Review

Membrane proteinase 3 expression on resting neutrophils as a pathogenic factor in PR3-ANCA-associated vasculitis

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Received on April 23, 2003; accepted in revised form on August 11, 2003.

Clin Exp Rheumatol 2003; 21 (Suppl. 32): S64-S68.

Key words: Proteinase 3, resting neutrophil, vasculitis, membrane expression, ANCA.

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-ANCAare 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 ANCAcorrelate, 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-ANCAfor 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

(which resulted in the development of anti-MPO antibodies cross-reacting with rat-MPO). Rats with both anti-GBM and anti-MPO antibodies developed necrotizing crescentic glomerulonephritis, whereas rats injected with anti-GBM antibodies only developed mild glomerulonephritis without crescent formation.

Recently, Xiao *et al*. (15) showed 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 and systemic necrotizing vasculitis in the latter mice. In addition, the authors demonstrated that direct intravenous injection of anti-MPO antibodies, derived from MPO-immunized MPO-deficient mice, into Rag2-deficient mice or in wild-type mice resulted in focal necrotizing and crescentic glomerulonephritis (15). These experiments strongly support the pathogenic role of MPO-ANCA in ANCA-associated vasculitis (16).

In vitro activation studies have shown that ANCA are capable of activating neutrophils (17-22). These studies demonstrated that neutrophils primed with a low dose of proinflammatory cytokines become further activated and subsequently degranulate upon ANCA binding. This degranulation may have pathogenic consequenses, since lytic enzymes and reactive oxygen species are released which have been shown to be able to induce apoptosis, cell lysis and endothelial detachment (22-25). In order to become activated there are some prerequisites that have to be fulfilled (22). First, the neutrophil needs to express PR3 on its membrane to allow ANCAbinding. The $F(ab')_2$ fragment of ANCA is supposed to bind to PR3 and the Fc part interacts with neutrophil Fc-receptors resulting in full activation, although $F(ab')_2$ fragments of ANCA alone have been shown to induce minor activation as well (19). Secondly, the neutrophil needs to be primed by a low dose of tumor necrosis factor-α (TNF-α) to induce translocation of PR3 to the membrane and, probably more important, to activate the NADPH oxidase system and allow the production of reactive oxygen species (ROS). Thirdly, the neutrophil needs an adhesive state as reflected in CD11b/CD18 expression (26). In recent years it has become clear that PR3 is not only expressed on the membrane after priming but can also be constitutively present on the membrane of non-stimulated neutrophils (27-29). Expression of PR3 on the membrane of resting neutrophils and the possible role of this phenomenon in PR3-ANCA-associated vasculitis will be the focus of this review.

Membrane proteinase 3 (mPR3) expression on the resting neutrophil

PR3 is a serine protease predominantly stored in the azurophilic granules, but can be found in the specific and secretory vesicles as well (30). As mentioned, neutrophils express PR3 on their 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 TNFα 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 bimodal expression was not seen for elastase or other neutrophil markers, sug-

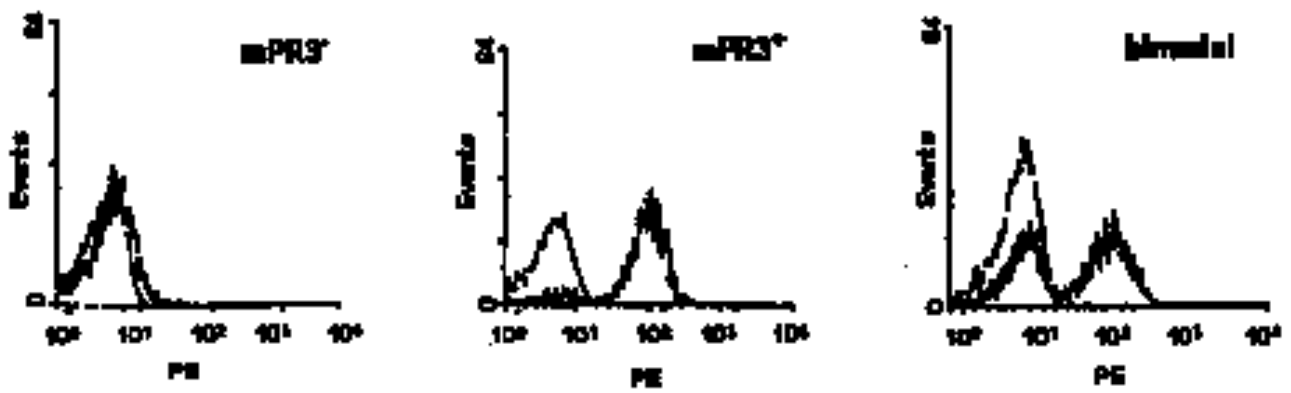

Fig. 1. Patterns of PR3 expression on the surface of resting neutrophils as analyzed by flow cytometry. The bold line represents staining with monoclonal anti-PR3 antibody, the thin line indicates non-specific binding of an isotype matched control. [Reprinted with permission from *The Journal of the American Society of Nephrology* and the authors (29)].

gesting 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 indivuals 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.

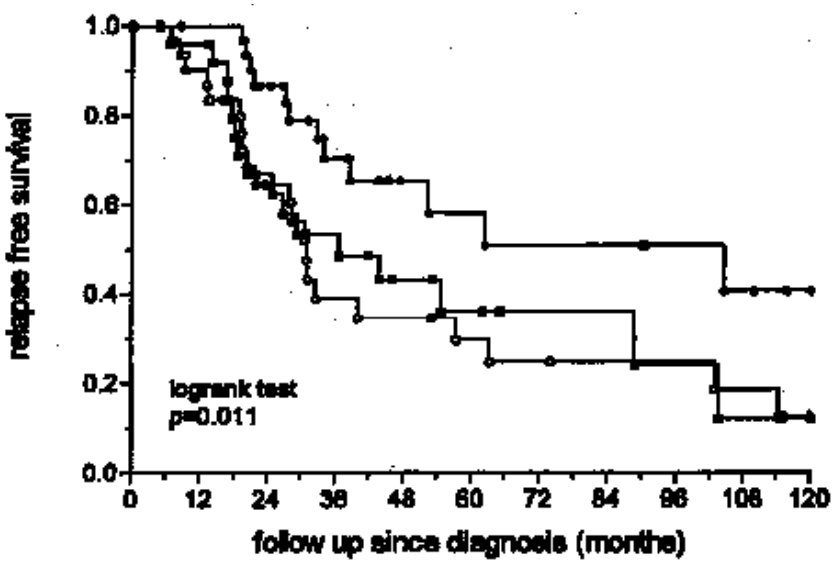

Fig. 2. Relapse free survival in WG patients with monomodal low (●, n =32), monomodal high (❍, n =31) and bimodal (■, n = 26) mPR3 expression. [Reprinted with permission from *The Journal of the American Society of Nephrology* and the authors (29)].

These *in vivo* observations further support 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 *susceptibilty 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 with 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 concomittant 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.

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