Interactions of antineutrophil cytoplasm autoantibodies with neutrophil proteinase 3 in systemic vasculitis
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Although studied for some time now, the pathophysiology of antineutrophil cytoplasm autoantibody (ANCA)-associated systemic vasculitis still remains a field of research with many unresolved questions. This group of diseases, manifesting as inflammation of small blood vessels, includes Wegener's granulomatosis (WG) and microscopic polyangiitis (MPA), characterized by ANCA specific for proteinase 3 (PR3) and myeloperoxidase (MPO), respectively. Anti-PR3 and anti-MPO autoantibodies have become an established and specific marker for WG and MPA, respectively, and their detection is helpful in both diagnosis and in follow-up of patients with systemic vasculitis. Several clinical observations suggest that ANCA may be involved in the disease pathogenesis. First, an increase in ANCA titer frequently precedes a relapse and titers decline when the patient comes into remission. Second, persistent presence of PR3-ANCA after induction of remission has been found to be associated with an increased risk to develop relapses. Thirdly, development of relapses can be successfully prevented using ANCA titer-based treatment. Despite this close association between ANCA titers and disease activity, the effector role of PR3-ANCA in the pathophysiology of WG has not been fully elucidated yet. Nevertheless, there is evidence that ANCA are not only an epiphenomenon associated with systemic vasculitis, but may contribute directly to the occurrence of vascular and glomerular lesions. In vitro studies have demonstrated that PR3-ANCA are capable of cross-linking PR3 and Fcγ receptors expressed on the neutrophil surface resulting in production of reactive oxygen species and the release of proteolytic enzymes, including PR3 and elastase. Subsequently, this may lead to detachment, cytolysis, and apoptosis of endothelial cells. To some extent, endothelial cell injury is dependent on the proteolytic activity of PR3 which, in normal conditions, should be inactivated by physiological inhibitors of PR3 such as α1-antitrypsin (α1-AT). Interestingly, PR3-ANCA has been shown to interfere with binding of α1-AT to PR3 which may prevent PR3 from this inhibition to display its full proteolytic activity. This functional characteristic of PR3-ANCA correlates with disease activity of WG, suggesting that intra-individual changes of PR3-ANCA on the epitope level occur during the time course of the disease.

In this thesis we addressed five questions emerging from the observations mentioned above. The first two concern the importance of the accessibility of PR3 for PR3-ANCA binding and neutrophil activation. First: does the level of PR3 expression on the surface of resting neutrophils determine the susceptibility of patients with WG to develop relapses? Second: is the level of PR3 present on the cell surface related to the extent of neutrophil activation by PR3-ANCA in vitro? The other three questions concern the interactions between PR3-ANCA and its target antigen at the epitope
level. Is there any inter-individual variability in PR3-ANCA epitope specificity at the moment of presentation of the disease? Does PR3-ANCA epitope specificity change during the time course of the disease within an individual? What can we learn about the epitopes recognized by PR3-ANCA using a recombinant form of PR3?

In Chapter 2 we summarize the literature contributing to our current view on the pathophysiology of ANCA-associated vasculitis. We review evidence for a phlogistic effect of ANCA that has emerged from clinical observations, in vitro studies, and in vivo experimental animal models. Next, we address the role of exogenous factors possibly involved in disease initiation and progression, such as Staphylococcus aureus and exposure to silica. We also mention genetic factors that may determine the susceptibility of an individual to develop ANCA-associated vasculitis. Shortly, in this chapter we tried to put together all the factors participating in disease development and stress the fact that ANCA-associated vasculitides are complicated autoimmune disorders in which the interplay of autoimmunity with genetic and environmental factors determines their clinical expression.

The detection of PR3-ANCA by the indirect immunofluorescence assay (IIF) is an established diagnostic tool for patients with WG. Due to several drawbacks, such as lack of antigen-specificity and inaccurate quantification, the IIF assay is usually performed in combination with PR3-specific solid-phase assays (PR3-ELISA). This technique is antigen specific and gives precise information on the PR3-ANCA titers. Most ELISA systems for PR3-ANCA detection use native PR3 isolated from human neutrophils or a crude extract of neutrophil granules as an antigen source. Since the isolation of highly purified PR3 from neutrophils is laborious, difficult and inefficient, an alternative for purified native PR3 is the use of recombinant PR3. In Chapter 3 we compare the technical performance of direct and capture ELISA using native PR3 and a novel proteolytically inactive recombinant PR3 produced in the baculovirus expression system to detect PR3-ANCA in patients with systemic vasculitis at the moment of diagnosis. The use of proteolytically inactive PR3 can be advantageous for the stability and reliability of an ELISA, since proteolytically active PR3 is able to degrade both the capturing monoclonal antibody and patient PR3-ANCA. Our study shows that the sensitivity of ELISA applying this recombinant PR3 is comparable to the sensitivity of ELISA using native PR3. Therefore, baculovirus PR3 is a promising alternative for native PR3 for the detection of PR3-ANCA. However, we suggest that the specificity of the assays using recombinant
PR3 should be further improved. Moreover, better validation of the test requires the introduction of a new, improved, standard. Capture ELISA not only can be used as a diagnostic method, but it may also provide information on the epitope specificity of PR3-ANCA, when capturing antibodies of different epitope specificities are used. In our study, we applied two capture anti-PR3 antibodies, 12.8 and PR3G-3, which are known to recognize different epitopes on the PR3 molecule. Recombinant PR3 was more frequently recognized by patient sera when captured by MAb PR3G-3, suggesting that MAb 12.8 might interfere with binding of some PR3-ANCA. Although, in capture ELISA using MAb 12.8, native PR3 performed better compared to recombinant PR3, this difference was not present when MAb PR3G-3 was used, further demonstrating the implications of the choice of the capturing antibody for the results of the assay. Moreover, it cannot be excluded that epitopes present on the recombinant baculovirus PR3 are not identical to the epitopes on native PR3.

We conclude that baculovirus PR3 is a promising alternative for PR3 isolated from human neutrophils and, in the future, can be of value for diagnostic applications. Nevertheless, further optimizing of sensitivity and specificity of ELISA using this recombinant antigen is necessary, as well as development of a suitable validation standard.

Several attempts have been made to characterize the interactions between ANCA and PR3 and it has been demonstrated that PR3-ANCA mainly recognize conformational epitopes on PR3. One of these studies showed that monoclonal antibodies raised against PR3 recognize four separate areas on this protein, suggesting that there could also be a variability in epitope specificity of PR3-ANCA from patients with WG. However, up to now, knowledge on the epitopes for PR3-ANCA has been scarce. In Chapter 4, we describe a study using the biosensor technology to investigate possible variability in epitope specificity of PR3-ANCA of WG patients at the moment of disease presentation. Performing mutual inhibition experiments, we found that, at the moment of diagnosis of WG, PR3-ANCA of different patients recognize a limited number of overlapping regions on PR3. This area seems to cover an immunodominant epitope, common for PR3-ANCA from all patients, irrespective of the size of the total area recognized by an individual autoantibody. These results are similar to the results of comparable experiments analyzing the interactions of MPO-ANCA-positive sera with MPO described elsewhere.
The second question addressed in our study was whether epitope specificity of PR3-ANCA within a patient is stable in time. Experiments with sera collected at the moment of disease presentation and at the time of relapse show that the individual epitope specificity of PR3-ANCA may change during the course of the disease, a phenomenon known as epitope shift. Epitope shift has recently been recognized as an important pathogenic mechanism responsible for the initiation and progression of autoimmune diseases, for instance systemic lupus erythematosus (SLE). Intra-individual changes in PR3-ANCA epitope specificity may reflect B-cell epitope spreading from a disease-inducing epitope to other areas of the autoantigen (intra-molecular epitope spreading). Moreover, changes of epitope specificity of PR3-ANCA during the course of the disease may be responsible for differences in functional properties of these autoantibodies between various stages of the disease. One of these properties is the ability of PR3-ANCA to interfere with the proteolytic activity of PR3 and with binding of the physiologic PR3 inhibitor, α1-antitrypsin (α1-AT), to PR3. The number of patients included in this part of the study was very small and, therefore, our observation should be further confirmed in a larger group of patients. To characterize these changes in more detail, analysis of PR3-ANCA specificity at the time of other relapses as well as during remission should be performed.

An additional important observation from our study is the fact that epitope specificity of polyclonal rabbit anti-PR3 antibodies is distinct from the epitope specificity of human PR3-ANCA. This suggests that the immune response against PR3 in rabbit develops differently than in humans. This fact should be taken into consideration especially when rabbit anti-PR3 antibodies are used to detect human PR3.

The study described in Chapter 5 started from the hypothesis that, in WG patients, the presence of PR3 on the surface of non-stimulated neutrophils enables interaction with PR3-ANCA and influences clinical manifestations of the disease. Originally, PR3 was found to be stored in azurophil granules of the neutrophil only, and was believed to translocate to the neutrophil surface upon priming with TNFα. A recent study using immunoelectron microscopy showed, however, that PR3 localizes also in the specific granules, secretory vesicles, and, even more interestingly, can be present also on the surface of resting neutrophils (membrane PR3 or mPR3). PR3 can be detected either on the total neutrophil population or on a subset of neutrophils. The existence of mPR3 and mPR3* neutrophil subpopulations within one individual, called bimodal expression of PR3, has been suggested to be genetically determined,
since the proportion of mPR3+ neutrophils is individually highly stable. The observation, that the mPR3+ neutrophil subset tends to be larger in patients suffering from vasculitis than in healthy individuals, led us to the hypothesis that an increased number of mPR3+ cells could be a risk factor for relapse in this disease. We analyzed mPR3 expression on resting neutrophils in large groups of healthy controls and WG patients in complete remission and related it to clinical manifestations and to the susceptibility to develop relapses. Although the number of patients with a bimodal mPR3 expression did not differ between patients and controls, WG patients had an increased percentage of mPR3+ neutrophils and an elevated level of mPR3 expression compared with healthy individuals. Within the group of WG patients, an elevated level of mPR3 expression was significantly associated with an increased risk for relapse and with an increased relapse rate, but not with the disease extent or particular manifestations at diagnosis or at relapse. We suggest that neutrophils of the mPR3+ phenotype are more susceptible to activation by PR3-ANCA than mPR3- neutrophils because the autoantigen is directly accessible on the cell surface, also without priming. As a result, increased numbers of mPR3+ neutrophils and/or an increased level of mPR3 expression might be associated with more severe disease.

The functional significance of membrane PR3 expression for neutrophil activation induced by PR3-ANCA was investigated in the study described in Chapter 6. We stimulated resting and TNFα-primed neutrophils with monoclonal anti-PR3 antibodies and measured early and late functional responses using two different read-out systems, namely actin polymerization and oxidative burst, respectively. Additionally, we investigated the influence of TNFα on mPR3 expression. TNFα caused upregulation of membrane PR3 compared to resting neutrophils and, in neutrophils displaying a bimodal mPR3 expression, influenced both subsets to a comparable degree without changing the percentages of cells present within these subsets. This further supports the hypothesis on the genetic background of the pattern of mPR3 expression. The most important observation from this study is the fact that the degree of actin polymerization, but not the level of oxidative burst in response to anti-PR3 antibody, correlates with the level of PR3 expression on the neutrophil surface. The anti-PR3 antibody-induced oxidative burst is priming-dependent and independent of the pattern of PR3 expression. There are inter-individual differences in the concentration of anti-PR3 antibody inducing the maximal response. However, the amount of intracellular reactive oxygen species produced in response to anti-PR3 antibody does not correlate with mPR3 expression on primed neutrophils. Shortly, our results strongly suggest that the presence of
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PR3 on non-primed neutrophils is not sufficient for the anti-PR3-antibody-induced oxidative burst. In contrast to the oxidative burst measured 30-60 minutes after stimulation, actin polymerization measured within 10-30 seconds after anti-PR3 antibody administration is priming-independent and strongly correlated with the level of PR3 present on the surface of resting neutrophils, but not with the pattern of mPR3 expression (monomodal / bimodal). Since there is a correlation between the level mPR3 expression and the level of the early response of neutrophils to stimulation by anti-PR3 antibody, it is possible that, in vivo, the presence of PR3 on the surface of resting neutrophils has implications for neutrophil susceptibility to activation by PR3-ANCA. This gives, at least a partial, explanation for the observation that PR3-ANCA positive patients with WG, who have a high expression of PR3 on resting neutrophils, are more susceptible to develop relapse than patients with low membrane PR3 expression.

In Chapter 7 we summarize the current knowledge on neutrophil activation by ANCA in systemic vasculitis. We discuss the binding of ANCA to their antigens, namely PR3 and MPO, and to Fcγ receptors on the neutrophil surface, and the role of these interactions in triggering of the functional responses. We address the importance of TNFα priming for up-regulation of surface molecules involved in activation by ANCA, and we focus on the factors determining the susceptibility of neutrophils to this activation. Next, we describe the signal transduction pathways involved in activation of neutrophils by monomeric ANCA as well as by ANCA-containing immune complexes. Although these signaling routes have not been fully dissected yet, there is accumulating evidence that spacial interactions of multiple molecules, including Fcγ receptors, β2-integrins, and, possibly, ANCA antigens in 'activation clusters' or 'rafts' may define the signal triggered by ANCA. Finally, we summarize the contribution of neutrophils activated by ANCA to tissue injury in vivo and we describe the consequences of activation for the fate of the neutrophil.

General conclusions

In this thesis we tried to analyze interactions between PR3-ANCA and its target antigen in the context of activation of neutrophils in ANCA-associated small vessel vasculitis.
The first part, devoted to characterization of PR3-ANCA epitope specificity, provides some new insights into the background of the longitudinal changes in the functional properties of PR3-ANCA. Epitope shift from a disease-inducing epitope to other areas of the autoantigen during the course of the disease may be responsible for the initiation and progression of WG, as has been suggested for other autoimmune diseases such as SLE. It would explain how some functional properties of PR3-ANCA, such as interference with the proteolytic activity of PR3 and with binding of the PR3 inhibitor α1-antitrypsin (α1-AT) to PR3, may change between active and quiescent phases of the disease. One of the remaining questions to be answered in the future is why and how the autoimmune response against PR3 is triggered. Taking into account our observation of intra-molecular changes in PR3-ANCA epitope specificity, it cannot be excluded that mechanisms such as molecular mimicry may be instrumental in the initiation of PR3-ANCA production. It is conceivable that, on a particular genetic background, an ordinary immune response against an as yet unidentified bacterial or viral protein might lead to the synthesis of antibodies cross-reacting with PR3 (inter-molecular epitope spreading) and by intra-molecular epitope shift turn into pronounced autoimmune responses against this protein.

Comparison of the performance of a recombinant baculovirus PR3 and native PR3 in ELISA brought us to two important conclusions. First, the recombinant PR3 tested in our study is a promising alternative for native PR3 and, after extended optimization and validation of the assay, may be useful for PR3-ANCA detection in patients with systemic vasculitis. Importantly, the choice of the capturing anti-PR3 antibody is one of the factors determining the results of capture ELISA. Second, it should be stressed that, despite an apparently close similarity of native PR3 and recombinant PR3, these two forms of PR3 are not identical.

The second part of this thesis focuses on the implications of PR3 accessibility on the neutrophil surface for the susceptibility of these cells for activation by PR3-ANCA. Our observations, both in vivo and in vitro, support the hypothesis that the level of PR3 expression on the cell surface determines 'activatability' of resting neutrophils in PR3-ANCA-positive individuals. A high PR3 expression on the surface of resting neutrophils in patients with WG seems to be a risk factor for recurrent disease and, in the presence of PR3-ANCA, may have important implications for early cytoskeletal changes in the neutrophil, even without necessity of priming. Since both the pattern and the level of mPR3 expression have been suggested to be genetically
determined, it further supports the hypothesis on the involvement of genetic factors in the pathophysiology of WG.

In conclusion, these studies have contributed to the understanding of the role of PR3 and PR3-ANCA in WG. They show evidence for intra-molecular changes in PR3-ANCA which may explain changes in the functional properties of these autoantibodies. Furthermore, they demonstrate the consequence of PR3 expression on the cell surface for neutrophil activation and stress the role of this expression as, possibly, one of the genetically determined risk factors for the development of systemic vasculitis.