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Review

Cell Surface Area to Volume Relationship During Apoptosis and Apoptotic Body Formation

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Key Words
Apoptosis • Apoptotic bodies • Apoptotic volume decrease • Surface area • Cell membrane

Abstract
Apoptosis is a programmed form of cell death culminating in packing cell content and corpse dismantling into membrane sealed vesicles called apoptotic bodies (ABs). Apoptotic bodies are engulfed and disposed by neighboring and immune system cells without eliciting a noxious inflammatory response, thus preventing sterile tissue damage. AB formation requires a total surface area larger than the apparent, initial cell's surface area. Apoptotic volume decrease (AVD) is a two-stage process leading to an isotonic, osmotic reduction in cell water content driven by net K^+ and Cl^- extrusion. In this article, the role of AVD is presented from a geometric point of view through the process of AB formation. AVD decisively contributes to (i) endowing the cell with the appropriate electrolytic environment for apoptotic execution; (ii) increasing the membrane surface area-to-volume ratio, along with the mobilization of membrane reservoirs (cell rounding, membrane folds and endosomal membranes), so that the cell corpse can be dismantled into ABs; and (iii) reducing plasmalemmal stretch, tension and stiffness, thus facilitating membrane bulging, blebbing and vesicle expansion ultimately leading to separation and release.

Introduction

Cell volume regulation is a tightly controlled, continuous and dynamic process that is essential for cellular homeostasis, integrity and normal function [1]. Unless the cell undergoes growth or division, cell volume must be maintained constant to allow physiological

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performance and cell integrity [2]. In such circumstances, swelling or shrinkage leads to dysfunction and sterile death. However, in specific scenarios, such as during apoptotic programmed cell death, cell volume varies both as a necessary and a permissive event [3]. Specifically, inhibition of volume decrease early halts the apoptotic process [4]. Governed by regulated membrane channels and transporters, cell volume is dictated by the transmembrane ionic flux equilibrium that drags water in or out of the cell following the osmotic gradient, whereas plasmalemmal stretch and tension have minimal impact on cytosolic pressure and thus on volume regulation [5]. Many studies have addressed the mechanisms implicated in cell volume regulation under different biological scenarios. In this brief article, we analyze, from a geometric point of view, the changes in cell volume in connection and coordination with cell and plasma membrane surface areas during apoptosis. Accommodation of cell volume to available membrane area is an inextricable determinant of this process, which will be analyzed in the following sections.

Apoptosis: from apoptotic volume decrease to disintegration into apoptotic bodies

Apoptosis is a programmed form of cell death. Apoptotic (or apoptosis-resembling) programs are found in most living beings, from some unicellular organisms to vertebrates [6–9]. It is believed that programmed cell death has played evolving roles through evolution (in embryogenesis, homeostasis, disease prevention and altruism) [10]. Thus, the apoptotic phenotype has also evolved accordingly to accommodate increasing biological demands [11]. For instance, an ultimate event in the apoptotic program is the dismantling of the dying cell into smaller, membrane bound vesicles, known as apoptotic bodies (ABs). ABs contain an aliquot of all cell constituents (and the debris generated from their controlled degradation) packed and sealed by intact plasma membrane fragments [11].

Cell dismantling into ABs is believed to pursue two specific biological goals in pluricellular organisms endowed with an immune system. On the one hand, easier engulfment and disposal by neighboring and immune system cells. On the other hand, prevention of immunological overstimulation that would result from an uncontrolled released of cell content. This is opposed to other forms of cell death characterized by swelling and plasmalemmal rupture [12]. Shedding of cell content to the interstitium or extracellular space is a strong immunological activator. Many cell components (i.e. specific molecules from all structures and organelles) [13] are known damage-associated molecular patterns (DAMPs). DMAPs intensely attract immune system cells and strongly activate the innate response leading to inflammation [14]. The immune and inflammatory response is a double-edged phenomenon. Even under controlled circumstances, this response has significant side-effects and energetic cost. When exacerbated or abnormally extended in time or in its natural evolution and resolution, the immune response results in severe tissue-injuring effects.

Cell dismantling into ABs is an essential and distinctive characteristic that biologically differentiates apoptosis from other cell death modes. All other phenomena observed during the initiation and execution of apoptosis serve the correct achievement of this final purpose. These phenomena prominently include the very early event known as apoptotic volume decrease (AVD), a process that reduces cell volume [15]. AVD, a universal characteristic of apoptotic cells [16], is not a passive consequence secondary to other events, but an active process with switch properties on the apoptotic program. Inhibition of AVD early halts the execution of apoptosis and thus inhibits cell death and AB formation [17–20]. AVD results from water extrusion osmotically driven out of the cell by net K^+ and Cl^- efflux [21]. In agreement with this, caspases (i.e. the core enzymes of apoptosis) are inactive at the normal intracellular concentration of K^+. Caspases are activated by cleavage of their zymogens, but they also require a low K^+ environment to function [18, 22]. Other components of the apoptotic machinery, such as nucleases responsible for DNA fragmentation [18, 22], and formation of the apoptosome [23] are also inhibited by normal K^+ concentration, and are only allowed to occur upon AVD.

Two consecutive waves of AVD have been shown to occur in some cell types, with distinct roles in the process of apoptosis [24–26]. In the early stage, Na^+ and K^+ gradients are reversed, resulting in intracellular accumulation of Na^+ and K^+ extrusion, and in a 20–40% drop of the cell volume taking place before mitochondrial cytochrome c release [25]. In a second round, cell volume further shrinks leveraging additional K^+ outflow. This second stage is dependent on cytochrome c release and caspase activation and, interestingly, also on proper cytoskeleton organization [25]. In fact, cytoskeleton-disrupting agents prevent this second round of AVD, along with DNA degradation and formation of ABs [24, 27]. A channel-mediated K^+ outward movement activated by cytochrome c independently of initiator caspase-9 activation is involved [28[. However, channels behind AVD might be different from one cell type to another. Some channel redundancy is thought to ensure AVD under a variety of conditions, reinforcing the importance of AVD in apoptosis [18–20].

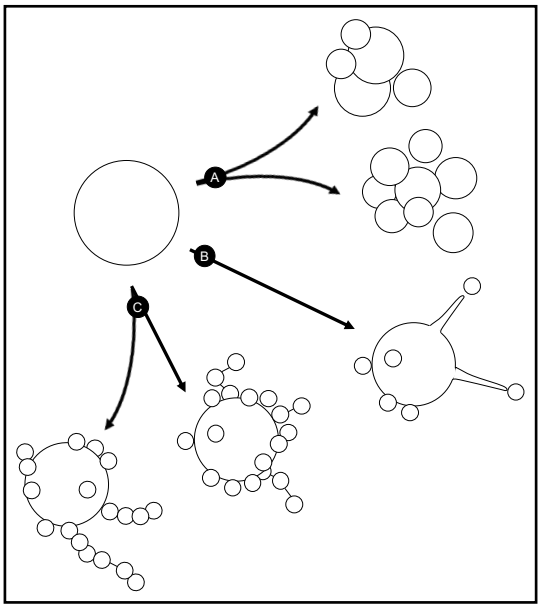

Fig. 1. Different modes of cell dismantling into apoptotic bodies. In the classical mode (A), cells are split into a number of relatively large apoptotic bodies. However, other processes have been recently identified, which lead to the formation of a higher number of smaller apoptotic bodies from cell membrane projections. These modes include the release of individual apoptotic bodies from the end of membrane spikes (B), or from apoptopodia (C); and the production of string-like groups of apoptotic bodies (beaded apoptopodia) resulting from the fragmentation of membrane protruding structures [29–31].

The process of cell dismantling into ABs not only occurs in the (classical) way depicted in Fig. 1. Newly identified forms involve highly regulated processes of cell projections from which one or multiple smaller ABs detach [29–31]. In fact, as shown in Fig. 1, cells sometimes divide into a smaller number of larger ABs of diverse sizes [32–37], and also into smaller ABs resulting from other, recently described processes of disassembly. These processes occur through structures known as apoptopodia, beaded apoptopodia [29, 31] and microtubule spikes, from a larger corpse [11]. The mechanism of cell disassembly determines AB size [29–31].

AB formation is a complex process regulated by coordinated morphological steps including apoptotic membrane blebbing (or zeiosis), protrusion formation, and eventual fragmentation [14, 30]. Blebs are formed when the plasma membrane delaminates from the cortical cytoskeletal network at specific locations. This process (as most apoptotic events) is orchestrated by caspases [12]. In fact, delamination occurs because of retraction of the actin-myosin II cortex (in a caspase and Rho-dependent manner). Delamination significantly alters membrane dynamics, thus facilitating the formation of membrane blebs [38, 39]. At delamination sites, membrane blisters are formed and expanded by the increased hydrostatic pressure produced by actomyosin-mediated cellular contraction. The size of blebs gradually increases during the progression of apoptosis, eventually forming large vesicles [40] that subsequently separate from the main cell body to form ABs [14, 41, 42]. At the final stages of apoptosis the actin cytoskeleton is degraded and phagocytosis of the apoptotic bodies ensues [39].

A role of apoptotic volume decrease in apoptotic body formation

A critical determinant for AB formation involves shape geometry. Packing intact cell volume into smaller spherical structures requires more surface area than apparently available [4]. Because of this, it has been hypothesized that a primary role of AVD is to reduce cell volume by excreting only immunologically inert constituents (mainly water and ions), so that the remaining content can now be packed with the available membrane in smaller vesicles [4]. The more and the smaller the ABs the cell is disaggregated into, the more membrane area is necessary to wrap the whole content (Fig. 2). We assume that smaller ABs are easier to clear than larger ones. Otherwise, the very process of cell dismantling into ABs would have no biological purpose. In such a case, the apoptotic cell would be engulfed and disposed as a whole by neighboring and immune system cells. Indeed, phagocytosis has been reported be most efficient for spherical particles sized 3 μm in diameter [43]. On this basis, we hypothesize that AVD early occurs to ensure a favorable plasma membrane surface area-to-volume ratio (Ω). This would allow the apoptotic cell's corpse to be later on divided into as many small ABs as possible. For the same shape, the larger the cell the lower the Ω, meaning less surface area per unit volume. *Vice versa*, the smaller the cell the higher the Ω, or the more surface area needed to pack each unit of volume. Fig. 2 shows a schematic analysis on the additional surface area needed to divide the initial cell volume into a number of ABs. In the exemplified case, the necessary membrane area is almost 3-fold the apparent surface area of the initial cell. Fig. 2 also graphically shows how the cell's initial volume cannot be packed into smaller vesicles with the initial surface area. Either volume must decrease, or additional surface area must be recruited for AB formation (or both).

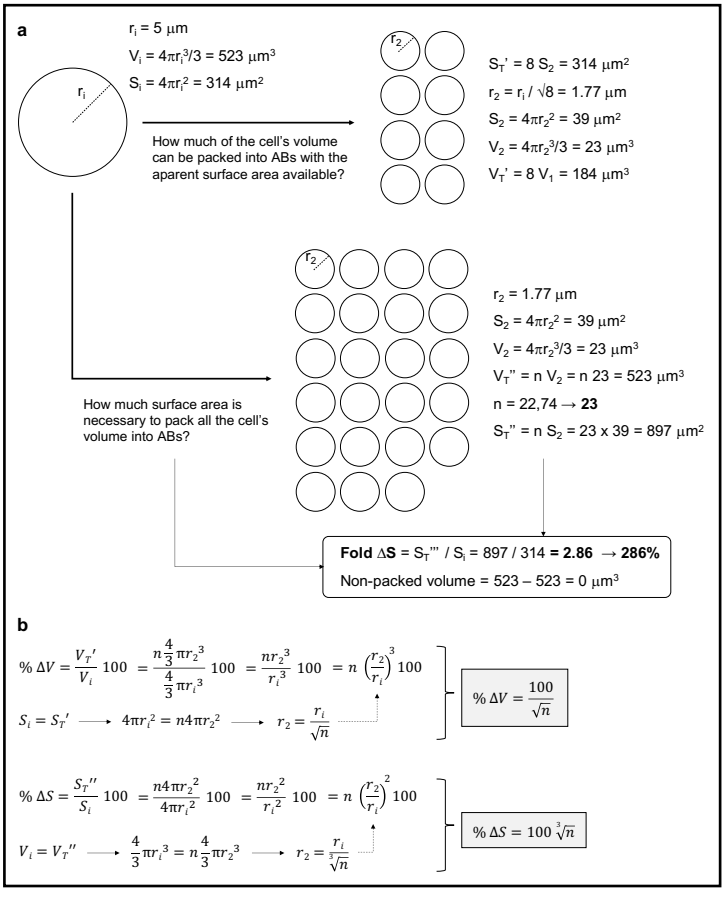

Fig. 2. a) Graphical representation of an exemplifying case (for conceptual purposes) showing the additional surface area needed to divide a spherical cell into apoptotic bodies of an arbitrarily selected size. Smaller apoptotic bodies would require increasing additional total surface area. b) Algebraic generalization (for sphere division into any number of smaller spheres, n) of the cases shown in a). The resulting equations indicate that the fraction of the initial volume that can be packed into smaller spheres (with the initial surface area) decreases as the number of spheres increases. The additional surface area required to pack the whole initial volume into smaller spheres increases along with the number of spheres. i (sub index), initial. n, number of apoptotic bodies. r, radius. S, surface area. T (sub index), total. V, volume. The apostrophe (') and double apostrophe ('') indicate total S or V in the new conditions.

Membrane reservoirs for apoptotic body formation

Whereas a reduction in cell volume increases Ω, thus allowing the formation of ABs, an increment in surface area would lead to an identical result. Membrane synthesis is highly unlikely to occur during apoptosis because of incompatible timeframe, also because the cell's metabolic and anabolic machineries are targeted and disabled by apoptotic executioners [44]. However, additional membrane reservoirs exist, which may provide a favorable surface to volume ratio for AB formation, as graphically summarized in Fig. 3.

A first source of extra Ω is provided by shape reconfiguration. During the initiation phase of apoptosis, cells detach from their surroundings tethers (i.e. contiguous cells and the extracellular matrix). During this process, cells rearrange the sub plasmalemmal cortical cytoskeletal structure (i.e. the cortex) and round up [45]. As shown in Fig. 4, because the sphere is the shape with the lowest Ω, the cell rounding process provides an automatic surplus of membrane for AB formation. Detachment induces membrane crumpling and bulging to accommodate the excess membrane surface around the spherical conformation. Reshaping results in an apparent cell's surface area which is smaller than the total membrane surface area [46]. In fact, although the magnitude of membrane surface area may be modified by membrane internalization and recycling through modulation of endo and exocytosis (see below), this process is slower and excess membrane is accommodated into wrinkled regions.

Membrane unfolding holds another Ω reservoir [47]. Under normal conditions, plasma membrane surface area is larger than the apparent cell's surface area, due to plasmalemmal folding into invaginations (e.g. caveolae, clathrin pits) and protrusions (such as microvilli lamellipodia, etc.) [48]. These membrane anfractuosities behave as pleats in a bellows, which flatten up upon stretch and re-gain folding when released. Membrane substructures are modeled by F-actin structures in the cortical cytoskeleton. Plasmalemmal folding and unfolding (i.e. formation and dissolution of invaginations and protrusions) is a regulated process determined by the level of membrane tension and the counteracting force provided by F-actin polymerization [49, 50] (Fig. 4). Membrane tension opposes membrane folding, whereas cortex contraction promotes corrugation. Membrane tension is dictated by cell swelling and shrinkage, and the opposing cortex force by F-actin assembly and disassembly [51]. This membrane reservoir is ready for immediate use when shape reconfiguration, endocytosis or cell division require rapid Ω increment [47]. In these cases, the gain in Ω is obtained at the expense of plasmalemmal flattening.

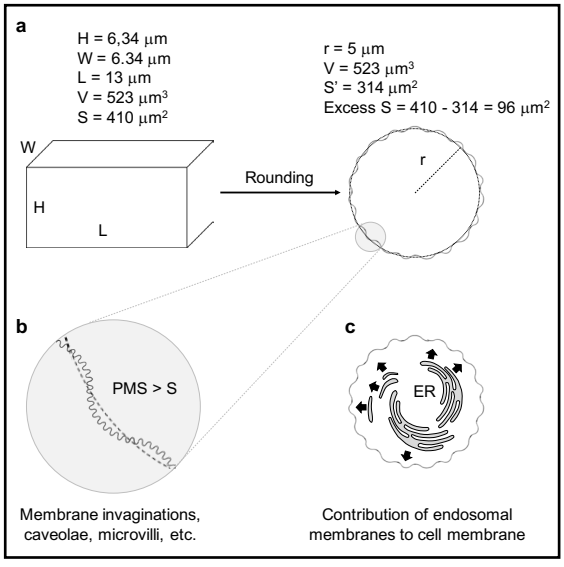

Fig. 3. Membrane and surface-to-volume reservoirs for apoptotic body formation. An illustrative case is fabricated, which starts with a cell (exemplified, for simplicity's sake, by a parallelepipedal structure) that changes shapes following detachment. a) Apoptotic cells very early detach from surrounding tethers and round up. Because the sphere is the geometrical shape with the lowest surface area-to-volume relationship, cell rounding generates an excess of membrane that is stored as membrane corrugations. b) Membrane folding into invaginations (e.g. caveolae, endocytic pits) and protrusions (e.g. microvilli) makes total membrane area several fold higher than the cell's surface area. c) Internal membranes from the endosomal pathway regulate plasmalemmal surface area through adjustment of endo and exocytosis. ER, endoplasmic reticulum. H, height. L, length. PMS, plasma membrane surface area. r, radius. S, surface area. V, volume. W, width.

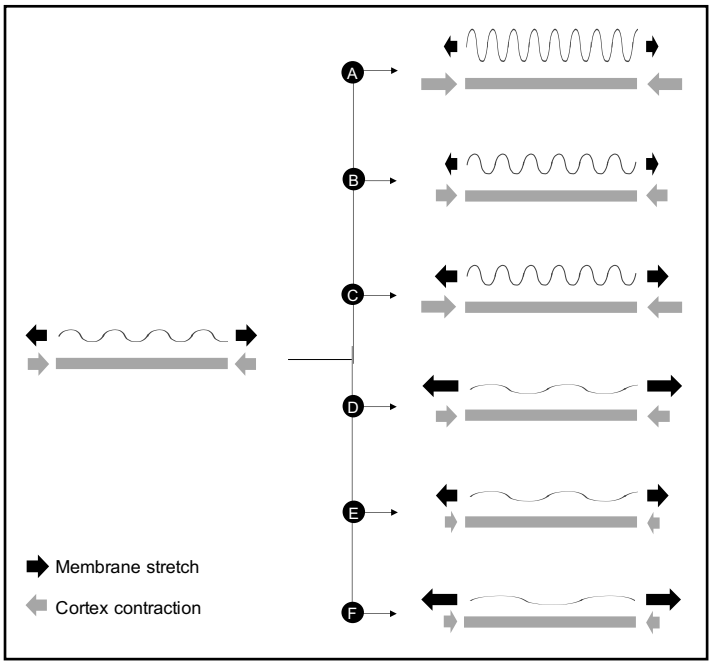

Fig. 4. Regulation of membrane folding by the opposing effect of membrane stretch and cortical cytoskeleton contraction. Further folding and corrugation may result from different combinations of reduced stretch and increased cortical contraction (as in A to C); whereas membrane unfolding and flattening occurs following increased stretch and cortical relaxation (as in D to F). Specifically, increased cortex contraction (C) or reduced membrane tension (B) individually augment membrane corrugation, an effect amplified by both events occurring simultaneously (A). On the contrary, higher membrane tension (D) or cortex relaxation (E) independently reduce membrane folding. When combined, membrane flattening is pronounced (F). Arrow length represents the magnitude of the force.

Depending on the cell type, it has been reported that membrane unfolding can increase surface area by as much as 3.6 fold, accommodating large increases in volume [2, 52].

Finally, a third (potential) reservoir is implemented by internal membranes. This reservoir is thought to be mobilized by membrane tension. Increased tension signals exocytosis to contribute additional membrane to the surface and, when tension decreases, increased endocytosis internalizes the excess membrane [53–55]. The exocytosis pathway is recognized as a second line reservoir, which is used when unfolding is exhausted, and membrane tension grows [47].

Integrated view of apoptotic body formation

As illustrated in the previous sections, blebbing and then AB formation require a higher Ω, which can be obtained from the reduced volume, from membrane reservoir mobilization, or from both. Evidence shows that plasma membrane stores exist in the cell, which can be used to form ABs, and that at least part of the membrane reservoir can be mobilized immediately by physical forces. On the other hand, along with cell rounding, AVD also provides a *de facto* increment in Ω, available for AB formation. The question remains to be solved, as to whether apoptotic cells use (or to which extent use) membrane recruitment to form ABs along with AVD. The level of AB membrane folding, compared to that in the original cells, needs to be studied.

Overall, AVD provides the apoptotic cell with a higher Ω and thus ensures the possibility of some AB formation independently of other potential reservoirs, whose contribution is presently incompletely understood. An inkling is provided by the observation that late apoptotic cells exhibit endoplasmic reticulum (ER) membrane epitopes in their plasmalemma, suggesting incorporation of ER membrane to the surface [56]. This would indicate that AB formation might exhaust readier membrane reservoirs provided by AVD, cell rounding and membrane unfolding, although this needs to be further investigated. The available Ω sets a limitation to AB formation, regardless of the form and mechanisms involved, and regardless

of the size and number of ABs. Yet, the smaller the ABs the more surface area is needed per unit volume packed. As more ABs are formed and separated from the main cell corpse, Ω in the main cell body becomes increasingly lower, until reservoir exhaustion. At that point in the course of apoptosis, no more ABs can be formed, and the remaining volume is retained in the larger corpse.

We also hypothesize that the strong switch effect of AVD on the execution of apoptosis, prevents cells incapable of eventually packing their content into ABs from proceeding through the apoptotic process towards a failed phenotype with noxious consequences. In fact, failure of immediate phagocytosis and quick clearance of apoptotic cells, generates immuno-activating signaling, shedding of immune-attracting micro particles, and secondary necrosis leading to deleterious inflammation and autoimmunity [57, 58]. In short, AVD concentrates the cell content by extruding the solvent (i.e. water) and a few other immunologically inert components (i.e. electrolytes) to facilitate its packaging into ABs, thus keeping it inaccessible to the immune system.

Besides incrementing Ω, AVD plays a second role in AB formation. Specifically, AVD is expected to reduce membrane tension and stiffness, as the opposed effects to swelling, which increases membrane tension [2] and stiffness [59] and thus facilitates bulging and blebbing (Fig. 5). The higher the membrane tension is, the higher the opposition to formation of protrusions. *Vice versa*, the lower the tension, the lesser the resistance to deformation. Cell shrinkage (i.e. as produced by a hypertonic environment) reduces cell membrane stretch and tension [2].

In summary, AVD is a critical and very early event in the course of apoptosis with switch-like regulatory properties. Prevention of AVD inhibits apoptosis progression and cell death. AVD is a two-stage process leading to an isotonic loss of up to 80% of the cell's volume and a net extrusion of potassium, which generates the appropriate electrolytic environment for apoptotic executors (i.e. caspases and the apoptosome) to function. Another role of AVD is to contribute to increasing the membrane surface area-to-volume ratio so that the cell corpse can be dismantled into intact membrane bound vesicles tightly packing cell debris, known as apoptotic bodies. These apoptotic bodies are cleanly phagocytosed and disposed by neighboring and white blood cells without overstimulating the immune system, thus preventing sterile inflammation and tissue damage. Finally, AVD also contributes to apoptotic body formation by reducing plasmalemmal stretch, tension and stiffness, thus facilitating membrane bulging, blebbing and vesicle expansion, separation and release.

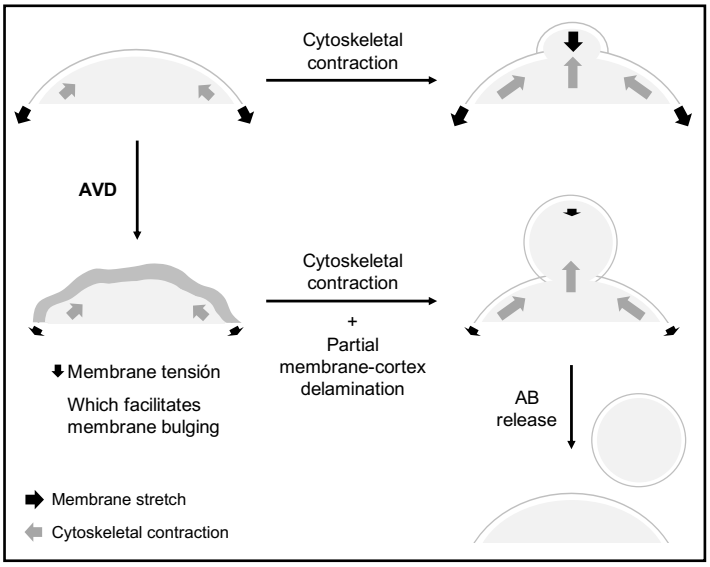

Fig. 5. Contribution of AVD to apoptotic body formation by facilitating membrane bulging, as a consequence of reduced stretch, tension and stiffness. In resting conditions, the level of cortex contraction and membrane tension generate an equilibrium of sufficient mutual compensation that prevents significant bulging and blebbing. Cortex contraction favors bulging by generating outward pressure on the membrane and corrugation. During apoptosis, the effect of cortex contraction is increased by a reduced opposition from membrane tension caused by AVD and cortex delamination. AB, apoptotic body. AVD, apoptotic volume decrease. Arrow length represents the magnitude of the force.

Disclosure Statement

The author declares no conflict of interests exist.

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