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Allosteric modulation of proteinase 3 activity by anti-neutrophil cytoplasmic antibodies in granulomatosis with polyangiitis

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ABSTRACT

Anti-neutrophil cytoplasmic antibodies (ANCA) with proteinase 3 (PR3) specificity are a useful laboratory biomarker for the diagnosis of Granulomatosis with Polyangiitis (GPA) and are believed to be implicated in the pathogenesis. It has been repeatedly suggested that disease activity of GPA is more closely related to the appearance and rise of PR3-inhibiting ANCA than to an increase of total ANCA. Previous studies on a limited number of patient samples, however, have yielded inconclusive results. To overcome the previous methodological limitations, we established a new ultrasensitive method to quantify the inhibitory capacity of PR3-ANCA using small volumes of plasma from patients with GPA. A large collection of longitudinally-collected samples from the Wegener Granulomatosis Etanercept Trial (WGET) became available to us to determine the functional effects of ANCA on PR3 in comparison to clinical disease manifestations. In these patient samples we not only detected PR3-ANCA with inhibitory capacity, but also PR3-ANCA with enhancing effects on PR3 activity. However no correlation of these activity-modulating PR3-ANCA with disease activity at either the time of enrollment or over the course of disease was found. Only patients with pulmonary involvement, especially patients with nodule formation in the respiratory tract, showed a slight, but not significant, decrease of inhibitory capacity. Epitope mapping of the activity-modulating PR3-ANCA revealed a binding on the active site surface of PR3. Yet these ANCA were able to bind to PR3 with an occupied active site cleft, indicating an allosteric mechanism of inhibition. The recently described signal ratio between the MCPR3-3 and MCPR3-2 capture ELISA was consistent with the binding of activity-modulating ANCA to the active site surface. Evidence for a shared epitope between activity-modulating PR3-ANCA and MCPR3-7, however, was very limited, suggesting that a majority of PR3-ANCA species do not inhibit PR3 by the same mechanism as previously reported for MCPR3-7.

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1. Introduction

Anti-neutrophil cytoplasmic autoantibodies (ANCA) reacting with proteinase 3 (PR3) have a high positive predictive value for the diagnosis of granulomatosis with polyangiitis (GPA), formerly known as Wegener's granulomatosis (WG), but their role in the pathogenesis of the disease and as a biomarker of disease activity and relapse risk remains a controversial issue [1,2]. When ANCA disappear during therapy, the likelihood of a relapse within the subsequent five-year period is reduced [3]. However, the persistence or increase of ANCA levels is not reliably associated with relapses [2,4]. Only less than 10% variation in disease activity was estimated to be attributable to changes in ANCA levels [2].

PR3-ANCA are directed to different non-overlapping epitopes on the surface of the native molecule and may vary between patients and over the course of the disease. Variable epitope recognition, binding affinities and association/dissociation rate constants of PR3-ANCA, all contribute to their heterogeneity and may result in functional differences. The functional effects of different PR3-ANCA are difficult to quantify in small plasma samples, and consequently their pathogenic potential and clinical associations are poorly understood. A few small studies have focused on a subset of PR3-ANCA which reduced the catalytic activity of PR3 and impaired its inhibition by the major plasma protease inhibitor, alpha-1-proteinase inhibitor (α1PI). Their results have suggested that disease activity of GPA may be more closely related to the appearance and rise of PR3-inhibiting ANCA than to total PR3-ANCA levels measured conventionally [4–6]. As PR3 is cleared by complex formation with α1PI and this complexation is dependent on the activity of PR3, inhibitory PR3-ANCA may delay the clearance of PR3 and contribute to the pathogenesis via this mechanism.

Genetic studies [7,8] and a recently published genome wide association study [9] further support this hypothesis. PR3- and MPO-ANCA-associated autoimmune vasculitis were found to be genetically distinct, and only the PR3-ANCA associated clinical condition was associated with certain risk alleles of the α1PI (serpinA1) and PR3 (PRTN3) loci. In particular, deficiency alleles of serpinA1, the S- and Z-variants, were identified as risk factors for ANCA-positive GPA, suggesting that an imbalance between PR3 activity and the α1PI inhibitor facilitates the development of GPA. Inhibitory PR3-ANCA may thus reduce the clearance of PR3 by α1PI, and consequently inhibitory PR3-ANCA and low levels of α1PI could promote the disease process in the same direction.

The question as to whether specific PR3-ANCA subsets and particularly those inhibiting PR3 activity are associated with relapses, specific organ involvement and overall disease activity, has remained unanswered, largely because the quantification of functional effects of PR3-ANCA could not be conducted efficiently on small plasma samples from large cohorts of patients.

Recent progress in the development of highly sensitive extended peptide substrates for PR3 and of optimized fluorophore-quencher pairs permitted us to establish a new ultrasensitive assay to quantify the inhibitory capacity of PR3-ANCA in small plasma volumes. Contamination of IgG preparations by α1PI is excluded by our protocol, and the inhibitory effect of PR3-ANCA is determined at very low levels of the active target antigen. Availability of plasma samples collected from a well characterized GPA cohort during the conduct of the Wegener Granulomatosis Etanercept Trial (WGET) [10], allowed us to explore clinical associations with inhibitory PR3-ANCA.

2. Material and methods

2.1. Patients

This study used plasma from patients with GPA obtained during the conduct of the WGET. 180 patients were enrolled into this trial [10]. All patients were diagnosed with GPA, fulfilling at least 2 of 5 modified criteria for the classification of GPA from the American College of Rheumatology. At enrollment all patients further had a Birmingham Vasculitis Activity Score for Wegener's Granulomatosis (BVAS/WG) of $\geq$3. 128 of these patients were diagnosed with severe disease and 52 patients with limited disease. Severe disease was defined as immediately threatening the life of the patients or organ function [11]. 179 baseline samples from these 180 patients were available for analysis in the present study. Furthermore follow-up samples from 13 patients with at least one relapse during the course of the study (n = 148) and from 8 patients without flares (n = 106) were assessed. The follow-up samples from the patients were obtained 6 and 12 weeks after enrollment and every 3 months thereafter [11].

2.2. Materials

Protein G Dynabeads, used for the purification of IgG from plasma, were purchased from Life Technologies (Carlsbad, CA, USA). Purified human neutrophil PR3 was obtained from Diarect AG (Freiburg, Germany) and recombinant human elafin from Proteo Biotech AG (Kiel, Germany). The catalytically inactive, conformationally intact variant of mature PR3, ΔhPR3S195A and proPR3 were produced as previously described [12]. The monoclonal antibodies (mAbs) MCPR3-1, MCPR3-2, MCPR3-3, MCPR3-7 and MCPR3-11 have been previously described [12–14]. WGM2 was purchased from Cell Sciences (Canton, MA), PR3-G2 and PR3-G4 were kindly provided by C.G.M. Kallenberg, Groningen, The Netherlands, and CLB-12.8 was purchased from Sanquin, Amsterdam, The Netherlands. The mAbs 1B10, 2E1 and 1F10 were purchased from HyTest, Turku, Finland and 4B12 and 7D12 were generated by E. Kremmer, HMGU, Munich, Germany. The FRET-substrate used for the activity measurements, 5-TAMRA-VADnVR-DYQ-diaminopropionyl-fluorescein (Dap-CF), was provided from EMC Microcollections (Tübingen, Germany).

2.3. Capture ELISA

PR3-ANCA binding to PR3 was assessed in three different capture ELISA tests. Immobilized MCPR3-2 or MCPR3-3 were used to capture mature PR3, whereas the proform of PR3 was captured with MCPR3-7 onto the plates. The capture ELISA was carried out as previously described [13].

2.4. Protein G purification of total IgG from patient plasma

For the purification of total IgG from patient plasma, the plasma was diluted with 1 volume of PBS, 0.05% Tween 20. One μl protein G Dynabeads was added per 2.5 μl patient plasma. After rotation of the mixture for one hour at room temperature the beads were washed three times with PBS, 0.05% Tween 20. IgG were eluted from the beads by adding nine tenth of elution buffer (0.1 M glycin/HCl, pH 2.5) of the original plasma volume. After incubation for one minute, the fluid was separated from the beads in a magnetic rack, and the pH of the elution fraction was neutralized by adding one tenth of the original plasma volume as