Epitope mapping of anti-proteinase 3 antibodies, implication for Wegener's granulomatosis

Geld, Ymke Maryse van der

Introduction

Anti-neutrophil cytoplasmic antibodies (ANCA) were first described in Wegener's granulomatosis (WG), a disease which is characterized by granulomatous inflammation in the upper and lower respiratory tract, systemic vasculitis affecting small blood vessels, and pauci-immune necrotizing crescentic glomerulonephritis. The close association between active WG and ANCA directed against proteinase 3 (PR3) suggested an autoimmune pathogenesis of WG. To date, the etiology and pathogenesis of ANCA-associated vasculitides, including WG, is not completely understood. It is firmly established that PR3-ANCA is a specific marker for WG and that the detection of these autoantibodies is helpful in the diagnosis and follow-up of WG patients. In addition, an increase in levels of PR3-ANCA precedes a relapse of WG in many cases, suggesting that ANCA may play a direct role in the pathogenesis of ANCA-associated vasculitides. Several *in vitro* observations further underline the hypothesis that ANCA are involved in the pathogenesis of WG. PR3-ANCA have been shown to activate primed neutrophils to the production of reactive oxygen species and to degranulation of lysosomal enzymes, including PR3. Furthermore, PR3-ANCA can interfere with the proteolytic activity of PR3 and with the complexation of PR3 to its physiological inhibitor α1-antitrypsin (α1-AT). Most importantly, the latter two functional characteristics of PR3-ANCA correlate with disease activity of WG. It has been described that changes in these functional characteristics of ANCA follow changes in disease activity more accurately than the previously mentioned changes in ANCA titers alone. This suggests that epitope specificity of PR3-ANCA is related to these functional characteristics of PR3-ANCA and to disease activity in WG.

Based on the above mentioned observations three questions were raised that we set out to address in this thesis. First, what are the epitopes on the PR3 molecule that are recognized by PR3-ANCA positive sera of patients with WG? Secondly, is there a spreading of epitopes recognized by PR3-ANCA positive sera in relation to changes in the disease activity of WG? And thirdly, is there a relation between the epitope specificity of PR3-ANCA positive sera and the functional activity of PR3-ANCA?

In **Chapter 2** we summarized the literature on the possible pathophysiological role of ANCA in ANCA-associated vasculitides. *In vitro* experimental work has extensively analyzed the effects of ANCA on neutrophils and monocytes. From those *in vitro* observations, we extracted four prerequisites for ANCA mediated endothelial cell damage; the presence of ANCA, expression of the target antigens for ANCA on primed neutrophils and monocytes, the necessity of an interaction between primed neutrophils and endothelium via β2-integrins, and finally, activation of endothelial cells. In this chapter we connected these prerequisites extracted from *in vitro* observed effects of ANCA to *in vivo* observations in patients with ANCA-associated vasculitides. We tried to explain whether *in vitro* effects may result into systemic inflammation, damage of blood vessels, and pauci-immune glomerulonephritis as seen in patients with ANCA-associated vasculitides.

Little is known about the epitopes on PR3 that are recognized by PR3-ANCA as elucidation of these epitopes has been hampered by the fact that the majority of PR3-ANCA recognizes conformational epitopes. Antibody binding to PR3 is abrogated by exposure of PR3 to reducing or denaturing agents. In order to elucidate the epitopes on PR3 recognized by PR3-ANCA, the 3D structure of PR3 has to be preserved. **Chapter 3** describes the characterization of monoclonal antibodies (mAbs) to PR3. These mAbs can be used as tools for epitope mapping studies of PR3-ANCA positive sera. Thirteen anti-PR3 mAbs, including nine widely used in diagnostic tests and four newly raised mAbs were tested. The antigen specificity was assessed by indirect immunofluorescence, immunoblotting, FACS analysis and antigen specific ELISA. The competition between the anti-PR3 mAbs in binding to PR3 was investigated by biosensor technology using the BIAcore. Interestingly, the anti-PR3 mAbs recognized a restricted number of four epitope areas on PR3 only. Two of the four newly raised mAbs recognized an epitope area on PR3 different from the established mAbs, thus enabling the detection of a broader range of epitope specificities of anti-PR3 antibodies. In preliminary studies some of the established mAbs were used in inhibition studies with PR3-ANCA positive sera. These mAbs were shown to bind to comparable epitopes areas as PR3-ANCA positive sera. Thus, these mAbs can serve as tools for epitope mapping. The restricted number of epitope areas recognized by mAbs on PR3, being a small and extensively folded protein, may suggest that the human anti-PR3 response in WG is also restricted.

As already mentioned PR3-ANCA mainly recognizes conformational epitopes on PR3. Conformational epitopes can best be identified by X-ray crystallography, nevertheless some epitopes defined by this method can also be identified by overlapping linear peptides. In **chapter 4** we used overlapping linear peptides of the entire sequence of PR3 to further define, possibly relevant, linear epitopes on PR3 recognized by PR3-ANCA of patients with WG. Sera of patients with WG as well as sera of healthy controls recognized five distinct peptide areas. Four of these peptide areas were significantly better bound by sera from patients with WG than by sera from healthy controls. Most interestingly, only sera of patients during their initial presentation of WG were responsible for the increased binding to these four distinct peptide areas. Two of these peptide areas were located near the active center of PR3, at the active site amino acids His^{44} and Ser^{176}. The other two peptide areas were located at the N-terminus and in the center of the PR3 sequence. All peptides recognized were surface accessible and coincided to some extent with earlier described epitopes that are recognized by PR3-ANCA. The amino acid sequences of the recognized peptide areas were highly specific for serine proteases but not specific for PR3 alone. Rabbit anti-PR3 antiserum recognized three distinct peptide areas, partly overlapping the areas recognized by PR3-ANCA positive sera, whereas none of the anti-PR3 mAbs recognized linear PR3 peptides.
Sera of healthy controls recognized peptides of PR3 but did not bind to the whole PR3 protein, whereas sera of WG patients did. These data suggest that the autoimmune response to PR3 may start from a universally present response to a restricted number of linear epitopes

and evolves into responses to conformational epitopes. Furthermore, it is suggested that upon relapse of WG epitope spreading of PR3-ANCA to more conformation dependent epitopes occurs resulting in PR3-ANCA that binds linear peptides with lower affinity.

In the previous chapter it was suggested that upon relapse of WG epitope spreading of PR3-ANCA occurs. In **chapter 5** we investigated whether these changes in epitope specificity of PR3-ANCA that occur during the course of the disease result in changes in functionality of PR3-ANCA. Sera of WG patients during active phase of disease or during remission were compared for their capacity to interfere with the proteolytic activity of PR3. The proteolytic activity of PR3 was determined with two different substrates; i.e. a small synthetic peptide substrate and a larger more physiological substrate, namely casein. Most of the PR3-ANCA samples inhibited the proteolytic activity of PR3. Especially with casein as a substrate, it was shown that, using a fixed amount of IgG, total IgG from patients with active disease had a higher inhibitory capacity towards the proteolytic activity of PR3 than IgG from patients during remission of WG. Furthermore, only for samples taken during active disease a correlation was observed between PR3-ANCA titer and their capacity to interfere with the proteolytic activity of PR3. For both substrates PR3-ANCA of patients during remission had a relatively higher inhibitory capacity towards the proteolytic activity of PR3 than PR3-ANCA of patients during an active phase. This may indicate that during disease exacerbation a shift in epitope specificity of PR3-ANCA occurs, which may have relevance for the pathogenicity of the antibodies in ANCA-associated vasculitides.

T cell-mediated immunity is thought to play an important role in the pathogenesis of granulomatous inflammation and vasculitis as found in patients with WG. The relevant epitopes responsible for this T cell-mediated immunity have not been identified yet. **Chapter 6** describes the *in vitro* proliferative responses to PR3 and linear peptides of PR3 by peripheral blood mononuclear cells (PBMCs) of patients with WG. The same set of peptides as used in the study described in chapter 4 was used. PBMCs of two WG patients and one healthy control proliferated to the whole PR3 molecule as well as to peptide pools. In addition, PBMCs from ten WG patients and from eight healthy controls who did not proliferate to the whole PR3 molecule did proliferate to pools of PR3 peptides. Although more WG patients tended to react to particular peptide pools, no significant difference was seen between WG patients and healthy controls. Thus, T cells of WG patients proliferate *in vitro* more frequently to PR3 peptides than to the whole PR3 protein. Interestingly, the highest levels of proliferation of WG patients and healthy controls were seen to peptides derived from the signal-sequence, the propeptide and the C-terminus of the PR3 sequence. These sequences of PR3 differ in various amino acids from HLE. The identified T cell epitopes might be specific for the PR3 sequence.
These data suggest that autoreactive T cells specific for peptides of PR3 are present in both WG patients and healthy controls. The proliferative response to whole PR3 was low in

patients with PR3-ANCA. A similar lack of response was seen in animals immunized with PR3. Possibly, the antigen that induces the T cell help for anti-PR3-specific B cells in WG patients is not PR3 itself but a protein complexed to PR3.

PR3 has been identified as the major autoantigen for ANCA in WG. For diagnostic purposes and to further study the pathophysiological role of PR3-ANCA in WG and to delineate the specific epitopes reactive with PR3-ANCA, highly purified PR3 is an important prerequisite. The usual method for its purification requires large amounts of blood and is laborious with relatively low yields. In addition, the presence of trace amounts of other autoantigens that maybe co-purified can never be ruled out completely. Therefore, we produced a recombinant form of PR3 and investigated whether this form of recombinant PR3 (rPR3) could be used for detection of PR3-ANCA in WG patients. Earlier studies attempting to produce rPR3 showed that expression of PR3 in bacteria is often accompanied by a loss of binding by PR3-ANCA positive sera. Therefore, we produced PR3 in the eukaryotic expression system of the methylothrophic yeast *Pichia pastoris* as well as in the bacteria *E. coli* as described in **Chapter 7**. *Pichia pastoris,* when grown on methanol as the sole carbon source, produces massive amounts of the *AOX1* (alcohol oxidase 1) gene product. The open reading frame of human PR3 without the pre-pro-peptide was cloned and expressed in *E. coli* (rcPR3) and in *Pichia pastoris* (rpPR3) behind the strong *AOX1* promoter. On SDS-PAGE the rcPR3 had the full length size of neutrophil PR3 and reacted with a polyclonal rabbit anti-PR3 serum. Unfortunately, only a minority of PR3-ANCA positive sera recognized this rcPR3. RpPR3 expressed with a 6-histidine tag was efficiently secreted into the culture supernatant. The secreted rpPR3 could be isolated through immobilized metal chelate chromatography. For three separate purifications this yielded 613 ± 122 μg rpPR3 per liter induced culture supernatant. Purified rpPR3 migrated as a single 32 kDa band on SDS-PAGE. RpPR3 proved to be proteolytically active and specific serine protease inhibitors like α1-antitrypsin and PMSF could inhibit its proteolytic activity. Seven out of ten sera from patients with WG recognized rpPR3 in an antigen specific capture ELISA. This suggests that the folding of rpPR3 approaches that of neutrophil PR3. Nevertheless, direct binding to the ELISA plates of any of the types of rPR3 lowered the binding of at least some WG sera. Since not all sera of WG patients recognized rpPR3 it cannot replace neutrophil human PR3 for diagnostic purposes at this stage.

As production of rPR3 which may replace neutrophil PR3 for diagnostic purposes proved more difficult than first expected we compared rPR3 produced in different expression systems on their recognition by anti-PR3 antibodies. **Chapter 8** describes the comparison between rPR3 produced in *E. coli* (rcPR3), *P. pastoris* (rpPR3), insect cells using the baculovirus system (rbPR3), the human mast cell line, HMC-1 (HMC-1/PR3-S176A), or the human epithelial cell line 293 (Δ-rPR3-S176A) as well as purified neutrophil PR3 on their recognition by anti-PR3 antibodies. All forms of rPR3 were recognized by rabbit serum raised

against PR3 on immunoblot, in capture ELISA and in direct ELISA system. By capture ELISA rcPR3 and rpPR3 were recognized by 11 (57%) and 13 (68%) of the 19 PR3-ANCA sera, respectively, whereas rbPR3, HMC-1/PR3-S176A, Δ-rPR3-S176A and neutrophil PR3 were recognized by all PR3-ANCA sera. Using the direct ELISA none of the PR3-ANCA sera recognized rcPR3, whereas rpPR3 and rbPR3 were recognized by 2 (11%) and 17 (89%) of the 19 PR3-ANCA sera, respectively. All thirteen anti-PR3 mAbs recognized neutrophil PR3 in the direct as well as in the capture ELISA. The rcPR3 was recognized by 2 mAbs in the capture ELISA but by none of the mAbs in the direct ELISA. The rpPR3 was recognized by 7 mAbs in the capture ELISA and only by 2 mAbs in the direct ELISA. All but one of the anti-PR3 mAbs recognized rbPR3, whereas HMC-1/PR3-S176A and Δ-rPR3-S176A were recognized by all anti-PR3 mAbs. Thus, our results demonstrate that rPR3 produced in a prokaryotic expression system is recognized only by a minority of anti-PR3 antibodies whereas PR3 produced in higher eukaryotic expression systems is recognized by most, if not all, tested antibodies to neutrophil PR3.
Reviewing the literature on rPR3 together with the data of this study several prerequisites were formulated for the production of rPR3 that is well bound by PR3-ANCA sera. Essential for the production of antigenic rPR3 is its secretion or localization in granules either by using a secretion signal or by using the leader sequence of PR3 itself. Cleavage of the N-terminal PR3 propeptide appears crucial for the expression of the complete array of epitopes recognized by PR3-ANCA. Finally, mammalian like glycosylation may contribute to the proper folding of PR3. In contrast, the C-terminal processing of PR3 does not appear to affect antigenicity.

Keeping the above mentioned prerequisites in mind we produced PR3 in a higher eukaryotic expression system. **Chapter 9** describes the expression of recombinant PR3 in insect cells using the baculovirus expression system. Two forms of rPR3 containing the propeptide of PR3 with or without a C-terminal His-tag (rproPR3-his and rproPR3, respectively) and two forms of rPR3 without the propeptide and with or without a C-terminal His-tag (rPR3-his and rPR3, respectively) were expressed in insect cells. By virtue of the honeybee mellitin signal sequence the four forms of recombinant PR3 were efficiently secreted into the culture medium of the infected insect cells. On SDS-PAGE these four recombinant PR3s migrated at approximately 32 kDa and were larger compared to neutrophil PR3. After four days of infection the production level of the rPR3s was approximately 2 μg/ml, which is comparable to another study on rPR3 expressed in insect cells. All four rPR3 variants were recognized by a rabbit serum raised against PR3 and by at least two anti-PR3 mAbs. All four rPR3 variants were recognized by seventeen of eighteen PR3-ANCA sera tested in a capture ELISA system. So, the presence of a C-terminal His-tag did not interfere with the antigenicity. The rPR3 containing a C-terminal His-tag, rPR3-his and rproPR3-his, could be isolated through immobilized metal chelate chromatography. Most interestingly both the mature and the proform of PR3 were enzymatically active. As almost all tested sera of WG patients

recognized these four rPR3 variants these variants of PR3 possibly can replace neutrophil PR3 for diagnostic purposes. Furthermore, these rPR3s, or substitution mutants of these rPR3s, can serve as important tools for studying epitope mapping and for studies on the pathophysiological role of PR3-ANCA in WG. In order to assess the use of recombinant PR3 expressed in insect cells in diagnostic tests, the recognition of this rPR3 by a large set of clinically relevant patient sera has to be determined.

PR3 is one of four serine protease homologues in the azurophilic granules of neutrophils and granules of monocytes. ANCA in patients with WG are mainly directed against PR3 and PR3, and not its homologues, is overexpressed in a variety of acute and chronic myeloid leukemia cells. Furthermore, cytotoxic T lymphocytes specific for a PR3 derived peptide have been shown to specifically lyse leukemia cells that overexpress PR3. In **chapter 10** we summarize the literature on PR3 and the characteristics of PR3 which may implicate PR3 in the pathogenesis of WG and as target for immuno-therapy in myeloid leukemia. We focused on the genetic localization and gene regulation of PR3, the processing, storage, and expression of the PR3 protein, and the physiological functions of PR3. Three main differences between PR3 and the other neutrophil derived serine proteases, that is human leukocyte elastase (HLE), cathepsin G (CatG) and azurocidin (AZU) were observed. PR3 is, apart from its presence in azurophilic granules, also present in the secretory vesicles and in specific granules of PMNs, and PR3 is expressed on the plasma membrane of resting neutrophils. Most importantly, this membrane expression is bimodal and the presence of a high percentage of neutrophils with PR3 membrane expression is a risk factor for vasculitis. Secondly, the role described for PR3 as a feedback regulator in myeloid differentiation has not been suggested for the other PMN derived serine proteases. Thirdly, PR3, both enzymatically active and inactive, binds to and enters human endothelial cells. Upon binding PR3 can activate these cells and induce apoptosis of endothelial cells. This apoptosis induction was ascribed to an enzymatically inactive C-terminal domain of PR3. The significance of the above mentioned differences between PR3 and the other neutrophil derived serine proteases in our understanding of the role of PR3 in the pathogenesis of WG or as target for immuno-therapy in myeloid leukemia has still to be determined. In conclusion, PR3 is a very intriguing protein with a large array of physiological and, possibly, pathophysiological functions.

General discussion

In this thesis we tried to address three questions. First, we wanted to elucidate the epitopes on the PR3 molecule that are recognized by PR3-ANCA positive sera of patients with WG. As already mentioned for the determination of the epitopes recognized by PR3-ANCA positive sera of WG patients it is important that the 3D structure of PR3 is preserved, which makes epitope mapping a difficult task.

Using linear peptides of the entire PR3 sequence four epitope areas were identified. Two of these epitope areas were located near the active center of PR3. The other two peptide areas were located at the N-terminus of the mature form of PR3 and in the center of the PR3 sequence. No epitopes were detected at the C-terminus of PR3 nor in the signalsequence or in the prosequence. Unfortunately, the amino acid sequence of the recognized peptide areas was not specific for PR3 alone. Also, in the study described in chapter 5 most of the PR3-ANCA IgG samples interfered with the proteolytic activity of PR3 but not with the proteolytic activity of HLE. This suggests that most PR3-ANCA positive sera recognize epitopes located at or near the active site of PR3. However, the active site of PR3 has large homology with murine PR3 and, to a lesser extent, with HLE. Still, PR3-ANCA do not react with murine PR3 or HLE. Along with the homology in primary sequence a difference in conformation of the protein could influence the recognition by antibodies. Since PR3-ANCA mainly recognize conformational epitopes this conformational difference might be of crucial importance for the antigen-specificity.

This importance of the 3D structure and of proper processing of PR3 for the expression of the complete array of epitopes recognized by PR3-ANCA hampered the production of an antigenic recombinant PR3. Not withstanding these problems we succeeded in the production of a recombinant PR3 which possibly can replace neutrophil PR3 for diagnostic purposes. By amino acid substitutions or deletion of small parts of this recombinant PR3, and still keeping its 3D structure intact, the epitopes recognized by PR3-ANCA could possibly be further delineated and narrowed down to specific areas on the PR3 molecule.

Thus, PR3-ANCA do not bind to one immuno-dominant epitope on PR3 but, possibly, in alliance with mAbs to PR3, recognize only a restricted number of epitope areas.

In Wegener's granulomatosis B cells producing PR3-ANCA need help from autoreactive T cells. The peptides responsible for the highest level of proliferative response of T cells were located in the signal-sequence, the propeptide and the C-terminus of PR3. Epitopes recognized by PR3-ANCA sera of WG patients do not correspond to epitopes recognized by T cells of WG patients and healthy controls. The signal-sequence, the propeptide and the C-terminus are removed upon processing of PR3, which may explain the low proliferative response to the PR3 molecule. However, autoreactive T cells to linear peptides of the PR3 sequence are present in both WG patients and healthy controls. A lack of response to whole PR3 was seen in most patients as well as in animals immunized with PR3. Possibly, the antigen inducing T cell help for anti-PR3-specific B cells in WG patients is not PR3 itself but a protein complexed to PR3. For further research on the epitopes recognized by T cells in WG

crude extracts of neutrophils or PR3 complexed to other substances such as anti-proteases may be of use.

The second question we wanted to address was concerned with spreading of epitopes recognized by PR3-ANCA positive sera in relation to changes in disease activity of WG. We have shown that only sera of patients during their initial presentation of WG recognized linear peptides significantly better than sera from healthy controls. This suggests that upon relapse of WG spreading of epitopes that are recognized by PR3-ANCA to more conformation dependent epitopes has occurred resulting in PR3-ANCA that bind linear peptides with lower affinity.

The third question concerning the relation between the epitope specificity of PR3-ANCA positive sera and the functional activity of PR3-ANCA. We have shown that, using a fixed amount of IgG, total IgG from patients with active disease had a higher inhibitory capacity towards the proteolytic activity of PR3 than IgG from patients during remission of WG. Furthermore, PR3-ANCA of patients during remission had a relatively higher inhibitory capacity towards the proteolytic activity of PR3 than PR3-ANCA of patients during an active phase. These data indicate that, during disease exacerbation, a change in epitope specificity of PR3-ANCA may lead to a change in functional activity. However, whether these changes in functional activity lead to a change in the pathogenicity of ANCA, such as their capacity to activate neutrophils to degranulate and produce oxygen radicals or the capacity to interfere with the inhibition of PR3 by α1-AT, still has to be determined. Experiments investigating this are currently performed.

In conclusion, this study has contributed to our understanding of PR3 as an autoantigen in WG. Tools have been generated to further explore the interaction between PR3 and PR3-ANCA. Recombinant PR3 and anti-PR3 mAbs can be used for diagnostic purposes. Recombinant PR3 can furthermore be used for studies aimed at a further characterization of the epitopes on PR3 that are recognized by PR3-ANCA. This, hopefully, will lead to elucidation of the pathogenesis of the associated disease, Wegener's granulomatosis.