The function and fate of neutrophils at the inflamed site: prospects for therapeutic intervention

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ABSTRACT – Neutrophils play a key role in the immediate non-specific immune response, and defects in their function increase host susceptibility to a range of infective agents. However, excess activation and/or delayed clearance of these cells from an inflamed site can lead to significant tissue damage. Neutrophil priming by agents such as endotoxin, granulocyte macrophage colony stimulating factor (GM-CSF), platelet activating factor (PAF) and tumour necrosis factor-α (TNFα) may play a pivotal role in modulating the adhesive and secretory properties of these cells. Priming also appears to affect the survival of neutrophils by delaying constitutive apoptosis. The unique signal transduction events that control neutrophil priming and apoptosis, and particularly the importance of the phospholipase C and phosphoinositide 3-kinase pathways, suggest opportunities for selective pharmacological intervention.

The polymorphonuclear leukocyte, or neutrophil, is the most abundant myeloid cell in the circulation and plays a fundamental role in the non-specific immune response. It is recruited rapidly to sites of inflammation or injury and plays a critical role in bacterial phagocytosis and killing. The importance of neutrophils in host defence is supported by the predisposition of patients with qualitative or quantitative neutrophil deficiency states (eg neutropenia, chronic granulomatous disease or leukocyte adhesion deficiency) to develop gram-negative and gram-positive bacterial infections. However, despite such overt benefits, the capacity of these cells to generate and release large amounts of histotoxic and pro-inflammatory agents (eg elastase, myeloperoxidase, arachidonic acid, leukotriene B4 (LTB4), reactive oxygen and nitrogen intermediates) indicates that any loss of control of neutrophil function can result in catastrophic collateral damage to host tissues. It is now recognised that tissue injury observed in a variety of clinical conditions, including acute respiratory distress syndrome (ARDS), ischaemia-reperfusion injury and rheumatoid arthritis results from inappropriate or uncontrolled neutrophil activation and/or a defect in the normal mechanisms that exist to remove these cells from an inflamed site. Therefore, understanding the cellular processes and mechanisms that regulate the function and fate of neutrophils, especially once these cells have accumulated at the inflamed site, may allow therapeutic intervention in a number of currently refractory inflammatory diseases.

Data derived from both in vitro and in vivo studies point to the importance of two cellular events in regulating the secretory capacity and longevity of the neutrophil; these processes are termed neutrophil priming and apoptosis (Fig 1). With regard to priming, it is not widely appreciated that circulating neutrophils derived from healthy donors are unable to degranulate or undergo respiratory burst activity when challenged by a secretagogue agonist unless they have first been in contact with agents such as endotoxin, TNFα or GM-CSF. This process, which requires the neutrophil to be 'primed' in order to acquire the capacity to undergo full receptor-mediated activation, offers a critical control point preventing inappropriate or premature cell activation. This has particular relevance in preventing activation of cells in the intravascular granulocyte pool, which may have catastrophic consequences.

The importance of neutrophil priming has been clearly demonstrated in a number of in vitro and in vivo models of neutrophil-mediated cell damage where priming appears to be a pre-requisite for tissue injury. However, in patients where there is evidence of systemic neutrophil priming (eg inflammatory bowel disease, systemic vasculitis, graft versus host disease or sepsis), while there is significant retention of primed cells within the pulmonary capillary bed, granulocyte migration into the lung interstitium and lung damage does not occur unless there is a secondary stimulus (eg infection). The hypothesis that priming is the 'master controller' of neutrophil function is supported by evidence suggesting that priming is also reversible, with neutrophils able to undergo a full cycle of priming, de-priming and re-priming. Priming also has a major impact on the survival of these cells by influencing their susceptibility.
to apoptosis. This review will outline the cellular events and signalling mechanisms involved in neutrophil priming and apoptosis, highlighting how such information may explain the effectiveness (or ineffectiveness) of current anti-inflammatory regimes and point to alternative therapeutic targets.

Functional consequences of neutrophil priming

Priming can be induced by incubating neutrophils with a variety of cell and bacterial-derived products (eg, PAF, TNFα, GM-CSF, interleukin-8 [IL-8], endotoxin), exposure to tonic or shear stress, or via adhesion to a number of biological or artificial surfaces. This results in an upregulation of neutrophil cell surface integrin expression and function, cell shape change (polarisation), reduced cell deformability, and an enhanced capacity of the cell to degranulate or release superoxide anions when subsequently challenged by a secretagogue agonist (Fig 2). Although in extreme circumstances neutrophils may be exposed to significant amounts of soluble TNFα or lipopolysaccharide within the circulation, the usual site for neutrophil priming is thought to be at the (activated) endothelial cell surface. This is where the neutrophil is exposed to cell-associated priming agents such as PAF and TNFα. Knowledge of the events involved in β2 integrin-mediated neutrophil adhesion and subsequent diapedesis also predicts that the majority of neutrophils that have migrated to the interstitial spaces at an inflamed site arrive in a primed and hence ‘alert’ state. However, little is known about the mechanisms that exist to prevent premature activation of neutrophils, especially at the point where these cells cross the delicate endothelial cell layer.

One suggestion is that the distinct collagen make-up of the basement membrane, in particular the presence of collagen type IV, may be crucial in this respect.

The potential pathophysiological relevance of neutrophil priming is illustrated by two important studies. First, Smedley et al demonstrated that the ability of N-formyl-Met-Leu-Phe (fMLP) or C5a-stimulated neutrophils to damage endothelial monolayers in vitro was potentiated when cells were initially exposed to lipopolysaccharide.

**Figure 2.** TNFα priming of fMLP-stimulated O2− generation in human neutrophils: concentration response and time course. Human neutrophils were incubated with 0–10 000 U/ml TNFα for 30 min prior to stimulation with buffer (open bars) or 100 nM fMLP (closed bars). Quantification of O2− release (A) during a 10 min incubation with fMLP by superoxide dismutase-inhibitable reduction of cytochrome c (data points represent mean ± SEM of three separate experiments each performed in triplicate) and (B) with lucigenin-dependent chemiluminescence (LDCL), monitored at 14-s cycle intervals in: control unprimed cells; TNFα-primed (100 U/ml, 30 min) unstimulated cells; unprimed fMLP (100 nM)–stimulated cells and TNFα-primed, fMLP-stimulated cells. Data are from a single experiment, representative of four. (Reproduced with permission from Condiffe et al, FEBS Lett 1998;439:147–51.)
(which itself was non-damaging). Second, intravascular administration of lipopolysaccharide in addition to fMLP in rabbits greatly enhanced neutrophil sequestration in the lungs and lung damage, an effect which was not seen when either agent was used alone. In this latter study, the lung damage induced by the combination of lipopolysaccharide and fMLP was completely abrogated by prior nitrogen mustard-induced neutropenia, and hence was entirely neutrophil dependent. It is likely that neutrophil priming and activation are both required for effective bacterial clearance at the site of infection and that such a response (eg in streptococcal pneumonia) does not in itself lead to uncontrolled neutrophil activation or tissue damage. It is currently speculated that the extent of vessel and organ damage may reflect the balance between appropriate 'physiological' degranulation and respiratory burst activity (such as that occurring within the intracellular phagolysosome) and 'inappropriate' extracellular release of granule contents and reactive oxygen intermediates. Despite the proposal (see below) that all tissue neutrophils, irrespective of their activation status, meet their fate by undergoing apoptosis and are removed by macrophage phagocytosis, we remain uncertain as to the absolute or relative importance of this disposal mechanism and whether other potential 'escape' mechanisms exist. Data suggesting that neutrophils can return to a fully de-primed state following PAF incubation, and that neutrophils sequestered in the pulmonary capillary bed following inhaled PAF challenge subsequently return to the central circulating granulocyte pool, indicate that some degree of 'back traffic' to the blood may be possible (Fig 3).

Mechanisms of neutrophil priming

While a considerable amount is understood regarding the assembly and activation of the multicomponent NADPH (or 'respiratory burst') oxidase that underlies superoxide anion generation, less is known about the upstream signalling pathways that trigger this process. The current model suggests that receptor-mediated activation of the NADPH oxidase requires the generation of at least two second messenger molecules, inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) [see 14 for refs]. These are both derived from a common substrate, namely PtdIns(4,5)P_2, and appear to have independent but essential functions in mediating respiratory burst activity. Hence, agonist-stimulated superoxide anion generation is completely abolished by pharmacological inhibition of the phosphoinositide 3-kinase (PI3K) responsible for PtdIns(3,4,5)P_3 generation and by intracellular ion chelators that block the Ins(1,4,5)P_3-induced rise in intracellular calcium, Ins(1,4,5)P_3 and PtdIns(3,4,5)P_3 are thought to co-operate in activating the Rho family GTPase Rac2 which is restricted in expression to haematopoietic cells. This activation of Rac2 may well

**Fig 3. Functional consequences of neutrophil priming.**

**PRIMED NEUTROPHIL**

- Minimal secretion and O_2^- release unless stimulated by secretagogue
- Up-regulated β_2^-integrin function
- Enhanced survival

**ACTIVATION**

eg fMLP, C5a, LTB_4, IL-8

**PRIMING**

eg LPS, TNFα, adhesion, GM-CSF

**CIRCULATING NEUTROPHIL**

? De-priming

**APOPTOSIS**

? Degrnuation, O_2^- release

Necrosis

?
involve the upstream guanine-nucleotide exchange factor Vav, which facilitates guanosine di/triphosphate (GDP/GTP) exchange and dissociation of Rac2-GTP away from its chaperone inhibitor guanine nucleotide dissociation inhibitor (GDI) Thereafter activated Rac2 is thought to promote phosphorylation and membrane translocation of the p67phox and p47phox subunits of the NADPH oxidase. This latter step may be dependent on activation of p21-activated kinase (PAK) and require phospholipase D (PLD)-derived phosphatidic acid.

To explain the molecular basis of neutrophil priming we need to either determine what is missing in the above cascade of events when unprimed neutrophils are stimulated with agents such as fMLP (where superoxide anion release is minimal), or understand the impact of priming agents on secretagogue-mediated signalling events. While this is an area of considerable controversy [see ref 2 for recent review] a few common themes appear to be emerging:

1. When analysed at a single cell level, priming appears to reflect the capacity of agents to recruit previously unresponsive cells to a responsive pool (ie priming is not a graded response shared by all cells)\(^1\).

2. The changes in cell surface receptor and G-protein expression that have been reported following priming appear to lag behind functional priming and can be clearly dissociated from the events that underlie the upregulation of secretory events. This suggests that these effects follow rather than drive the priming process and most likely reflect translocation of preformed receptors to the cell surface from intracellular vesicles.

3. Priming does not influence the ability of secretory agents to activate phospholipase C (as judged from analysing Ins\((1,4,5)\)P\(_2\) accumulation) and has little impact on the size of the calcium transient following secretagogue stimulation\(^2,14\).

4. While many priming agents are able to stimulate arachidonic acid, intracellular PAF and LTB\(_4\) generation, and to activate phospholipase A\(_2\), these events do not appear to play a direct role in neutrophil priming\(^17\). This suggests that priming may reflect a change in the PI3K/PtdIns(3,4,5)P\(_3\) and/or PLD/phosphatidic acid signalling pathways. Indeed, recent studies indicate that TNF\(\alpha\) can facilitate both receptor-mediated PtdIns(3,4,5)P\(_3\) and phosphatidylcholine-derived phosphatidic acid production in a manner that correlates closely with the functional activity of the NADPH. With PLD activation potentially lying downstream of PtdIns(3,4,5)P\(_3\), this suggests that priming impacts at, or at a site proximal to, the PI3K. Since neutrophils contain a unique G\(\beta\gamma\)-regulated p101/p110 isofrom of the PI3K\(^18\) in addition to other tyrosine-kinase regulated class 1A PI3-kinas (p110\(\alpha\), p110\(\beta\), p110\(\delta\)), this may enable the targeting of this pathway in a cell selective manner.

**Key Points**

| Isolation of human peripheral blood neutrophils under strict endotoxin-free conditions reveals that such cells are normally only poorly responsive to secretagogue agents such as N-formyl-Met-Leu-Phe (fMLP) |
|---|
| Exposure of neutrophils to agents such as tumour necrosis factor-\(\alpha\) (TNF\(\alpha\)), granulocyte macrophage colony-stimulating factor (GM-CSF), platelet activating factor (PAF) or endotoxin, agents that themselves fail to induce superoxide anion release or degranulation, causes a dramatic increase in the response of these cells to secretagogue agonists |
| This 'conversion' of the neutrophil from a quiescent, non-secretory phenotype to a highly polarised, motile and hyper-responsive state may determine the degree of an inflammatory response and thereby dictate the extent of collateral tissue damage |
| *In vitro* and *in vivo* studies also indicate that most priming agents also have a major impact on the functional longevity of neutrophils by delaying the in-built capacity of these cells to undergo apoptosis |
| Recent insights into the unique mechanisms that control neutrophil priming and apoptosis suggest that selective intervention may be possible and could spawn the development of a novel group of anti-inflammatory agents |

**Functional consequences of neutrophil apoptosis**

As stated above, one of the more recently identified and additional effects of neutrophil priming agents is their ability to modulate the rate of constitutive apoptosis *in vitro* (Fig 4). This event has been the subject of a number of recent reviews [eg 19] and is proposed to be a major mechanism underlying the normal resolution of acute granulocyte-mediated inflammation\(^1\). Under *in vitro* conditions, individual neutrophils appear to be selected to undergo apoptosis in a random manner, with the overall rate of apoptosis in any population of cells determined by cell density, their time in culture, the presence or absence of serum, and contact with artificial surfaces\(^20\). The executionary phase of apoptosis appears to be extremely rapid (ie start to finish within a few minutes) and leads to irreversible chromatin condensation, nuclear collapse, cytosolic vacuolation and cell shrinkage. The functional consequence of this event is that the neutrophil, while retaining the integrity of its cell membrane, is unable to respond to exogenous secretagogue or priming agonists, is immobilised and, from a secretary and synthetic viewpoint, is inert. Interestingly, intracellular adenosine triphosphate (ATP) levels appear to be maintained in the apoptotic neutrophil. The additional key property of the apoptotic neutrophil is that it becomes instantly recognisable to the inflammatory
and alveolar macrophage, an interaction that results in prompt phagocytosis and cell removal.

Evidence indicating the importance of this granulocyte 'suicide/cannibalism pack' has come from a number of in vitro experiments including:

- neutrophil apoptosis and macrophage engulfment in models of resolving inflammation (eg following intratracheal instillation of lipopolysaccharide in rats\(^2\), or challenge of sensitised horses with mouldy hay) are most evident at the time of maximal neutrophil removal and resolution of inflammation
- treatment of asthmatic patients with corticosteroids causes a decrease in total sputum eosinophil numbers and a corresponding increase in apoptotic eosinophil numbers\(^2\)
- observations that the bronchoalveolar lavage fluid of rabbits infected with *S. pneumoniae* contains a factor that stimulates neutrophil apoptosis, with opposite findings pertaining to *E. coli* infection which initiates a more chronic and severe pneumonia\(^3\). Similarly, the bronchoalveolar lavage of patients with ARDS has been reported to contain a factor that delays the rate of spontaneous apoptosis in vitro, leading to speculation that the duration and intensity of the neutrophil-mediated tissue damage observed reflects inhibition of the normal process of apoptosis and cell removal.

### Regulation of neutrophil apoptosis in vitro

A large number of studies have now been undertaken to examine the capacity of inflammatory mediators and other factors relevant to the inflammatory microenvironment to influence the rate of neutrophil apoptosis in vitro. A number of common themes have begun to emerge, most notably that neutrophil apoptosis can be modulated and is influenced by many agents found at the inflamed site. Examples of this are the capacity of GM-CSF, IL-8, lipopolysaccharide, C5a and IL-6 to inhibit, and TNF\(\alpha\) and Fas-ligand (Fas-L) to accelerate the rate of neutrophil apoptosis (Figs 4 and 5). It is important to stress that not all priming and secretagogue agonists share this ability to influence the rate of constitutive neutrophil apoptosis. For example, PAF and fMLP on their own have little if any impact on the rate of apoptosis when assessed in vitro at 6 and 20 hours\(^4\). It is also apparent that under certain circumstances, neutrophils can be fully activated and undergo vigorous degranulation and respiratory burst responses without this having any immediate or delayed impact on the apoptotic programme. This suggests that these acute secretory/degranulation events do not in themselves trigger apoptosis or impact directly on the fate of these cells. The intracellular events controlling granulocyte apoptosis are very different from those operating in many other and often closely related cell types, in particular the thymocyte and T-cell. For example, an increase in intracellular calcium or cyclic adenosine monophosphate (cyclic AMP) stimulates lymphocyte apoptosis yet inhibits this process in neutrophils\(^5\,\,^6\). There are also major differences in the regulation of eosinophil and neutrophil apoptosis. The rate of apoptosis in the eosinophil, although naturally

![Fig 4. Schematic diagram of factors that influence the rate of constitutive apoptosis in human neutrophils.](image)

![Fig 5. Ability of TNF\(\alpha\) and Fas-L to stimulate neutrophil apoptosis in vitro.](image)
slower, can be increased by agents that elevate intracellular calcium, and quite strikingly by corticosteroids, which delay this process in neutrophils. Although initially these differences were unexpected and difficult to understand, they have offered some intriguing insights regarding the differential sensitivity of eosinophil and neutrophil-mediated inflammatory events to corticosteroid therapy. Following the realisation that neutrophils have their own quite discrete set of apoptotic triggers and inhibitors, it has been shown that these cells also contain a unique array of molecules involved in the executionary events of apoptosis. Mature neutrophils do not express bcl-2 or certain other anti- and pro-apoptotic members of the bcl-2 family of proteins, including Bcl-X<sub>L</sub> and gelsolin, with gelsolin being an important target of the caspases involved in triggering apoptosis<sup>30</sup>.

An important observation obtained from in vitro studies is the contrast that exists between the natural 'high' rate of spontaneous neutrophil apoptosis observed under normal culture conditions and the difficulties that arise when trying deliberately to stimulate this process. Hence, although both Fas-L and TNF<sub>a</sub> can stimulate neutrophil apoptosis in a receptor and caspase-dependent manner (Fig 5), the effect is relatively small and, at least for TNF<sub>a</sub>, such pro-apoptotic effects are readily lost if neutrophils are pre-treated with PAF or allowed to become adherent prior to TNF<sub>a</sub> stimulation<sup>24</sup>. However, these effects contrast with the strong pro-apoptotic effects of certain pathogenic bacteria and viruses. Hence, neutrophils derived from HIV-infected individuals have a higher rate of constitutive apoptosis than neutrophils from non-HIV infected control subjects<sup>31</sup>, and ingestion of <i>E. coli</i>, <i>M. tuberculosis</i> and <i>H. somnis</i> is capable of stimulating neutrophil apoptosis in vitro<sup>19,32</sup>. If this occurs in vivo such an effect may explain, at least in part, the capacity of such pathogens to inhibit neutrophil function and evade normal intracellular killing.

In summary, the presence per se of neutrophils at an inflamed site does not necessarily equate with tissue injury, and additional controls determine the functional status and fate of tissue neutrophils. Of these, neutrophil priming and apoptosis, processes that are themselves intimately linked, appear to be the key factors dictating whether the initial inflammatory response is appropriate or inappropriate and whether such inflammation resolves or persists. The nature of the biochemical events controlling neutrophil priming and apoptosis suggests opportunities for selectively modulating these. Not surprisingly, it is apparent that many bacteria and viruses appear to be ahead of the game in learning how to divert such events to their own gain.

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**Stroke Rehabilitation**

**Patient and Carer views**

A Joint report by the College of Health in conjunction with the Research Unit of the Royal College of Physicians

prepared by Marcia Kelson and Carman Ford

As part of the preparatory work for the development of national guidelines for stroke, the College of Health were commissioned by the Intercollegiate Stroke Group to conduct focus groups and interviews with a range of patients and carers. The aim was to ensure that, as well as the research evidence, patients’ views and experiences informed the guideline recommendations.

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