ABSTRACT
Antineutrophil cytoplasm autoantibody (ANCA)-associated small vessel vasculitides are systemic diseases characterized by chronic inflammation of blood vessels. These vasculitides are associated with the presence of ANCA which are, in most cases, directed towards proteinase 3 (PR3) or myeloperoxidase (MPO). In vitro and in vivo data have suggested a pathophysiological role in the ANCA-associated vasculitides, particularly based on the capacity of autoantibodies to bind and activate neutrophils.
This review focuses on the role of constitutive expression of proteinase 3 on the membrane of resting neutrophils (mPR3). mPR3 can be expressed on the total population or on a subset of neutrophils and levels of mPR3 differ between individuals. The level of mPR3 on resting neutrophils and the percentage of mPR3 expressing neutrophils is stable in time for a given individual, suggesting a genetic determinant. Patients with ANCA-associated vasculitis have an increased constitutive expression of mPR3 on resting neutrophils compared to healthy controls. High levels of mPR3 on resting neutrophils are a risk factor for the development of relapses in patients with PR3-ANCA-associated vasculitis, probably by making resting neutrophils more susceptible for binding ANCA and induction of activation. As such, constitutive mPR3 expression on neutrophils seems another pathogenic factor in ANCA-associated vasculitis.

Introduction
The antineutrophil cytoplasm autoantibody (ANCA)-associated small vessel vasculitides (AAV) are a group of systemic diseases of unknown etiology affecting primarily the small blood vessels. These vasculitides are characterized by necrotizing inflammation of mainly the arterioles, venules, and capillaries (1). They are associated with ANCA which are in most cases directed against proteinase 3 (PR3) or myeloperoxidase (MPO), enzymes stored in the neutrophil azurophilic granules [reviewed in (2)]. 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) (1). PR3-ANCA occur in 80-90% of WG patients, to a lesser extent in patients with MPA and incidentally in CSS (2). MPO-ANCA are the dominant autoantibodies in MPA and CSS but occur in a minority of WG patients as well (2, 3).
A pathophysiological role of ANCA has been suggested not only by the close association of ANCA with these disorders, but also since titers of ANCA correlate, although not uniformly, with disease activity (3-8). In these studies an increase in ANCA titer frequently preceded relapses and a decline in titer was observed when remission was induced. In active WG, the sensitivity of PR3-ANCA for generalised and limited WG is respectively 80-90% and 55-96% (2). Moreover, most PR3-ANCA negative patients with active generalised WG are positive for autoantibodies to MPO or leukocyte elastase (9-11). Finally, patients persistently or intermittently positive for ANCA during remission have a higher risk of developing relapses (12, 13)
The pathogenic potential of ANCA is further supported by data from animal models. Heeringa et al. (14) injected rats with sub-nephritogenic doses of antiglomerular basement membrane (anti-GBM) antibodies with or without prior immunization with human MPO
Membrane PR3 expression on the resting neutrophil / A.P. van Rossum et al.

Plasma membrane in vitro after treatment with TNF-α, but also GM-CSF and TGF-β are capable of translocating PR3 (31,32). In vivo membrane PR3 expression is observed on neutrophils in patients with active ANCA-associated vasculitis (33, 34). Priming by TNF-α facilitates fusion of secretory vesicles and specific granules with the plasma membrane and their degranulation leading to a two- to three-fold up-regulation of PR3 expression on the neutrophil surface (26, 33). Besides from being translocated to the cell membrane by such a stimulus, PR3 can also be constitutively present on resting neutrophils. Resting neutrophils can express mPR3 either on the total population or on a subset of neutrophils (27-29). The percentage of neutrophils expressing mPR3 ranges from 0% to 100% of the total population of neutrophils (Fig. 1). Individuals can be categorized according to the pattern of mPR3 expression on their resting neutrophils into those individuals in whom all neutrophils express none or only minor levels of mPR3 (mPR3⁻), individuals in whom all neutrophils express substantial levels of mPR3 (mPR3⁺), and 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. The latter pattern is indicated as bimodal PR3 expression.

Interestingly, the phenomenon of bi-modal expression was not seen for elastase or other neutrophil markers, sug-

Fig. 1. Patterns of PR3 expression on the surface of resting neutrophils as analyzed by flow cytometry. The bold line represents staining with monoclonal anti-PR3 antibody, the thin line indicates non-specific binding of an isotype matched control. [Reprinted with permission from The Journal of the American Society of Nephrology and the authors (29)].
suggesting that bimodality is not a mere result of neutrophil activation (27). It has been shown that bimodal expression was neither due to an artefactual interaction of the anti-PR3 antibody with the neutrophil membrane, nor to neutrophil isolation procedures or the age of the neutrophil. In fact, the percentage of neutrophils expressing a specific level of mPR3 was shown to be stable for each individual over time (23, 25, 29). In addition, neutrophil degranulation induced by the chemotactic peptide fMLP in combination with cytochalasin B resulted in the increased expression of PR3, but the proportion of low and high PR3-expressing cells remained stable (27-29, 35). Taken together, the stability of the constitutive expression of PR3 on the neutrophil membrane suggests a genetic determinant. This hypothesis has been investigated in several studies (28, 35).

**Genetics of mPR3**

Studying a set of 30 monozygotic twins and 24 dizygotic twins Schreiber et al. showed that the percentage of mPR3 expressing cells was strongly correlated between monozygotic twins (r = 0.99), but not between dizygotic twins (r = 0.06), suggesting a strong genetically controlled expression of PR3 on the membrane (35). Furthermore, the intracellular PR3 content in neutrophils was not different between persons with low or high mPR3 expression, nor between cells with low or high mPR3 expression within a given individual. Witko-Sarsat et al. (28) studied the mPR3 phenotypes of 126 healthy individuals and distinguished three phenotypes: a low mPR3 phenotype in which 0 – 20% of neutrophils expressed mPR3, an intermediate mPR3 phenotype in which 21 – 58% of neutrophils expressed mPR3, and a high mPR3 phenotype in which 59 – 100% of neutrophils expressed mPR3. These phenotypes were present in 9%, 36% and 55% of the healthy population, respectively. Based on the phenotype distribution, an inheritance pattern regulation of two co-dominant alleles was hypothesized: individuals homozygous for low mPR3 expression, individuals homozygous for high mPR3 expression and heterozygous individuals in case of intermediate mPR3 expression. Gene frequencies based on this hypothesis fulfilled the Hardy-Weinberg law for a population at genetic equilibrium. Finally, this hypothesis was further supported by the inheritance pattern of mPR3 expression as observed in two representative families.

**mPR3 and its association with the neutrophil membrane**

The molecular organization of PR3 expression on the neutrophil membrane is still far from clear. Interaction of PR3 with the membrane does not result from charge interaction and does not depend on membrane sialic acid residues, nor is PR3 anchored to the plasma membrane via a glycosyl phosphatidyl inositol (GPI)-link as a flexible leash to interact with molecules outside the cell (30). It appears that the association of PR3 with the membrane is covalent, and may involve lipid interactions (36). Alternatively, Taekema-Roelvink et al. proposed that PR3 interacts with a 111-kDa receptor on endothelial cells that is specific for PR3 (37). This putative receptor has, however, not been cloned and characterized yet but may be present on the neutrophil as well.

**Clinical significance of mPR expression**

What is the clinical significance of constitutive mPR3 expression? Patients with PR3-ANCA associated vasculitis have an increased constitutive expression of PR3 on the neutrophil membrane compared with healthy individuals (28, 29, 35). In a study by Witko-Sarsat et al. (28) the frequency of mPR3expressing cells was significantly increased from 55% in healthy individuals (n=126) to 85% in patients with WG (n = 37). Interestingly, patients with rheumatoid arthritis had increased percentages of mPR3 expressing cells as well.

In addition, Schreiber et al. (35) confirmed that the percentage of the mPR3+ phenotype was significantly higher in WG patients than in a healthy cohort and showed that the total amount of mPR3, in terms of mean fluorescence intensity (MFI), was increased from 278 ± 206 for the healthy controls to 457 ± 310 for WG patients. Patients with idiopathic inflammation had increased levels (461 ± 257) of mPR3 as well.

We confirmed that the frequency of mPR3expressing cells and the level of mPR3 expression on resting neutrophils was higher in WG patients than in controls (29). Moreover, WG patients had comparable distributions of mPR3-phenotypes as healthy individuals: 29% of WG patients displayed a bimodal mPR3 expression compared to 30% in controls and 71% of WG patients showed a monomodal expression compared to 70% in controls. So, it appeared that not the distribution of mPR3 phenotypes seems relevant but the total level of mPR3 expression on neutrophils since bimodality was found in equal numbers in patients as in controls. Clinically, increased expression of mPR3 in vasculitis patients was associated with an increased incidence and rate of relapse, as shown in Figure 2 (29). Patients with high levels of mPR3 expression were significantly more at risk for relapse of vasculitis than those with low levels of PR3 on the surface of resting neutrophils. 38% of WG patients with low mPR3 expression relapsed, compared to 68% of WG patients with high mPR3 expression and 65% of those with bimodal mPR3 expression. Furthermore, the disease-free survival time between diagnosis and first relapse was significantly shorter in patients with high mPR3 expression and bimodal mPR3 expression (30.8 and 36.6 months respectively) compared to patients with low mPR3 expression (104.5 months). In another study by Schreiber et al. (35) patients with a Birmingham Vasculitis Activity Score (BVAS) below and above 2 were compared. This analysis revealed a trend towards higher levels of mPR3 expression in patients with BVAS above 2 (360 ± 270 MFI for BVAS <2 and 562 ± 315 MFI for patients with BVAS >2, P=0.08), whereas the mPR3+ phenotype percentage was not related.
Interestingly, actin polymerization and the authors (29).

Port the hypothesis that the more PR3 is expressed on the membrane of the resting neutrophil the more it is susceptible to binding of PR3-ANCA and, subsequently, under primed conditions, to become activated.

**mPR3 expression and in vitro susceptibility to activation**

Recently (unpublished results) we analyzed the functional significance of mPR3 expression for neutrophil activation induced by a monoclonal anti-PR3 antibody. We analyzed 12 healthy donors with different mPR3 expression levels and measured actin polymerization, as an early event in neutrophil activation, and oxidative burst, as a late event in neutrophil activation. The study clearly demonstrated a correlation ($r = 0.78$, $P = 0.0028$) between the level of mPR3 expression on non-primed neutrophils and the degree of actin polymerization in response to stimulation with anti-PR3 antibodies. Interestingly, actin polymerization could be triggered without priming by TNF-α. In contrast, when neutrophils were primed no correlation was seen between mPR3 expression and priming-dependent oxidative burst as well as actin polymerization. These observations connect membrane expression of PR3 in resting neutrophils to neutrophil activation, and further support the relation between membrane expression of PR3 and the susceptibility for relapses in patients with WG.

**Conclusion**

Taken together, the increased expression of membrane PR3 on resting neutrophils in patients with WG and the concomitant increased risk and rate of relapse strongly suggests a pathogenetic role for constitutive membrane expression of PR3. Thereby, a new pathogenetic determinant is introduced in the understanding of the pathophysiology of ANCA-associated vasculitis.

**References**

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