Early events in spontaneous neutrophil apoptosis


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Abstract

Neutrophils are very abundant, short-lived leucocytes and their death by apoptosis is central to homeostasis and the resolution of inflammation, yet the trigger for apoptosis is still a topic of debate. Depolarization of the mitochondrial membrane has been supposed to initiate neutrophil spontaneous apoptosis, as neutrophils gradually lose the anti-apoptotic protein Mcl-1 and Bax translocates and inserts into the mitochondrial membrane. However, other reports show that caspase 8 is required for neutrophil apoptosis, suggesting the involvement of DR (death receptor) signalling. As DR ligation is not required for neutrophil apoptosis, this raises the intriguing possibility that activation of caspase 8 during neutrophil apoptosis occurs via a novel mechanism. In the present paper, we discuss the current evidence for mechanisms occurring in neutrophil apoptosis, which could trigger DR signalling in the absence of DR ligation.

Neutrophils are very abundant, short-lived polymorphonuclear leucocytes that play a key role in the early stages of the inflammatory response to infection, particularly infections of rapidly dividing bacteria, yeast and fungi. Human neutrophils are produced at a rate of 1–2 × 10¹⁵ cells/day and they survive in the circulation for approx. 24–36 h before undergoing apoptosis [1]. Therefore the neutrophil turnover is rapid and the efficient removal of such large numbers of effete cells by apoptosis is a major homeostatic endeavour. During infection, neutrophil production is escalated by the action of cytokines such as GM-CSF (granulocyte/macrophage colony-stimulating factor), leading to significant neutrophilia. Peripheral blood neutrophils leave the circulation under the influence of chemotactic factors that include complement components (C5α) and chemokines (interleukin-8). After reaching the site of infection, they begin to phagocytose and kill ingested pathogens. To optimize neutrophil bactericidal function, lifespan is extended by a range of inflammatory mediators including cytokines, such as GM-CSF [2], and bacterial components, including lipopolysaccharide [3]. After killing of the ingested microbe, the neutrophil dies by apoptosis and is phagocytosed by macrophages, preventing loss of neutrophil contents and tissue damage. Once the infected site has been rendered sterile, the inflammatory response must be resolved by the removal of inflammatory cells, including neutrophils, again through the induction of apoptosis [4]. The correct regulation of the apoptotic programme is thus vital to ensure the maintenance of neutrophil numbers in the circulation, the efficient removal of invading pathogens and resolution of the inflammatory response.

Dysregulation of apoptosis will lead to the persistence of immune cells at inflammatory sites and the development of chronic inflammatory disease. Reduced neutrophil apoptosis has already been linked to several inflammatory conditions, including rheumatoid arthritis and acute respiratory distress syndrome [4–6]. Despite the obvious importance of spontaneous neutrophil apoptosis as a homeostatic mechanism in the immune system, our understanding of the mechanisms underlying the initiation of this process is incomplete. The rapid entry into apoptosis of neutrophils isolated from peripheral blood suggests that the apoptotic programme may already have been initiated in cells in the circulation, triggered possibly by their release from the bone marrow. Certainly, the process does not require de novo protein synthesis, as addition of cycloheximide to neutrophil cultures at concentrations that completely suppress protein synthesis (Figure 1A) does not delay neutrophil apoptosis (Figure 1B). Interestingly, cycloheximide did not accelerate neutrophil apoptosis either at these low concentrations, although it did increase death at higher concentrations (10 µg/ml; results not shown), as reported by others. This would indicate that neutrophils are not actively producing survival factors to stay alive in the blood. This does not exclude the possibility that entry into neutrophil apoptosis is due at least partially to the gradual loss of a survival factor such as Mcl-1 (see S.W. Edwards, this issue). To identify the potential triggers for neutrophil entry into apoptosis, it is pertinent to consider the molecular events known to occur during apoptosis and whose inhibition delays this process significantly.

Apoptosis can be initiated by two main pathways, namely the extrinsic, DR (death receptor) pathway and the intrinsic mitochondrial pathway [7]. In the DR pathway, ligation of a DR such as CD95, induces formation of a DISC (death-inducing signalling complex) consisting of the DR, an adaptor protein [FADD (Fas-associated death domain) for CD95] and an initiator caspase, predominantly procaspase 8 [7] or procaspase 10 [8]. Both the DR and the ligand exist as

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trimers and the ‘super clustering’ of DRs following ligation is required to promote aggregation of procaspase 8 molecules within the DISC [9]. The procaspase 8 molecules are thus brought into close proximity and cause autoproteolysis and generation of active caspase 8, which activates the downstream effector caspase 3 [10]. In the mitochondrial pathway, the activation of effector caspases is caused by an analogous death-inducing complex, the apoptosome, which is formed as a result of the depolarization of the mitochondrial membrane. Depolarization of the mitochondrial membrane, induced as a result of the presence of intrinsic factors such as loss of anti-apoptotic Bcl-2 family proteins, ROS (reactive oxygen species) or DNA damage, results in the release of proapoptotic factors such as cytochrome c. The latter associates with Apaf-1 (apoptotic protease-activating factor 1) and procaspase 9 to form the apoptosome. Caspase 9 is activated on the apoptosome and subsequently activates caspase 3 [11]. In addition, the work of Marcus Peters group [12] has shown that in certain cell types the generation of active caspase 8 after DR (CD95) ligation is insufficient to trigger caspase 3 activation and apoptosis. In these cells, DR signalling has to be amplified through the mitochondria by caspase 8 mediated cleavage of a Bcl-2 family protein, Bid [12], which inserts into the mitochondrial membrane and induces release of cytochrome c.

Several authors, including us, have shown that neutrophil apoptosis involves activation of caspase 3 [13,14], loss of mitochondrial membrane potential and release of cytochrome c [15]. However, neutrophils have also been reported to express a range of DRs including TNFR1 (tumour necrosis factor-α receptor 1) [16], receptors for TRAIL (tumour-necrosis-factor-related apoptosis-inducing ligand) [17], as well as both CD95 and CD95 ligand [18]. Involvement of DRs was initially ruled out as neutrophils in culture are not exposed to TRAIL or TNFα (tumour necrosis factor α) and antagonistic anti-Fas, anti-TRAIL receptor and anti-TNFα antibodies did not prevent spontaneous neutrophil apoptosis [17]. Furthermore, neutrophils from Fas (lpr) or Fas ligand (gld) deficient mice show a normal rate of spontaneous apoptosis [19]. These observations would tend to suggest that the mitochondrial pathway is predominant during spontaneous neutrophil apoptosis. Several authors have reported that freshly isolated human neutrophils do not express the anti-apoptotic proteins Bcl-2 or Bcl-XL [20–22], although they do have detectable levels of the Bcl-2 homologues Mcl-1 and A-1 [22,23] and the proapoptotic proteins Bak [24] and Bax [22]. Levels of Bax are maintained as neutrophils age in culture, but Mcl-1 expression decreases [22]. Moreover, neutrophils from A-1+/− mice have an accelerated rate of spontaneous apoptosis [23]. Interestingly, Bcl-2 is expressed in promyeloid cells but is lost as they differentiate towards neutrophils [25]. These results add weight to the suggestion that neutrophil apoptosis is actually triggered as a result of their maturation and exit from the bone marrow.

At this point, it would appear that the case for the mitochondria in initiating neutrophil apoptosis is overwhelming. However, there are also results that are not in accord with this hypothesis. Primarily, our own results (D. Scheel-Toellner, K. Wang, P. R. Webb, R. Craddock, H. M. McGregor, L. K. Assi, N. Parkes, L. E. Clough, E. Gulbins, M. Salmon and others, unpublished work) and that of others [14,27] have shown that neutrophil apoptosis is delayed by tetrapeptide inhibitors of caspase 8. As caspase 8 is activated only as a result of DR signalling, it is now necessary to revisit the role of DRs in neutrophil apoptosis. Intriguingly, preliminary results from Chilvers’ laboratory (E.R. Chilvers, personal communication) indicates that a preformed TNFRI DISC is present in freshly isolated neutrophils and this could clearly be the source of active caspase 8. There is a precedent for this proposal as spontaneous DISC formation has also been reported to occur in anokins [28], which is cell death resulting from the detachment of adherent cells from surrounding cells or stromal support matrix. Survival signals in this situation may therefore act to prevent formation of the DISC, which will occur spontaneously in their absence. Neutrophils are produced from precursors in the bone marrow and on their release into the circulation they lose contact with marrow survival factors, such as G-CSF and GM-CSF and die within 48 h. Therefore neutrophil apoptosis represents a
Disruption of lipid rafts delays neutrophil apoptosis

Isolated human neutrophils were cultured overnight in the absence or presence of methyl-β-cyclodextrin at the concentrations shown and apoptosis was assessed by observation of cell morphology of differentially stained cells. Results are means ± S.D. for three separate experiments; *P < 0.01.

Figure 2 | Disruption of lipid rafts delays neutrophil apoptosis

Classic example of death occurring as a result of the removal of a survival signal, and the presence of a preformed DISC in neutrophils may thus determine their short lifespan. There is a second example of DISC formation occurring in the absence of DR ligation: Hennino et al. [29] reported that the CD95 DISC was formed, independent of CD95 ligation, in germinal centre B cells during an antibody response. In these cells, the DISC also contained the caspase 8 inhibitor c-FLIPL, and apoptosis was only prevented if the B cells received a survival signal from CD40L [29]. It is possible that neutrophils also contain c-FLIP, and that release from the bone marrow removes the survival signals maintaining its expression; we are currently investigating this possibility.

If caspase 8 is the primary signal for neutrophil apoptosis and neutrophils have a preassembled DISC, then in the absence of DR ligation it is still necessary to identify a factor that would induce sufficient caspase 8 activation to bring about apoptosis. The coalescence of CD95-containing lipid rafts after ligation of CD95, also termed capping, is required for CD95 signalling and apoptosis [30,31] (Figure 2). Intriguingly, a recent paper by Huang et al. [32] have shown that ROS, generated by chemotherapeutic agents, induce clustering of CD95 and apoptosis in T-cell lines.

Neutrophil apoptosis is also influenced by ROS. Neutrophil spontaneous apoptosis is reduced in patients with chronic granulomatous disorder [33], an inherited condition in which the enzyme generating superoxide (NADPH oxidase) is non-functional. Antioxidants, such as N-acetylcysteine [33] and catalase [34], have also been reported to delay neutrophil apoptosis. ROS are generated as a by-product of oxidative metabolism and their toxic potential is limited by several mechanisms, one of the most important being the presence of GSH (glutathione), a powerful antioxidant and the major determinant of redox status in mammalian cells. Whether neutrophils lose the capacity to neutralize ROS as they age, possibly due to decreased GSH levels, remains to be established. However, this is an attractive hypothesis for a mechanism to limit neutrophil lifespan. A range of proinflammatory cytokines can increase the neutrophil lifespan and,

Figure 3 | Model for initiation of spontaneous apoptosis of neutrophils

A model is proposed in which accumulation of ROS leads to generation of ceramide and clustering of DRs. The clustering leads to activation of the DISC and caspase 8. Caspase 8 can then cleave the Bcl-2 family member Bid, leading to loss of mitochondrial membrane potential (Δψm), release of cytochrome c and formation of the apoptosome. Caspase 3 is the final downstream effector caspase mediating apoptosis.
in contrast, ingestion of microbes will trigger an oxidative burst and accelerate apoptosis. Both of these mechanisms could modulate apoptosis by influencing the levels of GSH, allowing for neutrophils to have a preset lifespan dictated by their GSH content but with the flexibility to modulate rates of apoptosis during infection.

In summary, we propose a model for spontaneous neutrophil apoptosis (Figure 3) in which cell death is initiated by the clustering of DRs, mediated possibly by ROS. The DR signal is amplified through the mitochondria leading to activation of caspase 3 and apoptosis. This model may well apply to other cells dying as a result of cytokine deprivation or loss of contact with neighbouring cells, or to other short-lived cells such as eosinophils. There are still several unanswered questions with regard to our proposed model of neutrophil spontaneous apoptosis. The requirement for DR signalling can only be truly tested if we can ablate all DR signalling. This could be done by knocking out FADD, which is the adaptor molecule in the DISC of all DRs so far examined. However, the FADD knockout mouse is embryonically lethal. As an alternative, we intend to look for the formation of other DR DISCs during neutrophil apoptosis and also to try to knockdown FADD in neutrophils using a small interfering RNA approach. The second aspect of our model, which requires investigation, is the mechanism of activation of the DISC, as this will identify the true initiating signal for spontaneous neutrophil apoptosis. The most probable candidate is ROS build-up and we intend to assess levels of antioxidants, such as GSH and thioredoxin, during neutrophil apoptosis.

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