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News and Commentary

Neutrophils: dead or effete? Cell surface phenotype and implications for phagocytic clearance

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Neutrophil apoptosis – not all cells are equal

The neutrophil granulocyte provides a versatile cellular defence against invading pathogens. This cell type is produced in large numbers in the bone marrow (10^{11} cells/day) and represents the principal leukocyte subset that is present in the circulation. Neutrophils can be rapidly recruited to sites of tissue injury or infection, transmigrating endothelial barriers to enter tissues. In terms of effector function, the neutrophil is principally a phagocytic cell, armed with a diverse repertoire of receptors for potential opsonins of pathogens, a large array of anti-microbial compounds, degradative enzymes and the ability to produce reactive oxygen species that ultimately allow destruction of infectious organisms.[1] Although the neutrophil may be thought of as a cellular 'thug' with little capacity for modulated destructive function, *in vitro* studies have revealed the exquisite control of every aspect of neutrophil behaviour, including adhesion, migration, phagocytosis and granule release. In addition, it is now apparent that neutrophils are also capable of limited production of important inflammatory mediators that may influence development or progression of an inflammatory response. Finally, the ultimate demise of neutrophils through the process of programmed cell death or apoptosis is also closely regulated.[2]

The interaction of the neutrophil with the local microenvironment occurs through the cell surface receptors that are expressed at the plasma membrane. Many of the key molecular mediators of adhesion, migration and phagocytosis are maintained within intracellular granule compartments, the azurophilic and specific granules. In response to exposure to a variety of stimuli derived either from pathogens (e.g. formyl methionyl-leucyl-phenylalanine) or from the host (a range of cytokines and growth factors), the neutrophil is able to rapidly mobilise these granule contents to the plasma membrane. Coupled with this, the neutrophil can control the levels of surface receptor expression through the rapid and specific proteolytic 'shedding' of the ecto-domain of specific receptors through the action of metalloproteases. Together these processes combine to generate a very different receptor profile that is optimal for destructive capacity. Indeed, the functional repertoire and cell surface receptor profile of neutrophils that have been exposed to these different stimuli is distinct and has been used to define neutrophil functional status.[3]

Regulation of apoptosis

We have been particularly interested in the molecular mechanisms that underlie the engagement of apoptotic programmes in neutrophils. As a terminally differentiated cell type, the neutrophil constitutively undergoes apoptosis when cultured *in vitro*. It is also clear that apoptosis is influenced by neutrophil functional activity, including adhesion and phagocytic activity. For example, apoptosis is delayed in the CD11b deficient animals due to reduced phagocytic capability and activation of the NADPH oxidase.[4] In view of the importance of neutrophil apoptosis in determining the numbers of neutrophils that will be present within inflammatory sites, many studies have investigated the signalling pathways that exert regulatory effects upon the rate at which neutrophils undergo apoptosis. These studies indicate that a wide variety of stimuli can control neutrophil survival during *in vitro* culture, including growth factors (e.g. GM-CSF), cytokines (e.g. TNF-α) and inflammatory mediators (e.g. prostaglandin E_2).[5,6]

The rates of neutrophil apoptosis that are derived from *in vitro* studies are likely to be too low to account for the rapid removal of large numbers of neutrophils from inflammatory sites *in vivo.* For example, in murine peritonitis models, 5–10 million recruited neutrophils are cleared within 24 h, whereas *in vitro* data relating to constitutive apoptosis would predict that a small proportion of that number would be removed. Under physiological conditions, neutrophils may be removed by a process of assisted suicide. In addition, there are other internal control mechanisms that can augment apoptosis of neutrophils including ligation of death receptors (e.g. Fas, TNFR and TRAILR). Thus, cellular interactions between neutrophils and phagocytes or stromal cells may engage the proapoptotic signalling receptors, allowing initiation of neutrophil apoptosis in close proximity to cells involved in their phagocytic removal.[7] In addition, apoptosis can be specifically accelerated following interactions of neutrophils with a number of pathogens including *Escherichia coli, Streptococcus pneumoniae, Mycobacterium tuberculosis* and *Neisseia gonorrhoeae.* Interestingly, mapping the neutrophil transcriptome following pathogen-induced apoptosis suggests that there may be pathogen-specific alterations in gene expression profiles associated with apoptosis.[8] The induction of neutrophil apoptosis by pathogens may represent a mechanism for ensuring that highly phagocytic neutrophils containing

pathogenic organisms are 'shut down' and subsequently cleared.

One possible explanation for the experimental evidence *in vitro* for involvement of many different molecular pathways in the recognition and internalisation of apoptotic cells[9] is that the assumption that all apoptotic cells are recognised in a similar manner by phagocytes is flawed. In the study of apoptotic cell clearance there has been a reluctance to address the heterogeneity of apoptotic cell populations that are used in studies of phagocytosis. In particular, distinct subpopulations of apoptotic neutrophils can be readily identified with monoclonal antibodies or by use of specific soluble extracellular proteins.[10] This may go some way towards explaining apparently contradictory findings, for example, the question of serum dependency. As an example, Table 1 shows data relating to the effects of serum on phagocytosis of apoptotic neutrophils. These data clearly show that the proportion of necrotic cells present in the target cell population may determine the extent of observed dependency upon the presence of serum for efficient phagocytosis. Furthermore, while we may be able to define *in vitro* culture conditions that yield reproducible apoptotic neutrophil phenotypes, it is likely that under conditions present at inflamed sites, neutrophils may have been exposed to multiple stimuli with either synergistic or antagonistic effects upon the apoptotic process and also upon cell surface phenotype.

Apoptosis-induced surface receptor alterations

Investigation of the surface receptor alterations that are associated with long-term exposure of neutrophils to cytokines (including GM-CSF and interferon-γ) revealed that for the glycophosphatidylinositol-anchored IgG receptor, FcγRIII (CD16), the overall levels of expression were much higher in GM-CSF-treated neutrophils.[11] Careful analysis indicated that two distinct subpopulations of neutrophils were present in cultured cells in terms of levels of CD16 expression. Subsequent studies demonstrated that the FcγRIII high expressing neutrophils had normal nuclear morphology, whereas those with lower levels of membrane FcγRIII displayed morphological alterations that were characteristic of apoptosis.[12] This observation represented one of the first demonstrations that specific alterations in membrane receptor expression accompanied the programme of morphological changes associated with apoptosis. Our data suggested that the nature of the membrane anchor was not responsible for the profound loss ($>90\%$ of surface receptors) of FcγRIII.

A number of other specific membrane receptors also exhibited marked down-regulation as neutrophils underwent apoptosis, notably CD62L, CD44, CD43 and TNFRI (CD120a).[13,10] In these studies it was difficult to detect neutrophils with an intermediate level of expression of these receptors when either single or dual immunofluorescence (with labelled annexin V to mark apoptotic neutrophils) profiles were examined. This observation would be consistent with the suggestion that the levels of receptor expression reflect a 'catastrophic' event rather than a gradual loss of receptors over time. Since of these receptors can be shed via the action of metalloproteases during neutrophil activation, it is possible that the intense membrane re-organisation (blebbing) gives rise to metalloprotease activity resulting in receptor loss. However, the importance of the cytoskeleton in regulation of these processes should not be underestimated.

It was notable that many surface receptors exhibit a smaller reduction in surface expression (by about 30%) on apoptotic neutrophils when compared with non-apoptotic neutrophils.[14] This may reflect the loss of plasma membrane that is associated with membrane blebbing in the earliest stages of apoptosis. Alternatively, studies from a number of laboratories have suggested that the disruption of the Golgi represents an early event in the apoptotic process.[15] Loss of the ER–Golgi–plasma membrane secretory transport pathway during apoptosis might be predicted to contribute to the net loss of receptors from the plasma membrane. A corollary of this suggestion is that equivalent or increased levels of receptor expression upon apoptotic neutrophils may require some mobilisation of intracellular stores of receptors during the blebbing phase. Interestingly, recent data suggest that bacterially loaded apoptotic neutrophils, in contrast with UV-induced apoptotic cells upregulate expression of heat shock proteins (HSP), notably HSP-60 and HSP-70.[16] The expression of HSPs by pathogen-induced apoptotic neutrophils has the potential to provoke pro-inflammatory cytokine production by macrophages and clearance of pathogen-loaded apoptotic neutrophils may influence the development of acquired immunity through processing and presentation of antigen in the context of costimulatory molecule expression. In addition, proteins that exhibit altered patterns of glycosylation, perhaps due to incomplete processing in the Golgi, become expressed on the plasma membrane during membrane blebbing. It is also possible that there is altered activity of carbohydrate modifying enzymes that accompany apoptosis, for example, sialidases. Analysis of the changes in protein glycoforms that are displayed by apoptotic neutrophils using a panel of labelled lectins did not reveal a global loss of binding of lectins derived from *Maackia amurensis* and *Sambrucus*

Table 1 Effect of serum as an opsonin on macrophage phagocytosis of apoptotic neutrophils

Stimulus	% Apoptosis	% Necrosis	%Phagocytosis−Serum	%Phagocytosis+Serum
None	42 ± 8	4 ± 2	20 ± 4	25 ± 4
Fas	68 ± 12	7 ± 2	24 ± 6	23 ± 6
UV	70 ± 13	21 ± 7	24 ± 5	45 ± 9

Neutrophils were cultured in the absence of serum to undergo spontaneous apoptosis (None), or induced to undergo apoptosis with the addition of CH11 (anti-Fas) antibody (Fas) or irradiation with ultraviolet (UV). Apoptosis and necrosis were determined by flow cytometry using annexin V and propidium iodide and phagocytosis determined by flow cytometric analysis. Results shown are mean$\pm$standard deviation for at least 3 experiments

nigra that bind specific sialic acid linkages. Nor did increased binding of lectins such as peanut agglutinin that recognises galactose occur, implying that carbohydrate modifications that accompany apoptosis do not involve mass desialylation.[10]

One of the important aspects of membrane changes associated with neutrophil apoptosis is that there is functional uncoupling, effectively isolating the apoptotic cell from stimuli that might trigger destructive responses. It is extremely likely that the activity of caspases and calpains may simply disrupt the assembly and localisation of signalling complexes that are required to translate receptor occupancy into a functional response. Furthermore, disruption of cytoskeletal integrity through cleavage of actin or other binding proteins such as ezrin, fodrin or band 4.1 would be predicted to inhibit neutrophil capacity for adhesion, migration and degranulation.[17] In addition to this somewhat nonselective loss of receptor function, we reported specific loss of β2 integrin ligand binding function despite maintained levels of surface expression.[13] Since the β2 integrins are known to show regulated ligand binding function through a process known as 'inside–out' signalling, it is possible that this observation also reflects dysregulation of key signalling pathways following apoptosis. However, an active conformation of either cell surface expressed or purified β2 integrins can be forced using the divalent cation Mn^{2+}. Surprisingly, our experiments revealed that β2 integrin function could not be promoted by Mn^{2+} on apoptotic cells, implying that the membrane lipid[18] and cytoskeletal alterations associated with apoptosis may pose additional restrictions upon receptor function that ensure a lack of neutrophil response to environmental signals.

Apoptotic cell subsets

In the course of screening monoclonal antibodies derived from several independent fusions for antibodies that bound specifically to apoptotic cells, it was notable that there were many clones producing antibodies that bound to subsets of neutrophils that had been cultured *in vitro*. Typical dual fluorescence profiles from a recent screen are shown in Figure 1. At least two distinct staining patterns can be identified. First, some antibodies bind a subset of apoptotic (annexin V positive) neutrophils that exhibit reduced forward/side scatter properties. Further analysis reveals that these cells are distinct from apoptotic cells with loss of membrane integrity and exposure of nuclear material (propidium iodide positive). The proportion of cells with this phenotype increases when neutrophils are cultured for prolonged periods or following conditions of stress, although evidence that they are derived from early apoptotic cells is lacking. Since these cells do not bind isotype-matched antibody non-specifically, one implication is that certain antigens become accessible to antibody when neutrophils lose membrane integrity.

A second identifiable staining profile observed is binding of antibodies to a subset of neutrophils that are annexin V positive, yet distinct from membrane permeable cells (Figure 1). We have previously described that this population represents 'late' apoptotic cells, with distinctive morphological appearance. Our data indicate that progression of neutrophils from early apoptosis to this late apoptotic phenotype is a relatively slow process. In contrast, lymphocytes rapidly acquire the late apoptotic phenotype following induction of apoptosis. Electron micrographs of affinity isolated late apoptotic neutrophils, show extensive nuclear degradation (see representative transmission electron micrograph in Figure 1). Whilst it was possible to select neutrophils from this population that exhibited intact plasma membranes, many of the cells showed one or more points of loss of integrity. Whether this represents the fragility of these cells during processing for electron microscopy is not clear. However, what is notable is that the extensive retention of intracellular granules within these neutrophils. Mechanisms for clearing late apoptotic cells may represent a backup pathway for ensuring that failure to clear early apoptotic cells does not lead to cells entering a necrotic phase. Surprisingly, given the potential importance of apoptotic cell removal in so many diverse processes, there have been few studies that have compared molecular mechanisms and functional consequences of phagocyte clearance of early and late apoptotic cells.

Interestingly, late apoptotic neutrophils have been shown to bind a number of plasma proteins that are importance in homeostatic regulation. Binding of these molecules does not appear to represent nonspecific protein binding capacity since other proteins (e.g. labelled bovine serum albumin) do not bind to these cells. These cells bind the matricellular protein thrombospondin and also bind C-reactive protein and C1q.[10,19] At present, it is not clear whether these bound proteins are accessible to phagocytes or not. However, the specific binding of proteins that have been implicated in the recognition and subsequent phagocytic clearance of apoptotic cells may have profound importance in determining how these cells may be removed if they are present at inflammatory sites. From the information we have relating to the potential for opsonisation of late apoptotic cells, it seems likely that their clearance may differ both in terms of phagocyte recognition pathways engaged and in efficiency of internalisation.

Apoptosis enabled receptors?

Recently, we have described a novel mechanism whereby apoptotic neutrophils become opsonised by immune complexes. This finding arose from the characterisation of a monoclonal antibody that exhibited a unique binding profile for neutrophils. After extensive characterisation, we found that this murine IgG1 antibody rapidly formed immune complexes in the presence of the antigen (a serum protein) and bound to apoptotic neutrophils via an interaction of the Fc portion to FcγRII.[20] Surprisingly, antibody–antigen complexes did not bind, or bound weakly to freshly isolated or cytokine/chemokine activated neutrophils, despite abundant expression of FcγRII on these cells. We believe that this alteration in ligand binding activity is the first example of a molecule that shows reduced expression on apoptotic cells, yet exhibits enhanced function. The molecular mechanism(s)

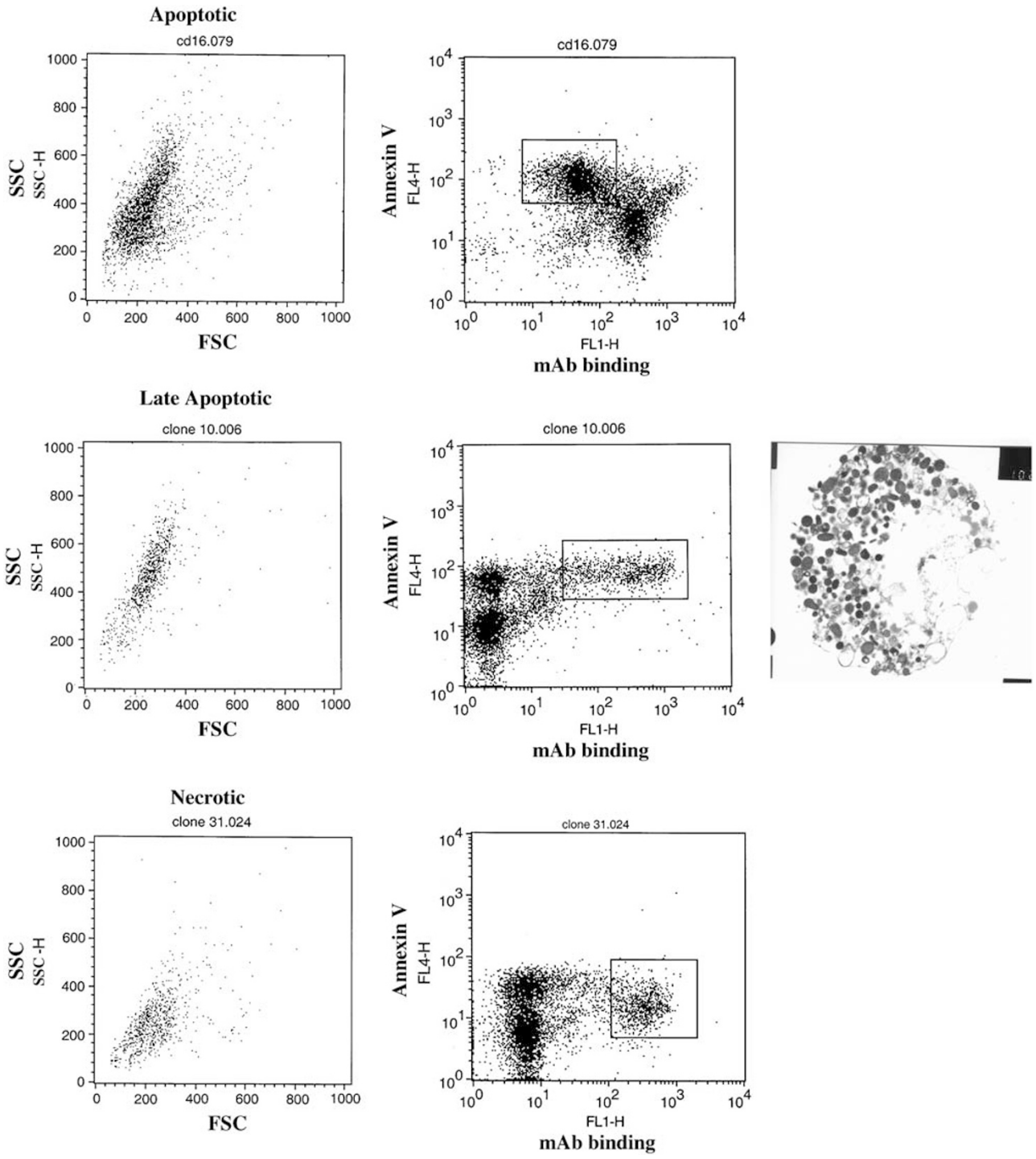

Figure 1 Subpopulations of *in vitro* cultured neutrophils defined by monoclonal antibodies. Human neutrophils were cultured *in vitro* for 20 h in Iscove's DMEM containing 10% autologous serum. Cells were labelled with murine mAb and FITC-conjugated $F(ab')_2$ anti-mouse IgG (FL1) together with annexin V APC (FL4); CD16 mAb 3G8 was used to define apoptotic neutrophils Dransfield et al[12], uncharacterised mAb (clone 10) as an example of binding to 'late' apoptotic cells, and clone 31 as an example of binding to necrotic cells. Representative histograms show laser scatter properties; forward scatter (FSC) and 90° scatter (SSC) for the populations defined by the mAb. Typical two colour immunofluorescence profiles are shown to illustrate gates used for laser scatter plots. A representative electron micrograph of a 'late' apoptotic cell affinity isolated using mAb BOB78[10] showing extensive nuclear degradation, lack of intact plasma membrane and retention of granule contents

responsible for this effect remains to be defined. It is possible that other, as yet unidentified, surface receptors may behave in a similar manner. Although the significance of opsonisation of apoptotic neutrophils by immune complexes during inflammation is not known, it is likely that subsequent phagocytic clearance will be affected.[21]

Summary

In conclusion, since the first observation that neutrophils undergo programmed cell death in 1989,[22] there has been tremendous progress in defining the underlying regulatory mechanisms and the consequences in terms of gene expression patterns, functional activity and membrane receptor alterations. However, the issues relating to heterogeneity of apoptotic cell phenotype discussed here have profound implications for future studies of phagocyte recognition, uptake and, crucially, phagocyte responses following phagocytosis of apoptotic neutrophils.

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