase 6-mediated cleavage of lamin A/C is required for complete chromatin condensation during apoptosis. The early onset and efficient cleavage of intermediate filaments proteins may be fostered by physical proximity as key effectors of the apoptotic machinery, including procaspase 3, bind cytoplasmic intermediate filaments.

After microtubules, actin, and intermediate filaments' depolymerization, apoptotic cells are devoid of the structured elements of the cytoskeleton. Then, microtubules are reorganized leading to the formation of the apoptotic microtubule network (AMN), which becomes the only element of the cytoskeleton during the execution phase of apoptosis (Figure 2).

The molecular mechanism involved in the early microtubule depolymerization during the execution phase of apoptosis is unknown although several hypotheses have been postulated. Microtubule dynamics is governed by several effectors, microtubule-associated proteins (MAPs), motor proteins such as kinesin, gradients Ran-GTP+ends proteins and proteins that bind to tubulin. These microtubule-associated proteins are in turn under the control of phosphatases and kinases. One of these regulatory kinases CDK1 is associated with cyclin B, a key enzyme for the entry into mitosis and essential for mitotic spindle formation. One of the activities ascribed to CDK1 is the interphase microtubule depolymerization at the beginning of mitosis. Among CDK1 substrates are numerous microtubular effectors such as MAP4 and XMAP215 that when are phosphorylated their ability to stabilize microtubules may be reduced. Furthermore, CDK1 can directly catalyze tubulin β phosphorylation, preventing its incorporation to microtubules. During apoptosis, increased CDK1 activity and other CDKs have been detected, suggesting that they could act as important regulators in the modifications of the microtubule cytoskeleton during the apoptotic process and the formation of AMN. CDK1 activity may also be responsible for actinomyosin ring contraction at the onset of apoptosis since previous studies have shown it can activate MLC kinase by phosphorylation. However, in PC12 cells, tubulin depolymerization at the onset of apoptosis has been associated

with tubulin deacetylation, activation of a PP2A-like phosphatase and dephosphorylation of Tau protein. However, both hypotheses are not mutually exclusive and that PP2A may regulate a cdc25 phosphatase which in turn dephosphorylates and activates CDKs.

4. Apoptotic microtubules during the execution phase of apoptosis

AMN formation in the execution phase of apoptosis has been reported in many cell lines (H460, A431, HeLa cells, primary human fibroblasts, and pig LLCPK-1 α cells) and in response to a variety of apoptotic inducers such as camptothecin (CPT), staurosporine, anisomycin, UV irradiation, TRAIL (TNF-related apoptosis-inducing ligand), and serum withdrawal. In addition, AMN has also been observed in enucleated cells and apoptotic bodies. These findings suggest that AMN is a critical player in the genetically programmed process of apoptosis.

Topologically, AMN is organized in the proximity of the plasma membrane, forming a cortical structure that gives a typical "cocoon" like form, which delimits most of the intracellular contents including the fragmented nuclei and active caspases. Furthermore, apoptotic microtubules extend from the body of the cell as slender spikes, suggesting its structural role in maintaining the cell morphology during the execution phase of apoptosis. This spatial organization of the AMN might give some clues about its functional role during the execution phase of apoptosis. This arrangement also suggests a kind of cortical barrier or cocoon-like structure that may act to preserve plasma membrane integrity and/or as a structural barrier for the degradation reactions inside the cell. Other functions of apoptotic microtubules have 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.

5. AMN visualization by live cell imaging

To exclude the possibility that the structures of the AMN could be due to artifacts during the fixation in the immunofluorescence microscopy protocol, AMN formation has been also studied by live cell imaging in pig epithelial cells (LLCPK-1 α) expressing GFP-tubulin and A431 cells expressing YFP-tubulin. In untreated interphase cells, microtubules are organized in long tubular polymers found throughout the cytoplasm growing from a central microtubule organizing center (MTOC) that corresponds to the localization of the centrosome. In cells undergoing apoptosis, this radial network organization is no longer preserved but is replaced by a cortical rearrangement of microtubules bundles enclosing the whole intracellular compartment corresponding to the AMN previously showed in fixed cells by immunofluorescence microscopy. Time-lapse imaging studies showed that the interphase microtubule network was depolymerized as the cells rounded-up in the early stages of the execution phase of apoptosis. However, microtubules were soon reorganized beneath plasma membrane as cells began retracting, forming the characteristic cortical cocoon-like structure of the AMN.

Under physiological conditions the cytoskeleton aids to maintain plasma membrane integrity. Changes to the cytoskeletal network could therefore alter membrane permeability. During the

execution phase of apoptosis, the cortical actin network and intermediate filaments, which normally give support to plasma membrane, become depolymerized. In this situation, tubulin repolymerization that forms the AMN would be the only cytoskeletal component present in the apoptotic cell for preserving both plasma membrane integrity and cell shape. The organization of the AMN beneath plasma membrane surrounding the whole cellular volume suggests that tubulin reorganization in the execution phase of apoptosis might have a protective role helping to maintain plasma membrane integrity and thus delaying the transition to secondary necrosis. In fact, it has been reported that AMN was observed in all genuine apoptotic cells but was disrupted in cells in secondary necrosis. Furthermore, colchicine treatment, which disrupts apoptotic microtubules, increases cell permeability and the release of the intracellular marker lactate dehydrogenase (LDH). In addition to a purely structural role, AMN disorganization by colchicine treatment might facilitate the caspase cleavage of important proteins localized in the plasma membrane such as calcium channels and cellular cortex proteins such as fodrin (spectrin α II), which could in turn accelerate secondary necrosis by inducing ionic disturbances and cellular breakdown.

6. AMN formation

As mention above, formation of AMN is a biphasic process: first, during the early phase, interphase microtubules rapidly depolymerized but these were soon replaced by extensive bundles of closely packed, new tubulin polymers after actin and intermediate filaments are disassembled.

The microtubule depolymerization phase correlated with the loss of peripheral centrosomal γ -tubulin, suggesting that the two events may be linked. Notably, although the core centrioles remain essentially intact throughout apoptosis, they are unlikely to direct the formation of the novel apoptotic microtubule array because this is not assembled with radial pattern and instead appears randomly throughout the peripheral cytoplasm. The mechanisms responsible for centrosome disruption and indeed for initial microtubule disassembly remain undetermined. One possibility is that certain pericentriolar proteins are cleaved by caspases but to our knowledge none has been identified as a caspase target. Interestingly, it has been demonstrated that the minus-end-directed motor cytoplasmic dynein is essential for the centrosomal localization of pericentrin and γ -tubulin in healthy cells. Cytoplasmic dynein function is arrested during the execution phase by caspase cleavage of the intermediate chains. Therefore, one possible explanation is that this reduces the concentration of pericentrin and γ -tubulin at the centrosome, thereby abrogating its capacity to nucleate microtubules.

What stimulates the reassembly of microtubules in the execution phase remains uncertain. AMN assembly takes place in the absence of γ -tubulin discrete complex, suggesting that the formation of AMN is produced by other unknown mechanism.

Although they are tightly bundled, apoptotic microtubules remain dynamic – as judged by time-lapse imaging of the plus-end tracking protein EB1 – suggesting that their assembly is regulated. AMN reorganization of the execution phase of apoptosis occurs even in the presence

of caspase inhibitors. However, 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.

Jon Lane's group has reported that active, GTP-bound Ran is indeed required to support apoptotic microtubule assembly and that release of RanGTP into the apoptotic cytoplasm serves as a trigger for microtubule nucleation. They showed that the RanGTP-activated spindle-assembly factor, TPX2 (targeting protein for Xklp2), escapes from the nucleus during the execution phase and associates with apoptotic microtubule bundles. Consequently, silencing TPX2 expression by siRNA abrogates apoptotic microtubule assembly. They propose that formation of the apoptotic microtubule array shares several features in common with mitotic and meiotic spindle assembly with a particular dependence upon RanGTP and the microtubule-binding protein TPX2. Together, these observations suggest that, like mitotic and meiotic cells, apoptotic cells utilize the RanGTPase pathway to stimulate the coordinated assembly of a specialized microtubule network. Although AMN lacks the morphological and functional precision of the spindle apparatus, it nevertheless represents an important example of regulated, non-centrosomal microtubule assembly and organization and further highlights that the apoptotic execution phase should be recognized as a dynamic, tightly controlled process of cellular demise.

In another approach, the study of apoptotic microtubules components has revealed that in addition to the expected alpha and beta tubulin subunits, they intensively recruit 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 evidence linking MAP4 with microtubule nucleation, bundling, and stabilization, this protein could play an important role in AMN formation and maintenance.

7. Intracellular calcium chelation disrupts AMN and increases the permeability of plasma membrane

The elevation of intracellular calcium plays a pivotal role in the induction of the biochemical processes that characterize the execution phase of apoptosis. Among these changes, an early translocation of phosphatidylserine to the outer leaflet of the cellular membrane seems to be a key step in apoptosis, which has been shown to depend on caspase-3 activity and cytosolic Ca^{2+} concentration. The resulting exposure of phosphatidylserine in the plasma membrane surface may serve as a "eat me" signal that triggers phagocytosis by macrophages.

Intracellular calcium levels are slightly elevated in genuine apoptotic cells with AMN. This slight elevation of intracellular calcium may play a role in the nucleation of the AMN because treatment with EGTA-AM that causes intracellular calcium chelation impairs AMN formation. These observations are consistent with a model whereby a slight increase in intracellular calcium, favored by the absence of actin and intermediate filament networks, triggers the reorganization of apoptotic microtubules in the execution phase of apoptosis.

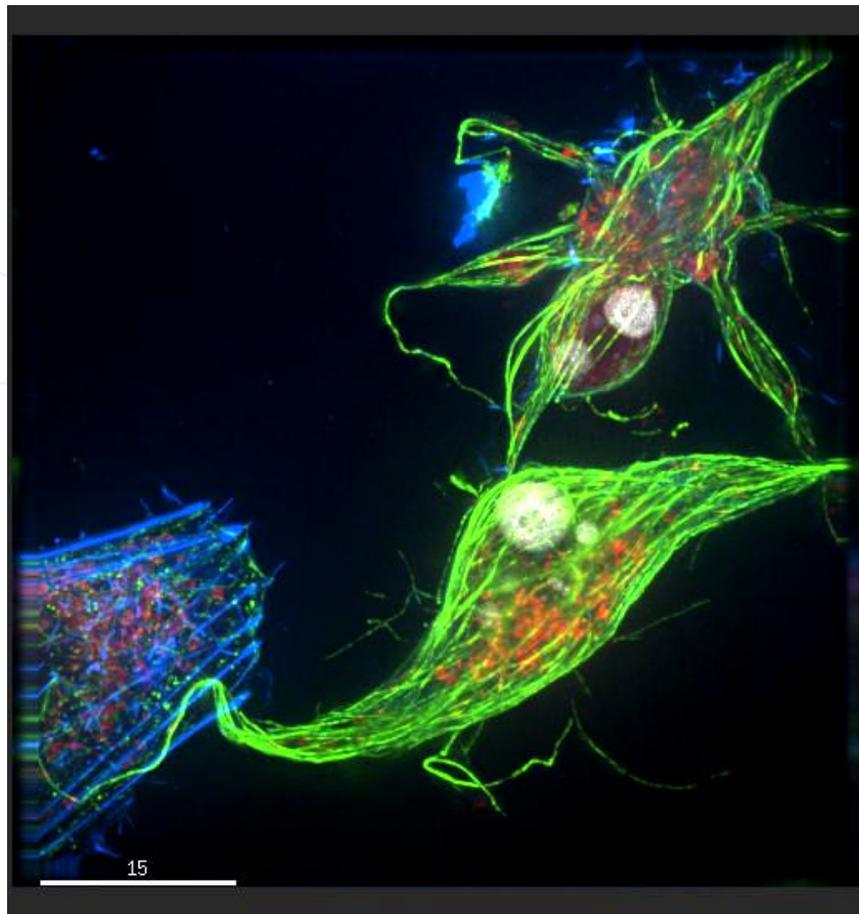


Figure 2. Human lung cancer apoptotic cells, H460: Green: anti-tubulin, AMN. Blue: actin. Red: Mitotracker, mitochondria; White: Hoechst, nuclei.

8. AMN disruption increases the permeability of plasma membrane

Given the critical role of apoptotic microtubules to maintain apoptotic cell integrity, any physical or chemical interference with AMN formation or stability might influence the process of genuine apoptosis and can induce a sort of derailed apoptosis with the release of toxic intracellular compounds that can have many pathological consequences in the context of multicellular organisms. Thus, as previously mentioned, the incubation of apoptotic cells with colchicine, an agent that depolymerizes microtubules, causes AMN depolymerization and increases plasma membrane permeability. In contrast, microtubules stabilization by taxol prevents both AMN disorganization and plasma membrane permeability.

Hypothermia or cold storage is widely used to protect cells and tissues against injurious processes. However, cold exposure can be deleterious for apoptotic cells given that microtubules which are essential to prevent plasma membrane permeability and secondary necrosis are cold labile. In contrast, AMN stabilization may preserve apoptotic cell integrity and prevents secondary necrosis.

Microtubules result from the polymerization of tubulin dimers in protofilaments that associate through lateral contacts. Microtubules are dynamic structures alternating growing and shrinking phases ended by catastrophes and rescues, respectively. *In vitro*, microtubule dynamics are under the control of the tubulin concentration and numerous other physico-chemical parameters. Among them, temperature plays a crucial role as microtubules depolymerize upon a temperature shift from 37°C to 4°C. This could be due to the modification of different dynamics parameters, especially the increase of catastrophe and the disappearance of rescue events at such temperatures. Because low temperatures depolymerize microtubules, cold exposition of apoptotic cells and re-warming to 37°C leads to AMN disorganization and secondary necrosis. In contrast, apoptotic microtubules' stabilization by taxol prevents AMN disruption and secondary necrosis after cold/warming exposition.

Furthermore, apoptotic microtubules' depolymerization by cold/warming exposure was associated with less-efficient removal of dead cells and increased production of pro-inflammatory cytokines by macrophages. Taken together, these results indicate that temperature has an essential role for the correct execution of the apoptotic process. Furthermore, cold exposition of apoptotic cells impairs the proper phosphatidylserine externalization and interaction with macrophages, indicating that temperature is also critical for the efficient clearance of apoptotic cells. Consistent with a role of AMN for proper phosphatidylserine exposure, phagocytosis of apoptotic cells with stabilized microtubules by taxol coincided with high phosphatidylserine externalization while it was reduced when apoptotic microtubules were absent by cold/warming exposure and phosphatidylserine externalization was low, confirming previous results published by Moss *et al.* (2006). These data also suggest that the induction of secondary necrosis in apoptotic cells by cold/warming exposure is capable of inducing inflammation through the increased production of pro-inflammatory cytokines such as IL-1 β and TNF- α .

Therefore, cold or hypothermia although often used as a means of reducing cellular alterations or injury during periods of storage or transport of biological material, may induce secondary necrosis in cells already undergoing apoptosis.

These findings can be relevant in order to preserve apoptotic cells by cold storage and avoid the toxic and pro-inflammatory events induced by secondary necrosis.

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

AMN indeed may work as physical barrier impeding active caspases to access and cleave critical proteins in the cellular cortex and plasma membrane, which are essential for plasma membrane integrity (Figure 3). AMN disorganization in apoptotic cells by a short incubation with colchicine allowed caspase-mediated cleavage of cell cortex and plasma membrane proteins such as α -spectrin, paxilin, Focal Adhesion Kinase (FAK), E-cadherin, plasma membrane Ca²⁺ ATPase-4 (PMCA-4), Na⁺/Ca²⁺ exchanger (NCX), integrin β 4, and Na⁺/K⁺ pump subunit β . These events were associated with increased cell permeability, calcium and sodium influx, and bioenergetics collapse, which in turn precipitate secondary necrosis. The essential

role of caspase-mediated cleavage of cortical and plasma membrane proteins after AMN disassembly was confirmed because the addition of both colchicine and Z-VAD (a pan-caspase inhibitor) blocked protein cleavage and significantly prevented plasma membrane permeability, cell detachment, LDH release, calcium and sodium overload, and bioenergetics failure.

Under physiological conditions, plasma membrane cytoskeleton supports plasma membrane. The significance of this supporting cytoskeleton network has been mainly demonstrated in erythrocytes in which deficiencies or defects in the cytoskeletal proteins spectrin or spectrin-associated proteins were associated with increased fragility and lysis of plasma membrane. Changes in the cytoskeletal network beneath the plasma membrane (as α -spectrin cleavage by caspases) may contribute to increase membrane permeability during the transition to secondary necrosis. Thus, spectrin-deficient spherocytes had decreased membrane mechanical stability, which probably contributes to cell lysis. It is thought that the spectrin skeleton acts universally to support the otherwise mechanically vulnerable cell surface bilayer. One way that membrane skeleton/bilayer interactions have been demonstrated is through the physiology of mechano-susceptible ion channels (channel whose gating is altered by abnormally high bilayer tension). These initially unresponsive channels become progressively more mechano-responsive under stretch and chemical reagents damage of the plasma membrane skeleton. The conclusion of these studies is that the intact membrane skeleton is mechano-protective and its perturbation may increase the ion permeability of the plasma membrane.

Likewise, focal adhesions are large, dynamic protein complexes through which the cytoskeleton of a cell interacts with the extracellular matrix. When cells adhere to the extracellular matrix, integrin receptors initiate signals to recruit more integrins and other cytoskeleton proteins (such as talin, tensin, vinculin, zyxin, and actinin), adapters (such as paxillin, Crk-associate substrate (p130CAS)1, and Crk), and kinases (such as FAK and Src) to their cytoplasmic tails, forming a "focal adhesion complex". Focal adhesions provide not only mechanical support to cells through the connection with the actin cytoskeleton and mechanically couple the cell with the extracellular matrix but also signals necessary for anchorage-dependent cellular responses such as proliferation, migration, and inhibition of anoikis, a type of apoptosis induced by cell detachment. Integrins, heterodimers formed by one beta and one alpha subunits, interact with extracellular proteins via short amino acid sequences, such as the RGD (found in proteins such as fibronectin, laminin, or vitronectin) or the DGEA and GFOGER found in collagen.

Hydrolysis of "focal adhesion complex" proteins by caspases after AMN depolymerization could break the membrane-cytoskeleton linkage and decrease the physical support leading to cell detachment. It has been reported that FAK, integrin β 4, and paxilin are cleaved in apoptotic cells when AMN was disorganized by colchicine treatment. Furthermore, this cleavage is a caspase-dependent process because it was blocked by z-VAD. These results suggest that disruption of FAK, integrin β 4, and paxilin may contribute to cell detachment and the morphological changes observed in apoptotic cells undergoing secondary necrosis.

In agreement with these results, it has been demonstrated that E-cadherins are also cellular cortex proteins targeted by caspases when AMN is depolymerized. Cadherins are transmembrane glycoproteins involved in cell-cell adherence. Recent developments indicate that

classical cadherins may act as adherence-activated signaling receptors. Previously, it has been showed that cadherins are also targeted during apoptosis. Specific cell–cell and cell–matrix contacts regulate cell growth in epithelial cells and disruption of these contacts induces apoptotic cell death. According to these observations, caspase-mediated cleavage of E-cadherins and the loss of cell–cell contacts are likely to represent an important process during the extrusion of apoptotic cells undergoing secondary necrosis.

The Na⁺/K⁺-ATPase (Na⁺/K⁺-pump) acts as an electrogenic ion transporter in the plasma membrane whose primary role is to maintain high intracellular K⁺ and low intracellular Na⁺ concentrations. Each cycle of Na⁺/K⁺-ATPase enzyme pumps three Na⁺ ions out of the cell, moves two K⁺ ions into the cell, and uses 1 ATP. Dysfunction of the Na⁺/K⁺-pump results in depletion of intracellular K⁺, accumulation of intracellular Na⁺ and, consequently, leads to membrane depolarization. Secondary, Na⁺/K⁺-pump failure increases intracellular free Ca²⁺ ([Ca²⁺]_i) due to activation of voltage-gated Ca²⁺ channels and reversed operation of the Na⁺-Ca²⁺ exchanger (NCX). Previous studies have shown that the intracellular Na⁺ concentration increases prior to a loss of plasma membrane integrity and that cell shrinkage in apoptotic Jurkat cells is accompanied by a net efflux of ions due to an inactivation of Na⁺/K⁺-ATPase. The latter authors also observed that Na⁺/K⁺-ATPase subunits were degraded in populations with reduced volume during apoptosis. Because active cellular volume regulation requires Na⁺/K⁺-ATPase activity, both events may act synergistically in the induction of cell shrinkage. The Na⁺-K⁺-ATPase is composed of two subunits. The α-subunit (~113 kD) is the catalytic subunit that binds ATP and both sodium and potassium ions and also contains the phosphorylation site. On the other hand, the smaller β-subunit (~35 kDa glycoprotein) is absolutely essential in facilitating the plasma membrane localization and α-subunit activation. Studies on purified enzyme also suggested that both subunits were essential for activity because any efforts to separate α and β resulted in inactive enzyme. It has been shown that Na⁺/K⁺-ATPase β-subunits but not α-subunits were cleaved by caspases after AMN depolymerization, which may contribute to ionic imbalance and increase plasma membrane permeability. Degradation of Na⁺/K⁺-ATPase β-subunit associated with mitochondria and plasma membrane depolarization during apoptosis has been previously reported. Consistent with this hypothesis, sodium levels were notably increased after AMN depolymerization by colchicine and partially restored when AMN was disorganized but caspases were blocked by z-VAD.

Changes in cytosolic calcium have an essential role during apoptosis by triggering the activation of Ca²⁺-dependent processes thereby inducing global intracellular and morphological modifications including phosphatidylserine externalization. However, AMN disruption by colchicine allows the caspase-mediated cleavage of key proteins involved in calcium extrusion such as PMCA-4 and NCX and, consequently, provokes calcium overload. PMCA are vital caspase substrates for the regulated subprogram leading to secondary necrosis. Cells expressing PMCA-4 mutants that lack the caspase cleavage site(s) prevent calcium influx during apoptosis and notably delay secondary necrosis. Furthermore, the Na⁺/Ca²⁺ transporter (NCX) also participates in calcium efflux in addition to PMCA. NCX has a low calcium affinity but high calcium transporting activity, which is required to rapidly eject large amounts of calcium. While the NCX contribution in regulating resting cytosolic calcium may be less

important than that of PMCAs, its function may avoid calcium overload in cells undergoing apoptosis. It has been reported that both PMC-4 and NCX are cleaved by caspases when AMN is depolymerized by colchicine treatment and suggest that inactivation of plasma membrane calcium transporters is a relevant process leading apoptotic cells to secondary necrosis.

Like many other insults, increased cytosolic calcium can trigger either apoptosis or necrosis. The final result of cell death is probably controlled by the concentration of cytoplasmic calcium. Whereas low to moderate calcium levels (200–400 nM) induces apoptosis, higher concentration of calcium (>1 μ M) is associated with necrosis. This may help to understand why an initial slight calcium increase is pro-apoptotic and favors AMN formation whereas the late calcium influx through the plasma membrane that cannot be expelled out of the cell is associated with secondary necrosis. Calcium overload can induce: 1) activation of calpains leading to more extensive disruption of the protein components of cytoskeleton, 2) activation of calcium-dependent phospholipases causing liberation of arachidonic acid and formation of lysophosphatides that alter membrane structures and disruption of membrane permeability with ensuing secondary necrosis and/or 3) Mitochondrial permeabilization and bioenergetics collapse.

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

Phagocytic clearance of apoptotic cells or efferocytosis consists of four main distinct steps: accumulation of professional phagocytes at the site where apoptotic cells are located, recognition of apoptotic cells through a number of binding molecules and receptors, engulfment by a unique uptake process and digestion of engulfed cells within phagocytes. The efficient phagocytosis of apoptotic cells by macrophages reduces the potential for an inflammatory response by ensuring that the dying cells are eliminated before their intracellular contents are released to the extracellular medium. Early apoptotic cells are targeted for phagocytosis through the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. The externalization of phosphatidylserine is an early event of apoptosis occurring while the plasma membrane remains intact and cells exclude membrane-impermeant dyes. Phosphatidylserine exposure has been reported to be a process that needs energy and depends on caspase activation but its mechanism is still not clearly understood. A combined effect of down-regulation of a phospholipid translocase activity and activation of a lipid scramblase may participate in phosphatidylserine translocation.

Consistent with a role of AMN for proper phosphatidylserine exposure, it has been shown that the phagocytosis of apoptotic cells with AMN is associated with high appearance of phosphatidylserine on the cell surface while it was reduced when AMN was depolymerized by colchicine treatment. Phosphatidylserine externalization and phagocytosis of apoptotic cells were restored when AMN was depolymerized in the presence of Z-VAD, suggesting that caspase-dependent plasma membrane permeabilization impairs proper phosphatidylserine externalization.

These findings confirm previous observations that in the absence of AMN (nocodazole treatment), the proportion of macrophages interacting with and engulfing apoptotic targets was markedly reduced compared with apoptotic cells with AMN. The ability of apoptotic cells to trigger their own engulfment by phagocytic cells before cell lysis is crucial to prevent tissue damage and inflammation associated with necrosis.

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

Microtubule polymerization *in vivo* is an energy-dependent process since the tubulin dimer contains bound GTP and GDP and the polymerization *in vitro* requires the presence of GTP and ATP. Therefore, a hypothesis has been proposed that AMN polymerization is dependent on the bioenergetic status of apoptotic cells.

In a normal genuine apoptotic process, ATP levels must be kept high to allow all the active processes occurring in apoptosis to proceed including AMN formation and maintenance. Live cell imaging and *in vitro* experiments have shown that AMN was organized predominantly in apoptotic cells with energized mitochondria and, on the contrary, was absent in apoptotic cells with depolarized mitochondria. These results suggest that AMN depends on polarized mitochondria and high ATP levels. Live cell imaging in pig LLCPK-1 α cells expressing GFP-tubulin also showed that AMN was maintained during the execution phase of apoptosis until a large mitochondria depolarization marked the onset of secondary necrosis. Overall, these results suggest that AMN is a genuine marker of apoptotic cells in the execution phase of apoptosis. In addition, mitochondria depolarization by using a mitochondrial uncoupler (FCCP) or mitochondrial inhibitors (rotenone, antimycin, and oligomycin) induced AMN disassembly that was associated with LDH release and increased calcium influx, indicating increased plasma membrane permeability. On the contrary, 2-deoxyglucose, an inhibitor of glycolysis, had no effect either on mitochondrial polarization, AMN organization or calcium influx. Furthermore, apoptotic microtubules' stabilization by taxol prevented both the increased calcium influx and mitochondrial depolarization. AMN stabilization by taxol prevented calcium influx even in the presence of mitochondrial depolarization by rotenone or FCCP. All together, these results indicate that apoptotic microtubules' stabilization is sufficient to preserve plasma permeability and cell integrity during the execution phase of apoptosis.

Currently, there is no doubt that apoptosis requires energy because it is a highly controlled process involving a number of ATP-dependent processes such as caspase activation, enzymatic hydrolysis of macromolecules, nuclear condensation, blebbing, and apoptotic body formation. Depletion of cellular ATP was found to cause shifting of the type of cell death from apoptotic cell death to necrotic cell death. Thus, it is now well established that intracellular ATP levels determine whether the cell dies by apoptosis or necrosis. Increased ATP levels during the execution phase of apoptosis may also explain why mitochondria can be in a hyperpolarized state. In fact, it has been suggested that mitochondrial hyperpolarization is the consequence of high intracellular ATP levels. Thus, high ATP levels during apoptosis have been shown by

other investigators. In one of the works, the authors made continuous measurements of cytosolic ATP levels throughout the apoptotic process. Their results showed that ATP levels within cells undergoing apoptosis were maintained higher than in control cells even as caspase activation and DNA fragmentation were occurring during the final stages of apoptosis. Also, they suggest that elevation of the cytosolic ATP level is essential for the apoptotic cell death process. These authors found that ATP levels were maintained at a higher level than the control value for over 70 min. A similar long-lasting ATP increase was observed also for cell death induced by $\text{TNF}\alpha/\text{CHX}$. In accordance with these results, it has been reported that AMN maintenance and mitochondrial hyperpolarization persisted in an interval of 50-158 min during the execution phase of apoptosis.

But, how are apoptotic cells able to keep up mitochondria with a high polarized state? The mitochondrial membrane potential reflects the energy stored in the electrochemical gradient across the inner mitochondrial membrane, which, in turn, is used by FoF1-ATPase to convert ADP to ATP during oxidative phosphorylation. Early mitochondrial hyperpolarization during apoptosis has been described previously. However, the precise mechanism(s) of mitochondrial hyperpolarization during apoptosis is not completely understood. Mitochondrial hyperpolarization has been reported to be mediated by the activity of oligomycin-sensitive mitochondrial FoF1-ATPase. The FoF1 complex can pump protons in reverse from the matrix, across the inner membrane into the intermembrane space under circumstances of high $[\text{ATP}]/[\text{ADP}] + [\text{P}_i]$ ratios. Under these conditions, the FoF1 complex consumes ATP resulting in extrusion of protons from mitochondria. The activity of oligomycin-sensitive mitochondrial FoF1-ATPase operating in reverse mode during apoptosis and neurodegeneration has also been reported previously. It has been suggested that reverse functioning of the FoF1-ATPase participates in cytoplasm acidification and ROS (reactive oxygen species) production during apoptosis. However, there are many reports demonstrating that dissipation of the mitochondrial membrane potential is a general feature of apoptosis irrespective of cell type and of the apoptotic stimuli. While there is controversy regarding the significance of mitochondrial membrane potential loss during apoptosis (e.g., whether this is a cause or an effect of outer membrane permeabilization), the outcomes are predictable. The drop of mitochondrial membrane potential during apoptosis is expected to induce an arrest of the import of most proteins synthesized in the cytosol, release of Ca^{2+} and glutathione from the mitochondrial matrix, entrance of extracellular Ca^{2+} from the medium (calcium influx), uncoupling of oxidative phosphorylation with cessation of ATP synthesis, oxidation of $\text{NAD(P)}\text{H}_2$ and glutathione, and, finally, hyperproduction of ROS by the uncoupled respiratory chain.

These contradictory findings (mitochondrial hyperpolarization and depolarization during apoptosis) can be explained as follows: after a period of genuine apoptosis during the execution phase of apoptosis characterized by mitochondrial hyperpolarization, AMN polymerization, and low calcium levels, apoptotic cells *in vitro* undergo a process of secondary necrosis characterized by mitochondrial depolarization, AMN disassembly, and high intracellular calcium levels, although maintaining some of the typical characteristics of apoptotic cells such as fragmented nuclei and residual caspase activity. Therefore, apoptotic cells can show mitochondrial hyperpolarization or mitochondrial depolarization depending on the time of

analysis. Interpretation of these changes can also be more difficult if the study is not made in a homogeneous synchronized population because examination of a population of apoptotic cells can show a mixed population of cells with mitochondrial hyperpolarization or depolarization. Another interesting conclusion of these works is that AMN disorganization in apoptotic cells seems to be a consequence of mitochondria depolarization rather than increased calcium influx.

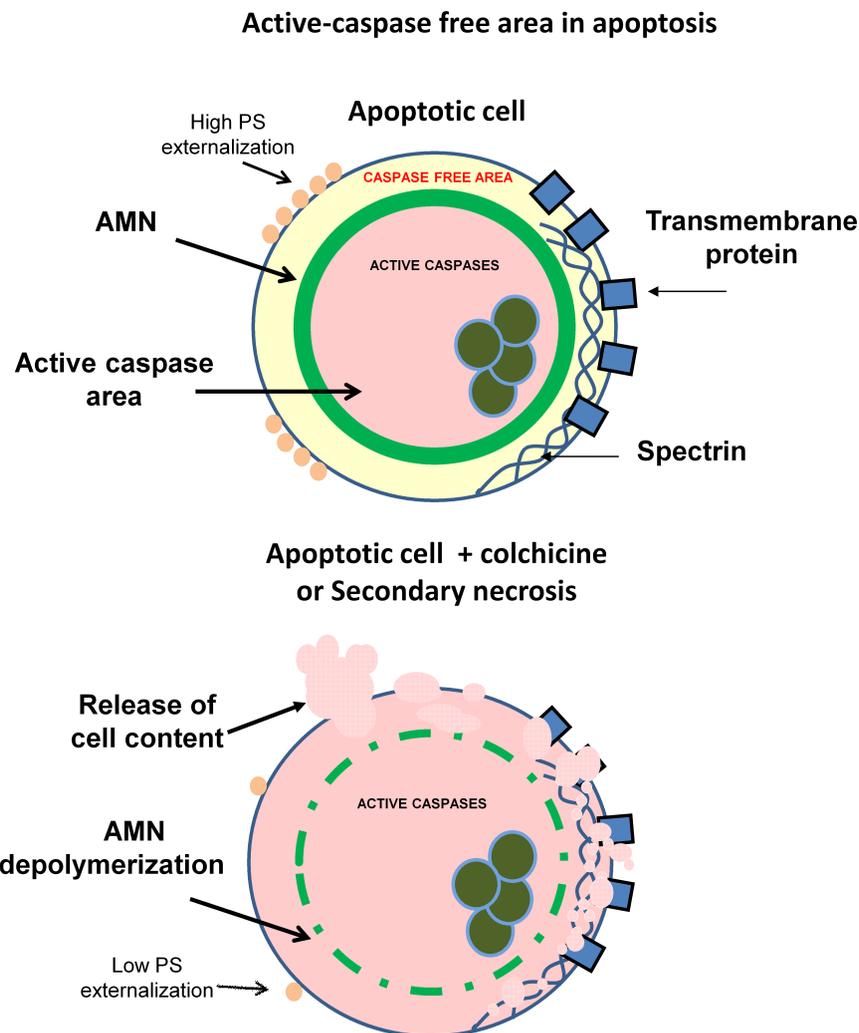


Figure 3. Scheme summarizing the main findings on AMN during the execution phase of apoptosis.

Furthermore, the observation of mitochondrial membrane potential fluctuations with cycles of hyperpolarization in the living cell imaging studies could reflect changes in the bioenergetic status of the apoptotic cell. When ATP levels are high, mitochondria are hyperpolarized by reverse flow of FoF1-ATPase that reduces ATP levels and permits the forward operation of FoF1-ATPase, which increases ATP levels again.

In summary, examining mitochondrial membrane potential during apoptosis, two features are striking: the cyclic changes of mitochondrial membrane potential associated with high ATP

levels and the presence of AMN and the final mitochondrial depolarization coinciding with AMN disassembly and increased plasma permeability in the transition to secondary necrosis. These findings also support the hypothesis that AMN is essential for preserving plasma membrane permeability and, therefore, cell bioenergetics during the execution phase of apoptosis.

12. Apoptotic cells can be stabilized

Given that apoptotic cells maintain the integrity of the plasma membrane and cellular cortex, an innovative method aimed at the long-term stabilization and preservation of apoptotic cells has been developed. This method consists of the combined treatment of apoptotic cells with taxol, Zn^{2+} , and coenzyme Q_{10} (CoQ). This experimental approach guarantees apoptotic cell integrity by preventing plasma membrane permeability and secondary necrosis for at least 96 hours in cell cultures.

The rationale for using this stabilizing combination is: a) the use of taxol, which as a microtubule stabilizing agent, prevents AMN depolymerization and subsequently the access of active caspases to the cellular cortex; b) the use of Zn^{2+} , which as a caspase inhibitor, avoids excessive degradation of cellular components and caspase-dependent cleavage of cellular cortex and plasma membrane proteins; and c) the use of CoQ, which as an antioxidant, protects against oxidative membrane damage which is increased in apoptotic cells.

Stabilized apoptotic cells can be seen as dying cells in which the cellular cortex and plasma membrane are maintained intact or alive. In a metaphorical sense, they can be considered as “living dead” or “zombie cells.” Stabilized apoptotic cells have many of the typical hallmarks of genuine apoptosis such as plasma membrane impermeability, integrity of plasma membrane and cellular cortex proteins, low intracellular calcium levels, plasma membrane potential, phosphatidylserine exposure, and the ability of being engulfed by phagocytes.

Recently, interest in apoptosis research has increased considerably for a number of reasons including development of new treatments, cell culture technology, metabolic engineering of mammalian cells, and gene therapy. Furthermore, apoptotic cells play important roles in biomedical research because due to their characteristics, they are being widely used to evaluate the cytotoxic effects of various drugs by quantifying apoptotic cells by flow cytometry. However, this determination is often affected by the process of cell manipulation (cell harvesting, cell centrifugation, cell pipetting) required for flow cytometry assays, which may disrupt plasma membrane and lead apoptotic cells to secondary necrosis. Thus, accurate and reliable apoptosis quantifications are particularly difficult in adherent cell cultures. Stabilization of apoptotic cells before cell harvesting permits a more accurate and reliable quantification of the actual number of apoptotic cells or the correct measurement of biochemical parameters of genuine apoptotic cells such as mitochondrial membrane potential, intracellular calcium concentration, pH, caspase activity, and many others without the interference of plasma membrane disruption.

In addition, apoptotic cells are currently utilized in various forms of clinical treatments, primarily with the objective of inducing immunological tolerance in the recipient individual. The stabilization of apoptotic cells before administration may help to guarantee that the inoculated apoptotic cells retain their characteristic features until they are engulfed by phagocytes. The inoculation of stabilized apoptotic cells can be additionally utilized for delivering compounds of interest such as therapeutic proteins (for protein replacement therapy) or drugs to recipient macrophages.

There are processes of cell death which by their nature hinder the correct AMN formation (e.g., mitochondrial toxics and cold exposure) and subsequently apoptotic cells are not able to keep up plasma membrane integrity and cells undergo secondary necrosis that may cause serious adverse effects. Apoptotic cells' stabilization may permit the development of therapies for the correct formation and stabilization of AMN and thus promote a more physiological and controlled sort of cell death. Taking into account the above arguments, there is a need to find drugs and protocols for apoptotic cell stabilization.

13. Conclusion

In summary, AMN plays an essential role in the preservation of plasma membrane integrity during the execution phase of apoptosis. In the context of multicellular organisms, the most critical aspect of apoptosis is that cell death occurs without the release of potential pathogenic or harmful intracellular molecules, and without inflammation or injury to adjacent cells. In this manner, the primary role of AMN could be to guarantee that the dying cell is confined to prevent damage to the surrounding tissues and the potentially devastating consequences to processes such as tissue homeostasis and elimination of damaged cells. It is therefore essential to know the effects of different physical or chemical agents on the correct formation of this structure allowing us to better understand their effects on the immune system response and the potential adverse reactions.

Furthermore, apoptotic cells can be stabilized for accurate detection and quantification of apoptosis in cultured cells. Stabilization of apoptotic cells might likewise permit a more secure administration of apoptotic cells in clinical applications and open new alternatives for the functional reconstruction of apoptotic cells for longer preservation.

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