Granulocyte apoptosis and inflammatory disease

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We have described a novel pathway available for the clearance of extravasated granulocytes whereby the cells undergo apoptosis, a process which controls the functional longevity of granulocytes in situ and the rate of which is modulated by external and internal control mechanisms. It leads to shut-down of the secretory processes and recognition of the intact senescent cell by a novel macrophage recognition mechanism which fails to stimulate the release of pro-inflammatory mediators. Thus, by contrast with a granulocyte fate involving necrosis, apoptosis is likely to represent an injury limiting tissue removal process for granulocytes which would tend to promote resolution processes. It is speculated that dysregulation of this process or an imbalance between it and necrotic pathways may be important in inflammatory disease pathogenesis. Whether or not this is the case, the apoptotic mechanisms available in neutrophil and eosinophil granulocytes provide opportunities for the selective induction of apoptosis in specific inflammatory cells in what may represent novel therapeutic approaches to inflammatory disease.

It has been recognised for centuries that the inflammatory response is an important and effective component of host defences against injury and infection. Indeed, it was considered as an entirely beneficial process; for example, as described by Metchnikoff: ‘a salutory response to some injurious influence’. In recent decades, however, it has become clear that, paradoxically, inflammation is also critically involved in the pathogenesis of a wide range of diseases that have assumed great importance in the developed world. These include: myocardial infarction/reperfusion injury, atherogenesis, chronic bronchitis and emphysema, asthma, and the arthritides and glomerulonephritides. In these and in many other important inflammatory diseases, persistent accumulation and activation of inflammatory cells is associated with the tissue injury, architectural disruption and excessive fibro-proliferative responses that lead to organ dysfunction and failure. Indeed, with current knowledge of the pro-inflammatory and fibrogenic capacity of inflammatory cells and their products, it is perhaps surprising that inflammation ever resolves! However, there are remarkable examples of the normal capacity of the acute inflammatory response to resolve completely and there is clear evidence that apoptosis plays an important role in these resolution processes. It is plausible, indeed likely, that failure of apoptosis and
subsequent clearance processes may represent hitherto unrecognised pathogenetic mechanisms in inflammatory disease, yet the proof of this concept will be difficult when inflammatory disease probably results from a quantitative imbalance between detrimental effects of inflammation and tissue protective mechanisms, rather than a single qualitative defect, and we do not yet possess definitive cellular markers of apoptotic and necrotic cells with which to dissect these processes \textit{in situ} in inflamed tissues. Nevertheless, whether or not such proofs are forthcoming, it should still be possible to harness the surface receptor-mediated mechanisms available for the selective induction of inflammatory cell apoptosis and subsequent tissue clearance as a new approach to the treatment of inflammatory disease.

In the remainder of this article, therefore, we will discuss the role of apoptosis in the normal resolution and control processes of acute inflammation, possible routes whereby disordered apoptosis may lead to inflammatory disease states and, finally, how the major mechanisms of apoptosis might be harnessed in the development of new therapeutic approaches to inflammatory disease.

\section*{Apoptosis and the normal resolution and control mechanisms of inflammation}

Over the past few years, we have been studying the normal resolution processes of inflammation in the hope that this might generate new insights into the persistent inflammatory states that characterise inflammatory diseases. Perhaps the most dramatic example of inflammatory resolution is provided by the inflammatory response in lobar streptococcal pneumonia. In this archetypal example of ‘beneficial inflammation’, the affected lobes of the lung fill with granulocytes and activated macrophages. There is clear evidence that this response not only saved the lives of more than 80% of patients in the pre-antibiotic era but, in more than 95% of cases, the process resolved completely with fewer than 2.5% of cases progressing to fibrosis\textsuperscript{1}. While the resolution of inflammation is undoubtedly complex, one simple pre-requisite is that extravasated inflammatory cells and their histotoxic contents must be effectively removed from inflamed tissues.

\section*{Removal of extravasated granulocytes from inflamed tissues}

The neutrophil granulocyte is the archetypal acute inflammatory cell; it is the first cell to migrate to the inflamed site, a number of subsequent
inflammatory events including monocyte emigration\textsuperscript{2} and the generation of oedema\textsuperscript{3} appear to depend on this initial response, and the neutrophil has been directly implicated in the pathogenesis of a variety of inflammatory diseases\textsuperscript{4}. Neutrophils contain a large number of agents with the capacity not only to injure tissues\textsuperscript{5}, but also to generate further chemotactic agents\textsuperscript{6} and to cleave matrix proteins into chemotactic factors\textsuperscript{7}, which would further amplify the inflammatory response. Eosinophil granulocytes also contain a number of injurious products that are common with the neutrophil as well as others, e.g. major basic protein and eosinophil peroxidase that appear to be eosinophil specific. They have been specifically implicated in the pathogenesis of allergic diseases such as asthma\textsuperscript{8}. Until recently, it had been widely assumed that extravasated granulocytes underwent disintegration (necrosis) at the inflamed site before their fragments were removed by local phagocytes\textsuperscript{9}. However, this perception was based on the study of diseased tissues rather than resolving ‘beneficial inflammation’ and, if this was the rule, healthy tissues would inevitably be exposed to large quantities of histotoxic granulocyte contents. Moreover, there is evidence, largely forgotten, from Metchnikoff’s work more than a century ago, of an alternative removal process by which intact neutrophils are ingested by macrophages\textsuperscript{10}. Although there have been numerous sporadic reports
over the intervening decades of neutrophils within tissue macrophages, the significance for the control of inflammation and the mechanisms underlying these observations have only recently been addressed. In 1982, Newman et al. showed that human peripheral blood neutrophils...
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after 'ageing' in culture became recognised and ingested by human monocyte-derived macrophages (but not by monocytes themselves), although the underlying mechanisms were obscure. Since then, we have shown that neutrophils constitutively undergo apoptosis (Fig. 1), that the rate of this process is controlled by internal and external influences, that it leads to the shut-down of neutrophil secretory processes and subsequent recognition of the intact senescent neutrophil by macrophages (Fig. 2A) in a 'silent' process utilising novel recognition mechanisms that fail to provoke macrophage release of pro-inflammatory mediators (Fig. 2B)\(^{12}\).

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Neutrophils derived from peripheral blood or harvested from inflamed joints remain intact and continue to exclude vital dyes and to retain their enzymatic content for up to 24 h in culture \textit{in vivo}. During this period, there is an exponential increase in the number of cells which demonstrate the classical cytological and ultrastructural appearances of apoptosis\(^{13}\). By contrast with many other cell types, this occurs constitutively in the neutrophil without the need for external stimulation and it is likely that this reflects the short constitutive half-life (6 h in the blood) of this end-cell. During prolonged \textit{in vitro} culture (<24 h), neutrophils increasingly undergo 'secondary necrosis', admit vital dyes, such as trypan blue, and begin to disgorge their granule contents. However, when neutrophils are cultured beyond 24 h in the presence of macrophages, the removal of apoptotic cells is so rapid and effective that no trypan blue-positive neutrophils are seen and there is no release of granule enzymes into the external medium. \textit{In vitro}, macrophages can recognise, ingest and destroy large numbers of neutrophils with remarkable speed, such that for electron-microscopical studies, it is necessary to fix macrophages within minutes of their interaction with apoptotic neutrophils in order to demonstrate recognisable neutrophils within phagosomes because, thereafter, the ingested cells become degraded. The rapidity and effectiveness of this process is probably part of the explanation why the importance of these mechanisms in inflammatory cell and tissue kinetics has not been fully appreciated until recently. Nevertheless, there are now several clear examples of apoptotic neutrophils and their uptake by macrophages in acute inflammation and its experimental models\(^{13-15}\).

Several lines of experimental evidence have emerged in support of the hypothesis that, by contrast with necrosis, apoptosis provides a
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granulocyte clearance mechanism that would tend to limit tissue injury and lead to resolution rather than persistence of inflammation:

1. Unlike necrosis, during apoptosis the cell membrane remains intact and the cell retains cytosolic enzymes and other potentially injurious and pro-inflammatory agents.

2. The neutrophil normally releases a proportion of its granule contents, including enzymes, on external stimulation with inflammatory agents such as formylated bacterial peptides. However, during apoptosis the cell loses the ability to degranulate with external stimulation, suggesting it becomes 'functionally isolated' from environmental stimuli which would otherwise trigger responses with the potential to damage tissue. Since free apoptotic cells are seen at inflamed sites, such a mechanism could be important in the control of inflammatory tissue injury, particularly if there is a significant delay between neutrophil apoptosis and subsequent phagocytic ingestion of the apoptotic cells. Apoptosis also causes major down-regulation of other neutrophil functions including stimulated phagocytosis and both selectin and integrin-mediated adhesion events. The mechanisms responsible for the rapid shut-down of function are poorly understood, but apoptosis is associated with specific alterations in the expression of a number of surface receptors that are related to effector function, including partial loss of fMLP receptors and greater than 90% loss of others including the IgG receptor FcRy.III or CD16. Although the precise mechanisms which underlie receptor regulation during apoptosis are unknown, there are striking parallels with activation-induced changes in receptor expression. Notably, those receptors which are shed by a proteolytic cleavage mechanism during activation, e.g. CD16, L-selectin, p55 and p75 TNF receptors and CD43 all show marked reduction in surface expression during early apoptosis suggesting that part of the activation programme may be engaged during apoptosis.

3. Large numbers of apoptotic neutrophils can be cleared by macrophages without 'leakage' of potentially injurious neutrophil contents into the surrounding medium.

4. Macrophages usually release pro-inflammatory mediators, e.g. thromboxane, enzymes and cytokines after ingestion of particles. However, even maximal uptake of apoptotic neutrophils or eosinophils fails to stimulate macrophage release of pro-inflammatory mediators. However, if granulocytes are cultured in vitro beyond apoptosis to the point at which they fail to exclude vital dyes, their subsequent ingestion provokes massive release of macrophage
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mediators, and a similar response is observed when apoptotic granulocytes are deliberately opsonised prior to the phagocytic interaction (Fig. 2B). These findings demonstrate that the 'silent' removal of apoptotic cells by macrophages is determined by the apoptotic phenotype and is mediated by the novel recognition mechanisms employed by macrophages for the phagocytosis of apoptotic cells.

Regulation of granulocyte apoptosis

It has become clear that the constitutive rate of apoptosis in neutrophils is under different controls than those described in stimulated lymphocytes and thymocytes. Hence, cycloheximide enhances the constitutive rate of apoptosis in neutrophils and agents that elevate $[\text{Ca}^{2+}]$, delay neutrophil apoptosis. A range of inflammatory agents, including LPS and GM-CSF, also inhibit neutrophil apoptosis and, in parallel, greatly enhance and preserve a number of neutrophil functions. Indeed, it now seems clear that apoptosis is the major mechanism controlling the 'functional longevity' of neutrophils at inflamed sites. Human eosinophils also constitutively undergo apoptosis, but at a much slower rate than neutrophils. As in neutrophils, GM-CSF profoundly inhibits the rate of eosinophil apoptosis, whereas IL-5 specifically inhibits eosinophil apoptosis, perhaps providing part of the explanation for the differential accumulation of eosinophils in allergic tissues.

Hypoxic conditions, which are observed in diseased tissues and at chronically inflamed sites, also inhibit apoptosis. Perhaps surprisingly, given that the neutrophil's physiological half-life in the circulation is only 6 h, the constitutive rate of neutrophil apoptosis can also be accelerated. Nitric oxide (NO) donors induce apoptosis perhaps explaining part of the beneficial influence of NO on inflammation. By contrast with other neutrophil priming agents, e.g. LPS and GM-CSF which inhibit neutrophil apoptosis, TNFα accelerates the rate of neutrophil apoptosis, particularly in the first 10 h of culture. Others first reported that ligation of Fas on the neutrophil or eosinophil surface can promote apoptosis, an observation repeated in our laboratory, and Fas-L appears to induce granulocyte apoptosis in vivo.

Although many inflammatory mediators, including the bacterial product LPS, exert inhibitory effects, neutrophil apoptosis is induced by phagocytosis of Escherichia coli. Moreover, in CR3 knockout mice, extravasated neutrophils show markedly delayed apoptosis ex vivo, and human neutrophil phagocytosis via CR3 appears to induce apoptosis by an NADPH-dependent mechanism. However, while the above CR3 dependent phagocytosis mechanism would not seem to confer great survival benefit to the ingested bacteria (since the apoptotic neutrophil is...
subsequently destroyed by macrophages), there are exciting historical hints that bacteria, like viruses in T cells, may have evolved mechanisms to subvert the inflammatory response by producing agents that rapidly induce 'neutrophil apoptosis', thereby limiting effective microbicidal activity. Three decades ago\textsuperscript{29}, it was found that a staphylococcal product caused neutrophil death. It was named 'Leukocidin' and, although not recognised as such at the time, electron microscopy showed classical intact apoptotic cells as a result of exposure of neutrophils to leukocidin. These early studies showed clearly that other cells, particularly lymphocytes, are not susceptible to such agents. Of great interest a recent study shows that leukocidin induces classical DNA fragmentation in neutrophils and its effects are resisted by prior GM-CSF treatment\textsuperscript{30}. It is possible that such naturally derived products, including bacterial toxins, may provide new therapies based on the induction of granulocyte apoptosis.

The mechanisms whereby some inflammatory mediators exert their powerful effects on the rate of neutrophil apoptosis are poorly understood. However, many chemotactic peptides signal via an elevation of intracellular $[\text{Ca}^{2+}]$, which we have shown exerts a major inhibitory effect on neutrophil apoptosis\textsuperscript{21}. Receptor-directed stimuli (such as the prostaglandins) as well as pharmacological agents (e.g. db-cAMP, forskolin) that elevate $[\text{cAMP}]$, have also been shown to inhibit neutrophil apoptosis by a PKA-dependent mechanism\textsuperscript{31}. The observation that the GM-CSF effect requires protein synthesis led to a search for neutrophil apoptosis-inhibitory proteins; agents such as Bcl-2 were plausible candidates since they inhibit apoptosis in myeloid cell lines\textsuperscript{32}. An exhaustive study was therefore undertaken to seek possible inhibitory roles of Bcl-2 and its associated proteins\textsuperscript{33}. However, we have not as yet detected expression of Bcl-2 or implicated its other family members in mature granulocytes, even when exposed to GM-CSF\textsuperscript{33}, LPS, or other important inhibitors of neutrophil apoptosis.

Although we did not expect to find major differences between the apoptotic control mechanisms of neutrophils and eosinophils (which are closely related in ontogeny), corticosteroids, acting directly via the same glucocorticoid receptor, clearly accelerate eosinophil apoptosis while profoundly inhibiting neutrophil apoptosis\textsuperscript{34}. While eosinophils behave in a similar manner to steroid-treated thymocytes, their apoptotic control mechanisms are not identical since increased [cAMP], inhibits eosinophil apoptosis but induces thymocyte apoptosis. These observations may be highly relevant to the known differential effects of corticosteroids on circulating and extravasated neutrophil and eosinophil numbers. They also suggest a potential underlying mechanism for a recent report that treatment of asthmatic patients with corticosteroids causes a wave of eosinophil apoptosis and macrophage engulfment of
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apoptotic eosinophils detected in airway secretions that is associated with clinical improvement. Perhaps, most importantly, identification of the intracellular mechanisms governing these divergent responses should provide new therapeutic targets for the selective induction of eosinophil apoptosis in allergic disease.

These observations suggest ways in which granulocyte apoptosis could be induced or promoted in what could represent a new approach to the treatment of inflammatory disease (see below). Moreover, differences between the control and neutrophil and eosinophil apoptosis could be harnessed to achieve cell selectivity.

Granulocyte apoptosis and the pathogenesis of inflammatory disease

The existence, under some circumstances, of remarkably effective resolution mechanisms for acute inflammation, and the association of inflammatory diseases with histological evidence of persistent inflammation suggests that some inflammatory diseases may arise as a result of failure of the normal resolution mechanisms. It can be seen from the illustrative diagram in Figure 3 that there are a number of steps in the

Fig. 3 Pathways for neutrophil fate in tissues.
mechanisms involved in the granulocyte apoptosis and clearance process where dysregulation could lead to the persistence of inflammation and excessive tissue injury that characterise inflammatory disease.

A primary imbalance between necrosis and apoptosis

A major wave of granulocyte necrosis would lead to the inevitable disgorgement of toxic neutrophil contents which would tend to exacerbate injury and amplify inflammation. Further, we have shown that macrophage uptake of necrotic granulocytes causes release of pro-inflammatory macrophage mediators, which would further amplify the response. While there are histological examples of diseases which might suggest that this mechanism has arisen, e.g. leukocytoclastic vasculitis in which there is major disintegration of neutrophils and disgorgement of their products, it is generally considered that inflammatory diseases are more likely to arise from quantitative imbalances between the detrimental effects of inflammation and host protective mechanisms, and in chronic diseases such imbalances may be subtle rather than dramatic. Furthermore, even in resolving ‘beneficial’ acute inflammation, in which apoptosis is clearly the major neutrophil removal mechanism, occasional necrotic cells are seen. Therefore, it could reasonably be hypothesised that in the evolution of chronic inflammatory states even a minor alteration of the balance between apoptosis and necrosis in favour of the necrotic process may lead to the persistent tissue damage associated with these diseases. Unfortunately, the studies to test such hypothesis will inevitably be extremely difficult for a number of reasons:

1. Single point counts of cells in ‘static’ histological sections are of no interpretative value without sensitive methods to measure cell influx kinetics and subsequent clearance kinetics (e.g. the rate of breakdown of apoptotic bodies within macrophages).

2. Such studies will need specific markers of the apoptotic cell and necrotic cell as well as markers to determine the cell type of origin from which the apoptotic bodies and necrotic cells are derived. DNA end-labelling techniques (e.g. TUNEL) have been used as markers of apoptotic cells in situ but apoptotic cells that have undergone secondary necrosis would also be positive and there are circumstances whereby the DNA from cells that have undergone primary necrosis might also be expected to be labelled by such techniques. Furthermore, it is not possible to determine, as yet, the cell type of origin of ‘late’ apoptotic bodies contained within macrophages.
Secondary necrosis of apoptotic granulocytes

Apoptotic cells are fragile and, even in static *in vitro* culture systems, in the absence of macrophages they undergo secondary necrosis and disintegration within a few hours (and it is possible that this process occurs more rapidly *in vivo*—pathway C in Fig. 3). Secondary necrosis of apoptotic cells is likely to occur if the subsequent apoptotic cell phagocytic clearance mechanism is ineffective for any reason. Again, a number of hypothetical situations could be predicted whereby this might occur.

1. If immature inflammatory monocyte/macrophages are present; since these cells are not very effective at recognising apoptotic cells (resident alveolar and peritoneal macrophages also do not recognise or ingest apoptotic neutrophils).

2. The macrophage clearance mechanism could be ‘mature’, yet overwhelmed by waves of granulocyte apoptosis. There are precedents for such mechanisms in murine models in which Fas is triggered with fatal results associated with evidence of massive apoptosis and secondary necrosis.

3. The surface mechanisms by which macrophages recognise apoptotic granulocytes could be inhibited by agents in the extracellular milieu of the inflamed site. For example, we have previously shown that macrophage recognition of apoptotic neutrophils is markedly inhibited in the presence of cations and by a reduction in local pH. It is of great interest that in chronically inflamed sites, a number of the agents that have been disgorged from granulocytes (e.g. a number of proteinases and major basic protein) are highly cationic. Furthermore, it has been known since the work of Menkin in the 1950s that low pH conditions exist at chronically inflamed sites.

Harnessing granulocyte apoptotic mechanisms to derive new therapies in inflammatory disease

Whether or not the above hypotheses are shown to account for the aetiology of some types of inflammatory disease, it should prove possible to harness the apoptotic mechanisms involved in the resolution of acute inflammation and to ‘drive’ such mechanisms in the more persistent inflammatory states that characterise disease processes. Evidence in support of such an approach is provided by experimental models in which Fas ligation attenuates inflammation and by the observation
that, in the treatment of human asthma with corticosteroids, clinical improvement is associated with evidence in airway secretions of the induction of eosinophil apoptosis and their uptake by macrophages. An effective strategy along these lines is likely to require a combination of approaches (see Fig. 4).

Receptor-mediated death signals

It is possible that, by utilising cell surface receptors that trigger death signals, it will be possible to achieve selective induction of apoptosis in specific inflammatory cells. For example, ligation of Fas promotes apoptosis in both neutrophils and eosinophils, whereas TNFα and corticosteroids specifically induce neutrophil and eosinophil apoptosis, respectively.

Inhibition of cell survival signals

If receptor-mediated induction of apoptosis is to be fully effective in vivo, it will be necessary to determine how to 'release the brakes' exerted on apoptosis by powerful survival signals, particularly GM-CSF and IL-5. The necessity of this additive or alternative approach is illustrated by our experiments in a rabbit model of streptococcal pneumonia in which neutrophil influx ceases by 16 h, but the neutrophil

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Fig. 4 Strategies for promoting the resolution of inflammation. Stimulating 'death receptors' on the surface of granulocytes and negating the influences of environmental 'survival factors' provide opportunities for selective manipulation of neutrophil and eosinophil removal mechanisms in tissues.
exudate takes 48–72 h to clear, whereas neutrophils cultured in vitro would all have undergone apoptosis within 24–36 h. Similarly, it is now widely accepted that, in allergic tissues, GM-CSF and IL-5 extend the lifespan and inhibit the clearance of eosinophils, and that this represents the major mechanism controlling the tissue load of these cells. Finally, neutrophils exposed in vitro to GM-CSF are not only inhibited in their constitutive rate of apoptosis (by up to 50–80%) but are resistant to external ‘death signals’ including Fas-L and TNFα and leukocidin, thus reinforcing the need to elucidate the mechanisms governing these inhibitory effects.

Promotion of phagocytic clearance mechanisms for apoptotic granulocytes

Any highly effective manoeuvre which results in the major induction of granulocyte apoptosis is likely to require a parallel strategy to promote the phagocytic clearance of apoptotic cells before they undergo secondary necrosis. It has been shown that macrophage clearance of apoptotic neutrophils can be enhanced by various cytokines and by activation of macrophage protein kinase C. Perhaps of more specific importance, we have recently found that macrophage clearance of apoptotic neutrophils is regulated independently following the induction of a novel recognition mechanism by ligation of macrophage CD44. Finally, other cells appear to be able to act as ‘semi-professional’ phagocytes and an alternative therapeutic strategy might be to provide such cells with the receptors necessary for the specific clearance of apoptotic granulocytes.

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