The importance of resolution of inflammation in the pathogenesis of ANCA-associated vasculitis

Division of Medical Sciences, University of Birmingham, Birmingham B15 2TT, U.K.

Abstract
The primary small-vessel systemic vasculitides are disorders that target small blood vessels, inducing vessel wall inflammation, and are associated with the development of anti-neutrophil cytoplasmic antibodies. Multiple organs are attacked, including the lungs and kidneys. Increasing knowledge of pathogenesis suggests that the antibodies activate neutrophils inappropriately, leading to endothelial and vascular damage. Cytokines, such as tumour necrosis factor, can facilitate damage by priming the neutrophils and activating endothelial cells. Apoptosis of infiltrating neutrophils is also disrupted by anti-neutrophil cytoplasmic antibody activation, and removal of these effete cells occurs in a pro-inflammatory manner, promoting persistent inflammation. The autoimmune response may be promoted by aberrant phagocytosis of apoptotic neutrophils by dendritic cells. Understanding the pathogenesis can help to rationalize existing therapies and indicate new approaches to therapy.

Introduction

ANCA (anti-neutrophil cytoplasmic antibody)-associated small-vessel vasculitides
ANCA-associated vasculitis is the most common primary systemic small-vessel vasculitis in adults. It affects arterioles, venules and capillaries, but may involve larger vessels. ANCA-associated vasculitis includes three major categories, defined by the Chapel Hill International Consensus Conference [1] as WG (Wegener’s granulomatosis), MPA (microscopic polyangiitis) and the Churg Strauss syndrome. These diseases share a common pathology with focal necrotizing lesions of blood vessels, which affect many different vessels and organs. WG is characterized by granulomatous inflammation of the upper/lower respiratory tract combined with necrotizing pauci-immune glomerulonephritis in 80% of the patients. Although PR3 (proteinase 3)-ANCAs are found in the majority of patients with systemic WG, they are often absent from patients with limited disease (restricted to the respiratory tract). MPA is characterized by pauci-immune necrotizing glomerulonephritis and small-vessel vasculitis. Without evidence of granulomatous inflammation, 90% of the patients have shown involvement of other organs [2]. The aetiology of these diseases is not clear. Strong circumstantial evidence exists, but there is no direct evidence that ANCA-associated vasculitides are mediated through autoimmune responses. The agents that provoke the autoimmune response are unknown, although certain drugs (e.g. propylthiouracil), infections (Staphylococcus aureus) and environmental toxins (silica exposure) have been suggested to play a role [3].

Anti-neutrophil cytoplasmic antibodies
These vasculitides are characterized by a circulating antibody ANCA, specific for PR3 or MPO (myeloperoxidase), present in the azurophil granules of the myelomonocytic cell lineage. PR3-ANCAs have high specificity for patients with WG, whereas MPO-ANCAs are more commonly found in patients with MPA [4]. Clinical and laboratory studies support a role for these antibodies in disease pathogenesis. Antibody levels often mirror disease activity and those patients who remain antibody-positive despite disease remission are at high risk of disease relapse [5]. Animal studies also support a role for ANCA in the disease. Transfer of splenocytes from MPO-deficient mice immunized with MPO to Rag2 knockout mice, which lack functioning B and T lymphocytes, led to severe necrotizing and crescentic glomerulonephritis. Also, direct injection of anti-MPO antibodies into wild-type or Rag2 knockout mice resulted in a similar pathology, providing strong evidence of a pathogenic role for ANCA [6]. In vitro studies shed light on the mechanisms by which ANCs exert their inflammatory potential.

Activation of neutrophils by ANCA
Priming of neutrophils with pro-inflammatory cytokines such as TNFα (tumour necrosis factor α) increases the surface expression of PR3 and MPO, allowing interaction with circulating ANCA. ANCA activation leads to a respiratory burst with release of reactive oxygen species, degranulation with release of proteolytic enzymes and increased production of nitric oxide, chemokines and cytokines such as IL-1 (interleukin-1) and IL-8 [3]. In a flow model mimicking events in the circulation, ANCs have been shown to promote the adhesion of neutrophils on to TNF-activated endothelial cells, which depends on the integrin receptor CD11b [7].

Vasculitic lesions may be initiated by the inappropriate activation of neutrophils by ANCA in the microvasculature.
Activation of neutrophils by ANCA requires both recognition of PR3 or MPO via the Fab region of the antibody and interaction with the Fcy receptors on the neutrophil surface [8,9]; FcyRII and FcyRIII are constitutively expressed on the neutrophil surface. F(ab)2 portions of ANCA binding to PR3 or MPO recruits G, proteins [10], whereas a tyrosine kinase Syk is activated when ANCA Fc tails bind to Fc receptors [10a]. Both whole ANCA IgG and the Fab portions stimulate the small GTPase Ras, which is sensitive to pertussis toxin and tyrosine kinase inhibitors (unpublished work). Further downstream, ANCA stimulate PI3K (phosphoinositide 3-kinase) and its downstream mediator PKB (protein kinase B). Both the G, protein- and tyrosine-kinase-dependent pathways are necessary for PI3K activation. Tyrosine kinases and PKC are also important in generating the NADPH complex [8]. Activation of neutrophil by ANCA differs from cross-linking Fc receptors on the neutrophil, both in its phospholipase D-independent form and because a different isoform of PI3K is recruited [11]. Generation of the respiratory burst also requires ligation of β2 integrins [12]. Recruitment of these pathways is responsible for the dysregulated and disturbed behaviour of neutrophils activated by ANCA which leads to a respiratory burst, with activation of NADPH oxidase and degranulation in the wrong place at the wrong time, and hence to endothelial damage. Once a vesiculic lesion begins, other inflammatory cells are quickly recruited, including mononuclear cells such as monocytes and T cells. T-cell-mediated immunity is supposed to contribute to the pathogenesis of ANCA-associated vasculitis. An advanced vasculitic lesion has many similarities to delayed hypersensitivity reactions that promote severe tissue injury [3].

Apoptotic neutrophils express autoantigens on their cell surface

The origin of ANCA present in the sera of patients with ANCA-associated vasculitis is perplexing, although apoptotic neutrophils may present a source of immunogen in this autoimmune disease. Neutrophils and monocytes constitutively express PR3 and MPO on their cell surface [13] and these are up-regulated after priming with pro-inflammatory cytokines [14]. Interestingly, patients with WG express increased PR3 on the neutrophil surface, and increased PR3 expression is a risk factor for disease and disease relapse [13,15]. Note that apoptotic neutrophils also express PR3 and MPO on the cell surface [16,17] and this is increased further in apoptotic neutrophils from patients with ANCA-associated vasculitis [17].

Casciola-Rosen et al. [18] have demonstrated that the autoantigen of particular importance in SLE cluster on the cell surface in membrane blebs. It has been suggested that, during apoptosis, these antigens are modified, either undergoing proteolysis or reacting with reactive oxygen species with the production of immunogenic epitopes [19]. The susceptibility of intracellular proteins to cleavage by granzyme B has been identified as a property that unites generation of a diverse range of autoantigens [20]. Anti-DNA antibodies found typically in SLE have a greater capacity to bind reactive oxygen species-modified DNA [21]. Relapses of ANCA-associated vasculitis are often triggered by infection, which is associated with an influx of neutrophils, the activation and generation of reactive oxygen species and increased neutrophil apoptosis, raising the possibility that altered immunogenic PR3 or MPO may be presented to immunologically competent cells. Studies are awaited to determine whether PR3 or MPO are modified during the apoptotic process, since ANCA recognize autoantigens expressed on both the fresh and apoptotic neutrophils. It may be noted that propylthiouracil has been linked to the generation of MPO-ANCA antibodies [22]. Propylthiouracil may act as a hapten-binding myeloperoxidase altering its configuration and may promote the development of autoantibodies in susceptible individuals [23]. Also, propylthiouracil increases thyroid follicular cell apoptosis [24], but its effects on neutrophil apoptosis are unknown. Propylthiouracil does accumulate in neutrophils [23], and increased apoptosis by propylthiouracil of neutrophils may also promote development of MPO-ANCA.

Antigen presentation

The induction and perpetuation of immune responses are probably dependent on the immune system receiving danger signals. Dendritic cells are potent antigen-presenting cells capable of stimulating primary immune responses [25]. Immature dendritic cells can ingest antigen, but express low levels of the molecules required for antigen presentation and T-cell stimulation. After antigen uptake, dendritic cells migrate to secondary lymphoid organs where they receive maturation signals, which include lipopolysaccharide, TNF and interferon-α. Immature dendritic cells can phagocytose necrotic and apoptotic cells, but the outcome with regard to the immune system varies depending on the type of cell death and whether maturation signals are received. If apoptotic cells are engulfed in the absence of a maturation signal, T-cell tolerance is induced [26]. However, during a viral infection, cells die by both apoptosis and necrosis. Both infected apoptotic and necrotic cells are ingested by dendritic cells, and maturation signals received from necrotic cells induce the expression of dendritic-cell co-stimulatory molecules, leading to the presentation of viral antigenic peptides from apoptotic cells, T-cell activation and immunity [27,28]. It has also been suggested that opsonization of apoptotic cells with β2-glycoprotein antibodies increases phagocytosis by dendritic cells and provides the necessary maturation signals to promote antigen presentation [29].

In WG, granulomas containing large numbers of freshly activated, apoptotic and necrotic neutrophils are present in the upper airways early in the development of vasculitis, raising the question as to whether interaction of these cells with dendritic cells promotes persistence of the autoimmune response. The effects of human apoptotic neutrophils on dendritic-cell maturation and antigen presentation have been examined. Studies performed in our laboratory [30] suggest
that uptake of apoptotic neutrophils has a tolerogenic effect on dendritic cells, with down-regulation of co-stimulatory molecules, as expected. Interestingly, necrotic neutrophils had a greater tolerogenic effect compared with apoptotic neutrophils and this was partially overcome by dangerous signals such as TNFα. Similar studies with necrotic cell lines have suggested that necrotic material is stimulatory and pro-inflammatory, so it is probable that special danger-type signals are needed to overcome the inhibitory effects of dying neutrophils. Opsonization of apoptotic neutrophils with ANCA, in contrast with βγ-glycosylphosphatidylinositol, had no effect on sustaining the immune response, since phagocytosis of these opsonized cells did not induce maturation of dendritic cells [30].

Interestingly, recent results from experimental animals have suggested that persistence of apoptotic cells in an inflammatory environment may result in the presentation of auto-antigens to the immune system, resulting in autoantibody production [31]. Injection of Brown Norway rats with syngenic apoptotic neutrophils resulted in the development of ANCA, although none of these rats developed disease. These studies did not address how immune tolerance was overcome in the absence of an adjuvant.

Failure to resolve inflammation and persistence of chronic inflammation: effects of ANCA

For successful healing, inflammation must be stopped by avoiding recruitment of new neutrophils and other inflammatory cells and by the removal of neutrophils already present [32]. Neutrophils migrating to inflamed sites are constitutively cleared by apoptosis. Apoptosis is characterized by stereotypical, morphological and biochemical changes, including activation of specific intracellular enzymes (caspases) that cleave multiple nuclear and cytoplasmic proteins. Apoptosis provokes specific cell-surface changes, such as externalization of phosphatidylserine, normally present on the intracellular region of the surface membrane, leading to a safe phagocytic clearance of the intact dying cell. Indeed, the macrophages that contribute to the phagocytosis of apoptotic cells release anti-inflammatory cytokines, such as transforming growth factor-β, promoting the resolution of inflammation [33].

Under other autoimmune conditions, particularly SLE, failure to remove these apoptotic cells promotes the development of an autoimmune disease [34]. In small-vessel vasculitis, this process appears to be affected. As stated previously, there is increased expression of PR3 and MPO on the cell surface, since neutrophils become apoptotic, allowing interaction with ANCA [17]. Binding of ANCA to these antigens on apoptotic cells does not lead to activation, unlike binding of ANCA with freshly isolated cells; however, binding of ANCA to these antigens does result in increased uptake by macrophages via Fcγ receptors [14,17]. Fc receptor-mediated uptake results in macrophage activation and these macrophages release pro-inflammatory cytokines, including IL-1, IL-8, TNFα and thromboxanes, in contrast with the normally occurring uptake of apoptotic cells by macrophages that is non-phlogistic [17,35]. Inflammatory clearance of apoptotic cells after increased production of IL-8, a strong chemoattractant for neutrophils, will recruit neutrophils and perpetuate inflammation.

Pathologically, ANCA-associated vasculitis is characterized by leucocytoclasis, and electron microscopy studies have suggested that there may be a defect in the clearance of apoptotic neutrophils. There is evidence of leucocytes with degraded nuclear material undergoing disintegration in tissues [36], and apoptotic cells have been observed in ANCA-positive renal vasculitis [37]. ANCA activation of TNF-primed neutrophil has been shown to accelerate constitutive apoptosis as measured by morphology and nuclear changes [38]. The process depends on the generation of reactive oxygen species, since neutrophils isolated from patients suffering from chronic granulomatous disease were incapable of generating reactive oxygen species and hence did not show accelerated apoptosis. However, nuclear and membrane changes of apoptosis were uncoupled owing to the fact that despite morphological changes of apoptosis, there was reduced expression of phosphatidylserine on the surface of apoptotic neutrophils that had been activated by ANCA. Phosphatidylserine is an important marker of apoptosis, allowing macrophages to recognize cells as apoptotic [39]. Failure to express phosphatidylserine is associated with decreased clearance of apoptotic cells by macrophages. Indeed, after ANCA activation, apoptotic neutrophils were less efficiently phagocytosed by macrophages, resulting in the development of secondary necrosis, a highly phlogistic event. Uncoupling of nuclear and membrane changes of apoptosis have previously been observed using mitochondrial inhibitors [40], but it is unknown whether mitochondria are disrupted after ANCA activation.

ANCA activation of primed neutrophils causes degranulation and release of serine proteases, which may also impair removal of apoptotic cells, resulting in progression to secondary necrosis. Elastase has been shown to cleave the phosphatidylserine receptor disrupting phagocytosis of apoptotic cells [41]. Elastase also acts directly on the apoptotic cell, disrupting clearance; it is not clear how this occurs, but possibilities include cleavage of glycoprotein ligands or direct blockade of externalized phosphatidylserine. In addition to the cleavage of a phosphatidylserine receptor, elastase promotes inflammation by acting directly on macrophage cells, promoting the release of TNF, MIP-2 (macrophage inflammatory protein 2) and IL-8 [42]. In the inflammatory environment present in patients with ANCA-associated vasculitis, a relative deficiency of anti-proteases has been suggested [43]. The reactive oxygen species released after ANCA activation and the acidic microenvironment can inactivate α1 antitrypsin, the main inhibitor of neutral serine proteases, promoting the inflammatory effects of proteases released after ANCA activation. Indeed, patients with ANCA-associated vasculitis who carry the deficiency allele for α1 antitrypsin have more severe diseases and a worse prognosis [44].
Elastase and PR3 can induce apoptosis on their own [45,46]. Elastase and PR3 enter into endothelial cells, activating ERK, c-Jun N-terminal kinase and p38 MAPK, promoting endothelial apoptosis, independent of their proteolytic function [47]. It is not clear how these proteases are taken up into the cell, but a specific receptor for PR3 has been described [48]. Elastase also promotes neutrophil apoptosis, but its role in ANCA-associated accelerated apoptosis is unknown [48].

**ANCA-induced apoptosis perturbed signalling**

ANCA-accelerated apoptosis depends on the generation of reactive oxygen species; however, it is not known how they interact with other death molecules to mediate apoptosis. We have preliminary results that suggest that activation of caspase 3 is important (unpublished work). Immuno-complex activation of neutrophils via FcγRII with generation of reactive oxygen species also accelerates neutrophil apoptosis [49]. Recently, an alternative pathway for immuno-complex-activated apoptosis that is reactive oxygen species-independent has been suggested, involving the up-regulation of Bax, a pro-apoptotic member of the Bcl family, and activation of caspase 3 [50]. It is not clear at present whether this pathway also plays an important role following ANCA activation of neutrophils. It is not clear how ANCA activation pathways generating reactive oxygen species and the pathways accelerating apoptosis interact, since many of the signalling pathways activated by ANCA are anti-apoptotic, e.g. PI3K with generation of PKB, PKC, etc. Future research should delineate signalling pathways involved in dysregulated apoptosis after ANCA activation of neutrophils to allow the development of new therapeutic targets.

**Conclusion**

ANCAs are not only important as diagnostic tools in the diagnosis of ANCA-associated vasculitis, but also have a crucial role in disease pathogenesis. These antibodies are important in triggering disease activation and also in preventing the resolution of inflammation by subverting the normal anti-inflammatory apoptotic processes. Dysregulation of apoptosis by ANCA may promote further inflammatory cell influx and may prevent the normal switching off of inflammatory macrophages by disrupting the normal process of cell removal, resulting in further scarring. Future research will shed light on the involvement of apoptosis in this challenging disease and, hopefully, delineate new targets for therapeutic intervention.

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