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Chapter 2

Activation, apoptosis and clearance of neutrophils in Wegener’s granulomatosis

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ABSTRACT

Wegener’s granulomatosis (WG) is strongly associated with the presence of anti-neutrophil cytoplasmic autoantibodies (ANCA). Within WG these ANCA are in most cases (80-90%) directed against the azurophilic enzyme proteinase 3, so called PR3-ANCA. A pathophysiological role for these autoantibodies, supported by numerous in vitro and in vivo studies, is particularly based on their capacity to bind and activate neutrophils and may, as such, potentially damage vessels.

In this review, the pathogenic potential of different developmental stages of the neutrophil in the pathogenesis of WG is discussed. After release from the bone marrow into the circulation, neutrophils can be primed by TNF-α and become attached to locally activated endothelium. Once attached to endothelium ANCA can fully activate these primed neutrophils. In this activation process, the degree of activation after stimulation with PR3-ANCA associates with the level of PR3 expression on the membrane of the neutrophil. Following activation, infiltrated neutrophils become apoptotic with further membrane expression of PR3. In WG patients clearance of apoptotic neutrophils can be disturbed, due to opsonisation of PR3-expressing apoptotic neutrophils with PR3-ANCA, thereby perpetuating inflammation by release of pro-inflammatory cytokines during clearance, or it may favour autoimmunity by PR3 presentation in an inflammatory environment. Furthermore, the presence of ANCA and the release of the vessel-related pentraxin PTX3 may lead to the persistence of late apoptotic neutrophils in tissues, thereby inducing leukocytoclastic lesions that are characteristic for patients with WG. All together, alive neutrophils as well as apoptotic neutrophils play a key role in different inflammatory phenomena seen in patients suffering from Wegener’s granulomatosis.
INTRODUCTION

ANCA-associated vasculitis

The antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV) are a group of autoimmune diseases characterized by necrotizing inflammation of arterioles, venules, and capillaries, although larger vessels may be involved as well. These autoantibodies are in most cases directed against the azurophilic granule proteins proteinase 3 (PR3) or myeloperoxidase (MPO) of the neutrophil designated as PR3-ANCA and MPO-ANCA, respectively. Based on histopathological and clinical manifestations the Chapel Hill international consensus conference defined three major categories of AAV: Wegener’s Granulomatosis (WG), microscopic polyangiitis (MPA), and Churg Strauss syndrome (CSS). PR3-ANCA are found in 80 - 90% of WG patients, to a lesser extent in patients with MPA and incidentally in CSS. In patients with MPA and CSS, MPO-ANCA are the dominant autoantibodies but they occur in a minority of WG patients as well.

These ANCA are supposed to play an important role in the pathogenesis of AAV. Their pathophysiological role is supported by clinical data, animal experimental models and in vitro data, which has been reviewed in various papers. Clinically, ANCA titers have been shown to correlate, although not uniformly, with disease activity. Specifically, an increase in ANCA titer frequently precedes a relapse whereas a decline in titer is seen when remission is induced. In animal models the pathophysiological role of ANCA has been supported by demonstrating that transfer of splenocytes from MPO-deficient mice immunized with MPO to Rag2-deficient mice, which lack functional B and T lymphocytes, led to the development of severe necrotizing and crescentic glomerulonephritis. Additionally, direct intravenous injection of anti-MPO antibodies, derived from MPO-immunized MPO deficient mice, into wild type or Rag2-deficient mice resulted in focal necrotizing glomerulonephritis. Finally, in vitro studies have shown that ANCA are capable of activating neutrophils leading to a respiratory burst and degranulation of noxious proteolytic enzymes which may induce endothelial injury.

Activated neutrophils within ANCA-associated vasculitis

The hypothetical model on how these ANCA activate neutrophils and harm the endothelium could be formulated as follows. First, neutrophils need to be primed with a low dose of tumor necrosis factor-α (TNF-α) to upregulate surface expression of the autoantigens PR3 and MPO in order to allow ANCA binding. However, probably more importantly, next to surface expression of autoantigens, priming facilitates clustering of FcγRIIa and β2 integrins and formation of the NADPH oxidase complex. Secondly, neutrophils need to have an adhesive state as reflected by CD11b/CD18 expression. Finally, when neutrophils are primed and firmly adhere to TNF-α activated endothelium, ANCA can fully activate neutrophils resulting in degranulation and reactive oxygen species (ROS)-production eventually leading to injury of the vessels. In this activation process, F(ab’)2 fragments of ANCA bind to their autoantigen, PR3 or MPO, whereas the Fc parts of ANCA interact with neutrophil Fcγ-receptors resulting in full activation although F(ab’)2 fragments of ANCA alone have been shown to induce minor activation as well.
In biopsies from WG patients it has been shown that neutrophils are really present and activated at sites of injury. These histological studies from the upper airways of Wegener’s patients demonstrated large numbers of activated, apoptotic and necrotic neutrophils in the early development of vasculitis. Furthermore, in renal infiltrates neutrophils are present next to monocytes. In addition, the activated state of neutrophils and extracellular localization of lysosomal enzymes was demonstrated in renal biopsies from patients with WG, and numbers of activated neutrophils present in renal biopsies correlated with renal tissue damage.

So, neutrophils seem to play an important role in the pathogenesis of ANCA-associated vasculitis. Therefore, the key role of neutrophils during their cycle of activation, apoptosis and clearance will be discussed in this review. In this the focus will be on neutrophils in PR3-ANCA-associated vasculitis.

**PR3 expression and activation**

PR3 is a serine protease mainly stored in the azurophilic granules of neutrophils, but it can be found in the specific and secretory vesicles as well. Moreover, PR3 can also be constitutively present on resting neutrophils. Priming of neutrophils with low dose of TNF-α facilitates fusion of secretory vesicles and specific granules resulting in a two- to three-fold upregulation of PR3 expression on the membrane of the neutrophil. PR3 can be expressed on the membrane (mPR3) of the total population or on a subset of neutrophils (Figure 1).

Individuals can be categorized according to their pattern of mPR3 expression on their resting neutrophils into those in whom all neutrophils express none to only minor levels of mPR3 (mPR3− individuals), individuals in whom all neutrophils express substantial levels of mPR3 (mPR3+ individuals), and so-called bimodal individuals in whom two subsets are present, that is a subset of neutrophils expressing none to minor levels of mPR3 and a subset expressing substantial levels of mPR3 (mPR3−/+. This pattern seems to be genetically determined. Interestingly, bimodal expression is not seen for elastase or other neutrophil markers. Various groups have studied the clinical significance of this differential mPR3 expression on neutrophils. Patients with PR3-ANCA associated vasculitis have an increased expression of mPR3 on their resting neutrophils as compared to healthy individuals. Furthermore, Rarok et al demonstrated...
that an increased expression of mPR3 in vasculitis was associated with an increased incidence and rate of relapse. In further in vitro studies, the functional significance of mPR3 expression for neutrophil activation after stimulation with anti-PR3 antibodies was analysed. We studied healthy individuals with different patterns of mPR3 expression and measured actin polymerization in neutrophils, as an early event in neutrophil activation after stimulation with anti-PR3 antibodies. It proved that even unprimed neutrophils can be activated by anti-PR3 antibodies, as measured by their F-actin polymerization. More importantly, levels of mPR3 expression were associated with the degree of F-actin polymerization after stimulation with anti-PR3 antibodies, further supporting the relation between mPR3 expression and susceptibility for relapses in patients with WG. Thus, increased mPR3 expression on neutrophils in WG is a risk factor for relapse, probably due to increased sensitivity for becoming activated by PR3-ANCA.

**Effects of apoptosis on PR3 expression of neutrophils**

The fate of neutrophils after activation is apoptosis. Apoptosis is a genetically programmed cell suicide, characterized by condensation of cytoplasm and intracellular organelles, cleavage of nuclear chromatin, and formation of apoptotic bodies with or without nuclear remnants. During induction of apoptosis several genes encoding proinflammatory factors, signal transduction mediators, adhesion molecules, and other inflammation-related proteins are down-regulated. Several groups have studied changes in the expression of the autoantigens PR3 and MPO on neutrophils and basophilic cells during apoptosis. When PR3 and MPO would be upregulated during apoptosis, this could provide an alternative mechanism for ANCA to interact with neutrophils. Gilligan et al were the first to show that, in the absence of priming, ageing neutrophils can translocate PR3 or MPO to the membrane during the apoptotic process, as assessed by increased ANCA binding. Using electron microscopy, translocation to the membrane of cytoplasmic granules, containing PR3, was demonstrated, suggesting that upregulation of the autoantigen was due to translocation of these granules. Four years later, Yang et al observed that incubation of neutrophils per se is already sufficient to translocate ANCA antigens. In that study, unstimulated neutrophils show increased ANCA antigen expression on the membrane after 4 hrs incubation, whereas those cells do not show signs of apoptosis. In addition, ageing of neutrophils for 24 hrs, by which 30% became apoptotic, resulted in increased expression of ANCA antigens. However, no significant difference in autoantigen expression could be found between apoptotic and non-apoptotic cells, implying that upregulation of these autoantigens was merely a result of priming by minor trauma resulting from in vitro incubation. In another study, apoptosis in neutrophils was accelerated by a low dose of TNF-α (10 ng/ml) in combination with the protein synthesis inhibitor cycloheximide. By this method, the pro-apoptotic effect of TNF-α is pronounced whereas anti-apoptotic effects exerted via the NF-κB pathway are blocked by cycloheximide. TNF-α-accelerated apoptotic neutrophils showed increased PR3 and MPO expression compared to TNF-α-primed alive neutrophils. However, increase of PR3 and MPO expression was only seen in a small proportion of apoptotic neutrophils, probably leaky neutrophils, whereas most apoptotic neutrophils showed no increase in cell surface expression. We may conclude from these studies that PR3
and MPO remain on the membrane of neutrophils during apoptosis and are accessible for ANCA. However, whether apoptosis intrinsically upregulates expression of these autoantigens on the cell membrane of neutrophils is not conclusive yet.

**Apoptotic neutrophils in vasculitis**
In ANCA-associated vasculitis neutrophils from the peripheral blood show increased apoptosis after incubation in vitro. In fact, these neutrophils are more primed, as assessed by increased ANCA-induced superoxide production, and they have increased expression of PR3 on their membrane in comparison to neutrophils of healthy controls. Ageing of neutrophils up to 24 hrs results in a decreased ability to produce superoxide upon stimulation with ANCA as well as with other potent stimuli. Accelerating apoptosis by TNF-α results in further abrogation of ANCA-mediated oxidative burst in neutrophils, which appears to be caspase-3 dependent. Also, ANCA itself have been shown to accelerate apoptosis of neutrophils. In a study of Harper et al, TNF-α-primed neutrophils incubated with IgG purified from ANCA-containing sera showed accelerated apoptosis compared to control IgG, a process which was dependent on oxygen radical generation. Interestingly, neutrophils in which accelerated apoptosis was induced by ANCA showed typical features of apoptosis such as shrunken nuclei and internucleosomal cleavage of DNA but no increased externalization of phosphatidylserine. Thus, ANCA induce increased apoptosis of neutrophils in AAV. These apoptotic neutrophils show upregulated surface expression of PR3, are, as such, accessible by ANCA, but cannot be stimulated to superoxide production by ANCA.

**Clearance of neutrophils in an inflammatory context**
Apoptotic neutrophils are recognized by phagocytic cells, resulting in their clearance by phagocytes. Clearance is a process of engulfment and digestion of apoptotic cells, mainly executed by macrophages in tissues. Apoptotic cells are recognized by their expression of certain surface membrane molecules, mostly altered carbohydrates or phospholipids. A crucial phospholipid in the recognition of apoptotic cells is phosphatidylserine. Normally, uptake of apoptotic cells by macrophages coincides with suppression of pro-inflammatory cytokines such as TNF-α and enhanced production of anti-inflammatory cytokines such as TGFβ, PGE2, and PAF through autocrine and paracrine mechanisms. In this way, clearance is safe and inflammation prevented. However, in ANCA-associated vasculitis autoantibodies can opsonize apoptotic neutrophils that express PR3 or MPO, leading into an enhanced uptake and production of pro-inflammatory cytokines as IL-1, IL-8 and TNF-α. As a result, clearance is unsafe and inflammation perpetuates. Another, potentially harmful, mechanism associated with opsonization by autoantibodies in an inflammatory context is the potential breakdown of tolerance, resulting in autoimmunity. Whether tolerance or immunity is established depends on the maturity state of the dendritic cells (DC). Immature DCs that internalize necrotic or apoptotic cells, needs maturation signals to mature. Maturation signals include lipopolysaccharide, interferon-α and TNF-α. Basically, T-cell tolerance is induced when apoptotic cells are engulfed in the absence of maturation signals. In contrast, T-cell activation and immunity may result when DCs ingest necrotic or apoptotic cells, for instance during a viral infection, leading to release of maturation signals that induce upregulation of MHC class II, and
subsequently presentation of (auto) antigenic peptides in a MHC class II-restricted manner to T-cells. Interestingly, opsonisation of apoptotic cells with anti-β2-Glycoprotein I (GPI) autoantibodies facilitated MHC class II antigen presentation by DCs. In addition, DCs that were challenged with anti-β2-GPI-opsonized apoptotic cells secreted interleukin-1β (IL-1β), TNF-α and IL-10, as such increasing immunogenicity of these apoptotic cells. Clayton et al. studied the effects of apoptotic and necrotic neutrophils on the phenotype of dendritic cells and their ability to stimulate allogeneic T cell proliferation. They showed that internalization of apoptotic and necrotic neutrophils by immature dendritic cells results in upregulation of CD83 and MHC class II, whereas CD40, CD80 and CD86 were downregulated resulting in a decreased ability to stimulate T cell proliferation. However, in the presence of TNF-α downregulation of these maturation markers after engulfment of apoptotic neutrophils was overcome to some extent, suggesting that apoptotic neutrophils in combination with cytokines at sites of inflammation may favor the development of autoimmunity. That apoptotic neutrophils under inflammatory conditions can be autoimmunogenic has been shown by a study of Patry et al. They showed that injection of syngeneic apoptotic neutrophils into Brown Norway rats resulted in the development of ANCA, although the rats did not develop disease. Thus, clearance of apoptotic neutrophils opsonized by ANCA will result in an inflammatory reaction, which promotes autoantigen presentation and, as such, augments the autoimmune response.

**Disturbances in the clearance of neutrophils**

In WG, 40 - 50% of the patients have cutaneous manifestations which are histopathologically, in most cases, defined as leukocytoclastic vasculitis (Figure 2). Leukocytoclastic vasculitis is characterized by infiltration and accumulation of unscavenged apoptotic or necrotic neutrophils or fragmented nuclei of neutrophils in the tissues around the vessels (leukocytoclasia), and swelling of endothelial cells accompanied by fibrinoid necrosis of vessel walls. Leukocytoclastic lesions are primarily found in the skin but other organs may be involved as well. Recent studies on the nature of the neutrophilic infiltrate showed strong positive staining with in situ nick end labeling techniques, thereby demonstrating DNA breakdown in the infiltrating neutrophils. In these lesions, only a minor part is typical apoptotic, whereas organelles and the majority of neutrophils shows breakdown of DNA with disintegrated cytoplasmic plasma membrane. The phenomenon of leukocytoclasia is interesting, since normally dying cells are quickly engulfed by phagocytes, particularly at sites of inflammation. Furthermore, accumulation of dying cells and the presence of nuclear debris in these lesions suggest that the removal of apoptotic or necrotic cells is incomplete. Pentraxins have been shown to be involved in the removal of dying cells. Classical pentraxins such as serum amyloid P (SAP) and C-reactive protein (CRP) bind to apoptotic cells in a calcium-dependent manner. Subsequently, opsonisation with CRP or SAP results in increased binding of the complement component C1q, thereby facilitating uptake. In addition, Bijl et al showed that SAP binds specifically to late apoptotic cells and regulate their clearance by macrophages. In contrast, another pentraxin, PTX3, seems to inhibit instead of...
facilitate uptake of apoptotic cells \(^{36,37}\). In view of the persistence of apoptotic neutrophils in leukocytoclastic vasculitis, the role of PTX3 in this phenomenon was further explored.

**PTX3 in vasculitis**

In vasculitic patients PTX3 levels correlate, independently from CRP, with disease activity \(^{38-40}\). PTX3 is produced locally at sites of inflammation under the control of pro-inflammatory signals, by endothelial cells, fibroblasts but also cells of the monocytic lineage \(^{41-43}\). PTX3 binds specifically to dying cells, either necrotic or apoptotic \(^{36}\), although binding of PTX3 to necrotic cells is less intense than to chemically or spontaneously induced apoptotic cells. Interestingly, human dendritic cells failed to phagocytose apoptotic cells in the presence of PTX3 \(^{36}\). In that same study, Rovere et al showed that PTX3 can bind to apoptotic neutrophils as well, however, only to late apoptotic neutrophils and not to vital or early apoptotic neutrophils. Subsequently, we showed that phagocytosis of late apoptotic neutrophils by macrophages was dose-dependently inhibited in the presence of PTX3, whereas SAP facilitated phagocytosis \(^{37}\). The inhibitory capacity of PTX3 on the clearance of late apoptotic neutrophils may explain for the persistence of apoptotic and necrotic cells at sites of leukocytoclastic lesions. However, PTX3 only inhibits the clearance of late apoptotic neutrophils. In other words, early apoptotic neutrophils should be normally engulfed by phagocytes, particularly in inflammatory tissue. So, where do these late apoptotic or secondary necrotic neutrophils come from. A possible
Role of neutrophils in WG

explanation comes from a study of Harper et al in which TNF-α-primed neutrophils incubated with IgG purified from ANCA–containing sera showed accelerated apoptosis compared to neutrophils incubated with control IgG. The former neutrophils showed typical features of apoptosis as shrunken nuclei and internucleosomal cleavage of DNA but no increased externalization of phosphatidylserine. Furthermore, these neutrophils were less well phagocytosed compared to apoptotic neutrophils incubated with control IgG, possibly due to defective phosphatidylserine expression. Taken together, ANCA and PTX3 both seem to be able to disturb normal clearance of apoptotic neutrophils by macrophages, as such being candidate factors in the development of leukocytoclastic phenomena in vasculitis.

CONCLUSION
Neutrophils are key players in the pathogenesis of Wegener’s Granulomatosis. At sites of injury neutrophils are present and their numbers correlate with damage. PR3-ANCA can activate these cells and, possibly, induce tissue damage. However, this is not all. The pathological potential of neutrophils continues after their death. Apoptotic neutrophils, expressing PR3 on their membrane, can be opsonized by PR3-ANCA resulting in unsafe, pro-inflammatory clearance by macrophages and presentation of PR3 or MPO by dendritic cells. In addition, in WG clearance of apoptotic neutrophils is sometimes incomplete as shown by the persistence of apoptotic neutrophils (leukocytoclasia). In these phenomenon, ANCA and a novel, vessel-related pentraxin PTX3 are supposed to play a role. In conclusion, the pathogenic potential of neutrophils may be exerted in WG during two stages of their lifetime: that is both in their vital and apoptotic state. Interaction of ANCA with vital neutrophils results in activation of these cells and tissue damage, whereas interaction with apoptotic neutrophils results in inflammatory clearance and boosting of the PR3- or MPO-specific autoimmune response.

REFERENCES


