Chapter 5

Human anti-neutrophil cytoplasm autoantibodies to proteinase 3 (PR3-ANCA) bind to neutrophils

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ABSTRACT

Background. Recently, the in vivo pathogenic role of anti-neutrophil cytoplasm autoantibodies (ANCA) in ANCA-associated vasculitis has been challenged by Abdel-Salam et al. In their report, they observed that ANCA directed against proteinase 3 (PR3) cannot bind to their target autoantigen PR3 on circulating neutrophils (PMN). Here we present evidence that human PR3-ANCA do specifically bind to PMN that express PR3 on their membrane.

Methods. PMN were isolated from donors showing bimodal membrane PR3 expression on their PMN (n=3). TNF-α-primed PMN or PMA-stimulated PMN were incubated with serum or plasma from PR3-ANCA positive patients with Wegener's granulomatosis (WG) (n=8) or healthy controls (n=8). Binding of IgG in serum or plasma samples to PMN was assessed by indirect immunofluorescence.

Results. Binding of IgG in undiluted plasma or serum from PR3-ANCA positive WG-patients to PMN was significantly increased compared to plasma or serum from healthy controls. Dilution of plasma and serum showed concentration-dependent binding of IgG. Double staining for PR3 and IgG demonstrated that IgG in plasma or serum from PR3-ANCA positive patients only bound to those PMN that expressed PR3 and not to PMN that lacked PR3 expression on their membrane.

Conclusion. PR3-ANCA in undiluted serum or plasma from PR3-ANCA positive WG patients bind to TNF-α-primed and PMA-stimulated PMN that express PR3 on their membrane. Therefore, the hypothesis that PR3-ANCA can bind and activate primed PMN is still the most attractive explanation for the contribution of PR3-ANCA to the pathogenesis of Wegener's granulomatosis.

Key words. proteinase 3, ANCA, neutrophil, vasculitis, Wegener's granulomatosis
INTRODUCTION
In ANCA-associated vasculitis, ANCA have been suggested to be pathogenic as ANCA titers correlate with disease activity 1-3 although this has been questioned as well 4. Experimentally, a single injection of anti-mouse MPO antibodies in mice induced focal, necrotizing and crescentic glomerulonephritis, supporting their pathogenic potential 7. The proposed pathogenic role of ANCA is further supported by observations demonstrating that in vitro ANCA can activate primed polymorphonuclear leukocytes (PMN) leading to their degranulation and oxygen radical production 6;7. In patients with Wegener's granulomatosis ANCA most frequently target proteinase 3 (PR3). Binding of ANCA to their target antigen on PMN is a prerequisite for activation of PMN by ANCA, as has been shown in many studies 6-9.

Recently, Abdel-Salam et al suggested that, in vivo, PR3-ANCA cannot bind to their target autoantigen proteinase 3 on PMN 10. They showed, by indirect immunofluorescence, lack of ANCA-bound circulating PMN in patients with active WG. In addition, no specific binding of IgG in PR3-ANCA-containing plasma to phorbol-myristate-acetate (PMA) activated PMN could be detected. Only purified IgG fractions from PR3-ANCA positive patients showed specific binding to PR3. This binding, however, was greatly reduced when low concentrations of serum, purified IgG or albumin were introduced. Based upon these observations the authors suggested that PR3-ANCA have a low affinity towards membrane-bound PR3. Therefore, they stated that PR3-ANCA cannot be potent activators of PMN and, as such, cannot contribute substantially to the pathogenesis of WG. As this conclusion challenges the relevance of many studies on ANCA-associated vasculitis, we undertook the present study in order to further explore binding of PR3-ANCA to PMN. The results are fully compatible with binding of human PR3-ANCA to PMN.

METHODS
Patient sera and plasma samples
Serum (n=8) and paired plasma samples (n=8) of PR3-ANCA positive patients were selected based on moderate to high titres of PR3-ANCA (ranging from 1:160 to 1:640, as determined by indirect immunofluorescence (IIF)) and were stored at –20°C until use. PR3-ANCA positive patients all had Wegener's granulomatosis based upon the Chapel Hill criteria 11. PR3-ANCA specificity was assessed by PR3-specific enzyme linked immunosorbent assay, as described previously 12. Control serum and plasma samples were obtained from 8 different healthy individuals. The study was approved by the local Ethical Committee.

Neutrophil isolation and priming
Neutrophils of 3 healthy individuals were isolated from heparinized blood by centrifugation on Lymphoprep (Nycomed, Oslo, Norway). All these individuals showed bimodal expression of PR3 on their PMN. Bimodal distribution of PR3 expression on PMN is a well established phenomenon 13;14 representing the presence of two subsets of PMN within one donor: a subset of PMN expressing PR3 on their membrane (mPR3+ PMN) and a subset of PMN lacking expression of PR3 on their
membrane (mPR3-PMN\textsuperscript{13,14}). Erythrocytes were removed twice by hypotonic lysis with ice-cold ammonium chloride. Thereafter, cells were washed with ice-cold Hanks’ Balanced Salt Solution (HBSS) without Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (GIBCO / Life Technologies, Breda, The Netherlands) and resuspended in HBSS with Ca\textsuperscript{2+} and Mg\textsuperscript{2+} (1.10\textsuperscript{7}/ml). This was followed by priming with 10 ng/ml TNF-\alpha for 15 min at 37\degree C or stimulation with 1 \mu g/ml phorbol-myristate acetate (PMA; Sigma, Zwijndrecht, The Netherlands) for 20 min at 37\degree C. In some experiments, Fc\gamma-receptors on PMN were saturated by incubation with goat derived heat-aggregated IgG (0.5 mg/ml) for 15 min at 4\degree C. PMN were subsequently washed with PBS.

\textbf{Assessment of binding of PR3-ANCA}

1.10\textsuperscript{6} neutrophils were incubated with 100 \mu l undiluted serum or plasma and 10 \mu g/ml mouse anti-human PR3 monoclonal antibody (PR3.G-3) \textsuperscript{15} for 30 min at room temperature. In control experiments an irrelevant IgG\textsubscript{1} monoclonal antibody was used as isotype control (IQProducts, Groningen, The Netherlands). In addition, experiments were repeated with another mouse anti-human PR3 monoclonal antibody (PR3.12.8) \textsuperscript{9,15,16} instead of the anti-PR3.G-3 MoAb. After incubation, cells were washed twice with 3 ml PBS and incubated for 30 min at room temperature with rabbit- anti-human IgG-FITC F(ab’)\textsubscript{2} antibodies (1:50, Dako Cyomation, Glostrup, Denmark) and with PE-conjugated goat anti-mouse antibodies (1:50, Southern Biotechnology Associates, Inc., Birmingham, USA). Subsequently, cells were washed with 3 ml PBS and fixed in 1% paraformaldehyde containing PBS on ice. Flowcytometric analysis was performed on an ELITE flowcytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). PR3-restricted binding of IgG from sera or plasma was assessed as follows. First, subpopulations of PMN showing either surface expression of PR3 or lacking PR3 expression were detected based on mouse anti-PR3 MoAb binding. These subpopulations were subsequently gated as mPR3\textsuperscript{+} or mPR3\textsuperscript{−} PMN, respectively. Negligible binding of IgG in plasma or serum from PR3-ANCA positive patients was observed on the mPR3\textsuperscript{−} PMN subpopulation. Mean Fluorescence Intensity (MFI) of binding of IgG in plasma or serum to the gated mPR3\textsuperscript{−} PMN subpopulation was subtracted from MFI of binding of IgG in plasma or serum to the gated mPR3\textsuperscript{+} PMN subpopulation.

\textbf{STATISTICS}

Statistical analysis was performed using the non-parametric Mann Whitney test and Graph-Pad Prism, version 3.0 (GraphPad Prism Software, San Diego, CA, USA).

\textbf{RESULTS}

TNF-\alpha-primed PMN of a donor with bimodal PR3 expression were incubated with undiluted serum or plasma samples from PR3-ANCA positive patients and healthy controls. In these experiments, PMN were co-incubated with mouse anti-PR3 MoAbs to assess PR3-restricted binding, as described in Materials and Methods. In Figure 1, a representative dot plot of double-staining is presented showing that binding of IgG in serum or plasma from PR3-ANCA-positive patients is restricted to those cells that
express PR3 on their membrane. Binding of IgG in undiluted serum (mean MFI 30.4 ± 9.1, n=8) or undiluted plasma (mean MFI 58.1 ± 9.2, n=8) from PR3-ANCA positive patients to primed PMN was significantly increased in comparison to undiluted serum or plasma from healthy controls (mean MFI 4.3 ± 0.7, n=8 and mean MFI 2.9 ± 0.6, n=8, respectively; p=0.003 and p=0.001, respectively) (donor # 1) (Figure 2). Comparable results were obtained when using another PMN donor (donor # 2, Figure 2).
Figure 3. Concentration-dependent IgG binding in serially diluted plasma samples from PR3 ANCA-positive patients to TNF-α-primed PMN. Plasma samples of PR3-ANCA-positive patients (n=4) were serially diluted in PBS. TNF-α-primed PMN were incubated for 30 minutes at room temperature with the respective diluted samples. PMN were co-incubated with mouse anti-PR3 MoAb to assess PR3-restricted binding, as described in Materials and Methods. Subsequently, cells were washed twice and incubated with FITC-conjugated rabbit anti-human IgG and PE-conjugated goat anti-mouse IgG. Results were compared with that of undiluted plasma samples from healthy controls (n=4). On the y-axis mean fluorescence intensity (MFI) values of the binding experiments are depicted. Values are given as mean ± standard error of the mean (SEM).

Furthermore, four plasma samples from PR3-ANCA-positive patients were serially diluted and showed concentration-dependent binding of IgG to primed PMN (Figure 3). To explore differences in IgG binding to PR3 on PMN due to different pre-incubation procedures, TNF-α-primed PMN as well as PMA-stimulated PMN were used. Binding of IgG in undiluted serum or plasma samples from PR3-ANCA-positive patients (mean MFI 26.9 ± 11.8, n=8 and mean MFI 30.9 ± 7.7, n=8, respectively) to PMA-stimulated PMN was also significantly increased as compared to serum or plasma samples from healthy controls (mean MFI 2.6 ± 0.8, n=8, and mean MFI 3.9 ± 1.2, n=8, respectively; p=0.0093, and p=0.0006, respectively) (Figure 4).
Finally, to exclude binding of IgG to Fcγ-receptors, we saturated Fcγ-receptors on PMN using goat derived heat-aggregated IgG (0.5 mg/ml). Also after saturating Fcγ-receptors, binding of IgG from plasma samples of PR3-ANCA positive patients (mean MFI 69.3 ± 6.9, n=3) was significantly higher as compared to plasma samples of healthy controls (mean MFI 16.7 ± 4.7, n=3; p < 0.05) (Figure 5).

**Figure 5.** Binding of IgG from plasma samples of PR3-ANCA positive patients is not dependent on Fcγ-receptor interactions. Fcγ-receptors on TNF-α-primed PMN from a donor with bimodal PR3 expression were saturated by incubation with goat derived heat-aggregated IgG (0.5 mg/ml) for 15 min at 4°C. Cells were subsequently washed in PBS. Saturated and non-saturated PMN were incubated for 30 min at room temperature with undiluted plasma samples from PR3-ANCA positive patients (n=3) and healthy controls (n=3). Samples were co-incubated with mouse anti-PR3 MoAb to assess PR3-restricted binding, as described in Materials and Methods. Subsequently, cells were washed twice and incubated with FITC- conjugated rabbit anti-human IgG and PE- conjugated goat anti-mouse IgG. On the y-axis mean fluorescence intensity (MFI) values of binding experiments are depicted. Values are given as mean ± standard error of the mean (SED).

**DISCUSSION**

In this study we demonstrate that PR3-ANCA in undiluted serum or plasma bind to TNF-α-primed or PMA-activated PMN. In addition, we show that the amount of binding of PR3-ANCA to primed PMN expressing PR3 on their membrane is dilution-dependent.

Our results seem to be in contrast with the findings of Abdel-Salam et al who showed that binding of IgG in plasma from PR3-ANCA positive patients to stimulated PMN did not differ significantly from binding of IgG in plasma derived from WG patients negative for PR3-ANCA or healthy donors. PR3-ANCA positive patients were defined as patients with titres of ≥ 1:160 (anti-PR3 > 100 U (ELISA)), whereas PR3-ANCA negative patients had titres of ≤ 1:20 (no detectable anti-PR3 by ELISA) 10. Furthermore, the authors observed no correlation between ANCA titres and levels of IgG binding to PMA-stimulated PMN. In our study, we used serum or plasma samples from PR3-ANCA positive WG patients with PR3-ANCA titres ranging from 1:160 to 1:640 and we observed a dose-dependent binding of IgG from PR3-ANCA positive patients to primed PMN.

Abdel-Salam et al used PMN stimulated with 1µg/ml phorbol myristate- acetate (PMA) as an upregulator of PR3 expression10. In most studies, effects of IgG binding have been studied in vitro using TNF-α-primed PMN 6;7;9;17. Therefore, we compared
IgG binding in serum and plasma from PR3-ANCA positive WG patient to TNF-α-primed and PMA–stimulated PMN in parallel. We observed significant binding of IgG in serum and plasma samples from PR3-ANCA positive WG patients both to PMA-stimulated PMN and to TNF-α-primed PMN.

In order to proof that binding of IgG from serum or plasma from PR3-ANCA positive patients was restricted to mPR3-expressing PMN, we used donors who showed bimodal PR3 expression on the membrane of their PMN. Bimodal distribution of PR3 expression on PMN is a well established phenomenon \cite{13,14}, representing the presence of two subsets of PMN within one donor: a subset of PMN expressing PR3 on their membrane (mPR3+ PMN) and a subset of PMN lacking expression of PR3 on their membrane (mPR3-PMN)\cite{10,13,14}. Furthermore, bimodal expression of PR3 is an unique and stable feature of certain individuals, and is genetically determined \cite{13,14,18}.

Therefore, we performed double staining experiments using mouse anti-PR3 MoAb together with IgG from serum or plasma of PR3-ANCA- positive patients in order to show that IgG from PR3-ANCA positive patients bind to the same PMN as anti-PR3 MoAbs do, that is to mPR3+ PMN. The results led us to conclude that IgG from PR3-ANCA positive patients bind to mPR3+ PMN, implying binding to the same antigen as anti-PR3 MoAbs do. This conclusion is strengthened by the fact that bimodal expression is only observed for PR3 and not for elastase or other neutrophil markers \cite{13,14}.

Abdel- Salam et al state that PR3-ANCA are low-affinity antibodies. Next to the lack of specific binding of ANCA to PMN in vivo and in vitro, this statement was build upon the observation that binding of isolated IgG from plasma of PR3-ANCA-positive patients to PMN was inhibited by addition of 1:10 diluted serum, human IgG, albumin, or by increasing the reaction volume \cite{10}. However, the concentration of isolated IgG that they used in their binding experiments (10 - 40 µg/ml) was rather low as compared to physiological levels of IgG in serum which are around 10-15 mg/ml. Another argument against the low affinity nature of ANCA is the functional interaction of PMN with ANCA-containing serum, already demonstrated in 1990 by Falk et al \cite{6}. They showed that incubation of TNF-α-primed PMN with 1:10 diluted ANCA-positive serum leads to activation of these cells resulting in degranulation and production of oxygen radicals \cite{6}.

In conclusion, in the present study we demonstrate that PR3-ANCA in undiluted serum or plasma from PR3-ANCA positive WG patients can bind to PMN that express membrane-bound proteinase 3. Therefore, the hypothesis that PR3-ANCA can bind and activate primed PMN leading to degranulation and oxygen radical production is still the most attractive in explaining the contribution of PR3-ANCA to the pathogenesis of Wegener's granulomatosis.

REFERENCES


