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REVIEW ARTICLE

Ca^{2+} influx shutdown during neutrophil apoptosis: importance and possible mechanism

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INTRODUCTION

Neutrophils are essential components of the natural immune system and form the first line of defence against bacterial and fungal infections. They are terminally differentiated cells and have half-lives in the circulation of only 8–20 hr. Their nuclei are invaginated to form a number of defined lobes with thin connecting filaments.[1] The DNA is condensed within these nuclear lobes and is randomly distributed into the separate lobes[2,3] and probably cannot be transcribed. These cells also have little endoplasmic reticulum (ER), but can produce some protein from existing mRNA.[4] They have few mitochondria and the cells generate ATP glycolytically. The long-term survival of these cells is thus insecure, and ultimately limited by the extent of their glycogen stores. One route towards death would thus occur as ATP levels diminished and ion pumps, especially plasma membrane Ca^{2+}-ATPase, became progressively ineffective until cell death proceeds by necrosis. However, it may result in the release of hydrolases and proteases either by necrotic lysis or by Ca^{2+}-driven exocytosis. Moreover, extracellular superoxide generation may also be triggered by Ca^{2+} influx.[5–8] Thus, potentially disastrous extensive damage to the tissues may occur. It thus seems unlikely that the elimination of the millions of neutrophils that die physiologically would be by the necrotic route. Furthermore, neutrophil necrosis is unlikely to be the route for neutrophil clearance in inflammation. Instead, at the resolution of inflammation the neutrophil activity is reduced by an increase in the rate of neutrophil apoptosis. While our understanding of this physiological process of apoptosis as a cell biological phenomenon has increased over the last decade, the unusual role of apoptosis for neutrophils and its consequences are only now being understood.

For example, in many cells, apoptosis is characterized by nuclear condensation and invagination, a morphological event that occurs in neutrophils as they mature. In some respects then the mature neutrophil has already embarked on the route towards apoptosis. This is also evident as neutrophils undergo apoptosis spontaneously, without the need for external stimuli. In fact, external stimuli may modify the rate of this constitutive apoptosis, either by accelerating or delaying cell death, but cannot prevent it.

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THE GOALS OF NEUTROPHIL APOPTOSIS

As neutrophils have the potential to inflict harm, whether oxidative or proteolytic, it is important to the host that their activity is tightly regulated. This is especially the case in inflammation, when large numbers of neutrophils may accumulate within a single organ. In theory, the potential for neutrophil aggression could be limited by: (i) switching down the signalling or response capabilities of the neutrophils, (ii) inducing neutrophil death, or (iii) a combination of the two. The apoptotic process in neutrophils aims to reduce the number of viable and activated cells without releasing the potentially harmful enzymes of the neutrophil. Signalling shutdown may thus be an early step in achieving this goal, with morphological changes of apoptosis being necessary for end stage clearance. Apoptotic neutrophils, as in other cells, ultimately fragment to form 'apoptotic bodies', which can be internalized by other cells. Each apoptotic body forms by a mechanism that at no time causes the plasma membrane to be disrupted. Ultimately, these 'apoptotic bodies' may be phagocytosed by macrophages, perhaps recognizing them by Phosphatidyl serine (PS) expression[9,10] or by lack of a CD31 'release/don't eat me' signal.[11] However, signalling shutdown may occur before this and be more important in limiting neutrophil activity. Apoptotic neutrophils are non-functional, being unable to move by chemotaxis, generate a respiratory burst or degranulate[7,12] and there is a down-regulation of a number of immunoglobulin superfamily members[10] and cell surface receptors[12] on neutrophils during apoptosis. Hence, it has been suggested that the loss of the functional capacity of neutrophils undergoing apoptosis may be the result of a decrease in the number of surface receptors, preventing them from transducing signals. Indeed, part of the process of signalling shut-down may involve a decrease in the number of adhesion molecules or other receptors.[13,14] However, receptor numbers usually vastly exceed that required for efficient signalling. For example, only 0·1% of formylated peptide receptors need be occupied for a maximal signal transduction.[15] It is now emerging that this functional inability of neutrophils undergoing apoptosis may instead be the result of shutdown of a key component of the Ca^{2+} signalling pathway which controls their activation mechanism.

UNREGULATED AND REGULATED NEUTROPHIL APOPTOSIS

Neutrophils undergo constitutive apoptosis both *in vivo* and experimentally. However, the rate of apoptosis can also be accelerated or delayed experimentally. This suggests that although the neutrophil apoptotic programme is running, its rate can be regulated.[16,17] For example, in acute inflammation, the neutrophil numbers within tissues may be extremely high both because of targeted influx from the circulation and also by slowing of the constitutive apoptotic pathway as reported in sepsis or systemic inflammatory response syndrome (SIRS).[18,19] This decreased apoptosis is thought to promote an unbalanced tissue load of neutrophils with resultant uncontrolled release of toxic metabolites exacerbating tissue injury in acute respiratory distress syndrome, SIRS, acute pancreatic inflammation and burn injury.[18,20,21] Although delayed neutrophil apoptosis correlates with the severity of clinical sepsis[19,22] and multiple organ dysfunction syndrome[23] the mechanism for the delay is unclear. However, experimentally bacterial products and cytokines released during sepsis can delay neutrophil apoptosis[24] and glucocorticoids, which are commonly given in these conditions, also inhibit apoptosis of human neutrophils.[25]

Perhaps the clearest trigger for accelerated apoptosis is via the fas (APO-1, CD95) receptor. Fas ligand[17,25] or cross-linking fas, on the neutrophil surface leads to a significant increase in apoptotic rate.[26–29] Activation of the fas receptor results in the formation of the death-inducing signalling complex (DISC) that contains CD95, the CD95-associated death domain containing molecule, FADD and procaspase-8. As a result of association with this complex, procaspase-8 is cleaved, generating caspase-8. Caspase-8 is then released and activates a cascade of protein-cleaving caspases. This cascade has been seen in a number of cell-types. In some cell types, caspase-8 cleaves Bid, a pro-apoptotic member of the Bcl-2 protein family; which in turn leads to activation of the apoptogenic function of mitochondria through the release of cytochrome c, resulting in the cleavage of other caspases downstream of the mitochondria.[30] It has been unclear so far whether this route is important in neutrophil apoptosis, but in the remainder of this review, we will highlight the recent evidence that points to a molecular explanation for Ca^{2+} signalling shutdown in fas-triggered apoptotic neutrophils and which points to an involvement of mitochondria.

CA^{2+} INFLUX IS IMPORTANT FOR NEUTROPHIL ACTIVITY

In neutrophils, as in most other cell types, Ca^{2+} signalling is important for a number of cellular activities, including the generation of oxidants and the release of proteases. Ca^{2+} signalling in neutrophils is initiated by the release of Ca^{2+} from storage sites. However, this event is coupled to the opening of Ca^{2+} influx channels on the plasma membrane.[31,32] While the initial Ca^{2+} release event is a necessary prelude, it is the influx of Ca^{2+} into the cytosol that is responsible for many of the neutrophil responses.[33] The influx of Ca^{2+} through channels in the plasma membrane raises the concentration of Ca^{2+} just under the plasma membrane to significantly higher levels than in the bulk cytosol[34] and is sufficient to activate degranulation[35] and calpain activation.[36] In neutrophils, as in other cell types, the route to Ca^{2+} influx can follow a conventional route, namely activation of phospholipase C β (or possibly γ), generation of inositol-1,4,5-trisphosphate (IP_3), which diffuses through the cytosol to the 'vestigial endoplasmic reticulum' (vER) near the neutrophil nucleus[37] releasing stored Ca^{2+}. Following this, store-operated Ca^{2+} channels (SOCs) in the plasma membrane are also opened and Ca^{2+} influx results. Although this process of 'capacitative Ca^{2+}entry' or 'store-operated Ca^{2+} entry' (for a review, see 38) is common to a number of non-excitable cells[39] the mechanism by which it occurs has yet to be fully resolved and may involve different channels in different cell types.[38,40] There may be two mechanisms, one involving conformational coupling of IP_3 receptors (IP_3R)[41] with a Ca^{2+} channel located in the plasma membrane[42] and another involving a diffusible second messenger released in response from an internal organelle which causes Ca^{2+} channel opening. Although the molecular identity is unknown, the latter factor, termed Ca^{2+} influx factor (CIF) may be a small membrane permeable phosphorylated molecule ($MW < 1000$)[43,44] and CIF activity can be isolated from Ca^{2+} store depleted neutrophils.[45] The characteristics of this store operated Ca^{2+} influx are perhaps best documented in the related myeloid cell type, the basophil, where the Ca^{2+} influx response generates a characteristic Ca^{2+}-release activated current, I_{CRAC}.[46,47]

CA^{2+} INFLUX SHUTDOWN DURING APOPTOSIS

In T cells, Ca^{2+} influx through SOCs is also important and is required for cell activation, cytokine synthesis, and proliferation. Lepple-Wienhues *et al*. (1999) have shown that fas-stimulation of T cells blocks Ca^{2+} influx triggered by Ca^{2+} store release. They further showed that the block of Ca^{2+} influx was lacking in fas-defective lymphocytes.[48] In neutrophils, we have shown that there is a similar uncoupling of Ca^{2+} influx from the Ca^{2+} store release Ayub *et al*. unpublished observation (2003). As fas-treated neutrophils progress towards apoptosis, Ca^{2+} influx in response to formylated peptide becomes increasingly diminished although the Ca^{2+} release component remains the same. This phenomenon cannot therefore be explained by loss of receptor function, but must result from an uncoupling of part of the stimulus-response linkage. The inability of SOCs to open was an early event occurring before detectable externalization of PS or gross morphological changes. It was unlikely to be the result of proteases mediated damage to the Ca^{2+} channel protein, as this shutdown was insensitive to caspase inhibitors. This may mean that unlike the irreversible step of cell-death, the Ca^{2+} influx shutdown may be reversible. However, it is clear that uncoupling Ca^{2+} influx from transduction by activating stimuli would have a profound effect on the responsiveness of neutrophils and render them unable to mount potentially pathogenic actions such as extracellular oxidant and protease liberation which depend on high subplasma membrane Ca^{2+} and are triggered by Ca^{2+} influx.

There are several suggestions for the mechanism by which fas ligation uncouples Ca^{2+} channel opening. The effect of fas activation on Ca^{2+} influx was prevented in T cells lacking in acidic sphingomyelinase, and was restored by transfection with

the enzyme.[48] This shed light both on the mechanism of capacitiative Ca^{2+} entry and on how the signalling shutdown is achieved.[49] Fas may not be the only member of the superfamily of tumour-necrosis-factor (TNF) receptors to inhibit Ca^{2+} channels in the plasma membrane through activation of acid sphingomyelinase. TNF-receptor ligands TNF-α and neural growth factor have also been reported to attenuate store-operated Ca^{2+} entry in thyroid and mast cells, respectively.[50,51] However, this is not the only plausible mechanism. The production of IP_3 that is triggered by ligation of CD3 in lymphocytes is also inhibited by fas-ligation.[52] Also, it has been suggested that there may be an effect on membrane potential, a negative membrane potential providing the electrical component for Ca^{2+} entry. It has been shown that activation of fas or the addition of ceramide inhibits N-type K^+ channels through activation of a protein kinase, $p56^{lck}$.[53] Inhibition of K^+ channels would cause membrane depolarization and consequently reduce the electrochemical gradient and inhibit Ca^{2+} influx. However, valinomycin, a K^+ ionophore, which would restore K^+ flux across the cell, failed to prevent the block on Ca^{2+} entry induced by fas ligation on T cells.[48] It should be noted that fas ligation itself has been reported to increase cytosolic free Ca^{2+} in some cell types[54,55] and that in Syrian hamster embryo cells reduced capacitative calcium entry was reported to result in increased apoptosis.[56,57]

THE LINK BETWEEN MITOCHONDRIA IN APOPTOSIS AND CONTROL OF CA^{2+} INFLUX

There would therefore seem to be no clear explanation at present for the mechanism of Ca^{2+} influx shutdown during apoptosis. However, the work from Hoth and Parekh's laboratories have provided an exciting possible explanation, which links the well-established roles of mitochondria in apoptosis with a new role in signalling Ca^{2+} influx.

Hoth's group have showed that in T-lymphoid cells[58] and Parekh's group in the myeloid cell line, basophilic leukaemia[59,60] that 'energized' mitochondria play an important role in the opening of the SOCs. As mitochondria are known to take-up Ca^{2+} from the cytosol and are often placed close to the endoplasmic reticulum where Ca^{2+} release is occurring, it was thought that they assisted in emptying storage sites of their Ca^{2+} by acting as a Ca^{2+} 'sink' or lowering local cytosolic Ca^{2+} below a level that may activate the SOCs. However, these effects alone are unable to account for the role of mitochondria in Ca^{2+} influx. Although, SOC opening induced experimentally by thapsigargin can occur in the absence energized mitochondria, Parekh's group have shown in basophils, that mitochondrial depolarization effectively uncouples Ca^{2+} influx from Ca^{2+} store release. These authors ruled out effects of changes in intracellular ATP, oxidants, cytosolic acidification, nitric oxide or the permeability transition pore and their data[61] suggests a new role for mitochondria as the generator of the signal for Ca^{2+} influx. One possibility (see Fig. 1) is that released Ca^{2+} is taken up by nearby mitochondria and that the elevated intramitochondrial Ca^{2+} generates the signal for Ca^{2+} influx (e.g. CIF). Because the uptake of Ca^{2+} into the mitochondria is dependent on its energized state (i.e. membrane potential), one way in which Ca^{2+} influx could be blocked would be for the energized state of mitochondria to be altered. As pointed out earlier, in many cell types there is a clear involvement of mitochondrial metabolism in apoptosis, culminating in the release of cytochrome c. Although neutrophils have few mitochondria, many of the molecule family members of this mitochondrial pathway have been identified in neutrophils.[62] These include Bcl-2 family proteins Al, Mcl-1, Bcl-X_L, and Bad. Furthermore, although mitochondria do not normally play an important role in ATP production in neutrophils, these cells being fully active at low oxygen levels are essentially anaerobic, yet they often have membrane potentials.[63] The few mitochondria that exist in neutrophils therefore have the required property for Ca^{2+} uptake and provide them with an important role in interorganelle signalling. Furthermore, recently it has been shown that an early event in the progress towards neutrophil apoptosis, which precedes externalization of PS, is loss of mitochondrial membrane potential[63] this would provide the link to Ca^{2+} signalling shutdown (Fig. 1). As Edwards' group have shown that depolarization of the mitochondrial membrane in itself does not alter the rate of apoptosis[63] it is unlikely that mitochondrial depolarization drives apoptosis. It would make teleological sense for Ca^{2+} shutdown in neutrophils to precede and perhaps be independent of total commitment to apoptosis, as neutrophil

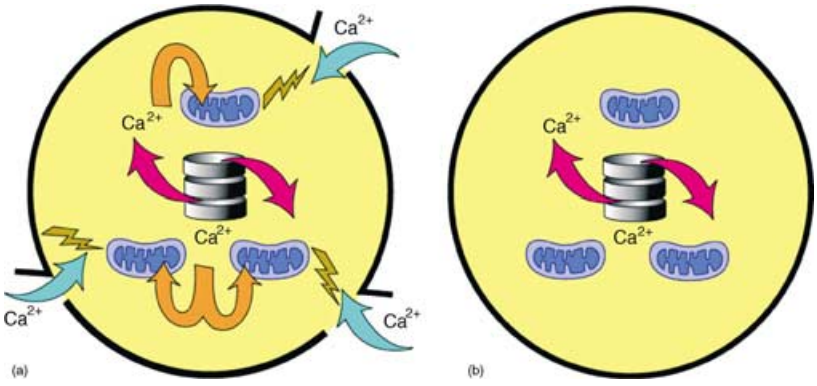

Figure 1. (a) Signalling competent neutrophil: showing (i) release of Ca^{2+} from vER; (ii) uptake by energized mitochondria; and (iii) signalling Ca^{2+} influx. (b) Neutrophil approaching apoptosis with non-energized mitochondria; showing (i) release of Ca^{2+} from vER, (ii) no uptake by non-energized mitochondria, and (iii) lack of signalling Ca^{2+} influx.

inactivation may be the main 'purpose' of neutrophil apoptosis in inflammation.

CONSEQUENCES OF CA^{2+} INFLUX SHUTDOWN

The apoptotic shutdown of the signalling pathway to Ca^{2+} influx in neutrophils may have important implications for their behaviour in the large number of inflammatory conditions encountered in clinical practice. It may be speculated that a failure of the shut-down mechanism could give rise to inappropriately prolonged activity, which may result in inflammatory tissue damage. The Ca^{2+} signalling shutdown mechanism may be of more importance for neutrophils than for other cell types as these cells are probably unique in their potential pathogenic effects. It may therefore also be possible to utilize this physiological pathway to trigger this 'off signal' pharmacologically. If it could be hijacked and used to prematurely inactive neutrophil sensitivity to stimuli, this may have therapeutic benefit. Clearly, it is important to understand the mechanism of Ca^{2+} influx shutdown during apoptosis in neutrophils before this knowledge may be beneficially exploited in future. The recent discoveries outlined here may be the first steps towards this understanding.

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