Progranulin-autoantibodies in sera of rheumatoid arthritis patients negative for rheumatoid factor and anti-citrullinated peptide antibodies

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Abstract

Objective

Previously we discovered antibodies against progranulin (PGRN-abs) in a protein array-based screening of sera from various rheumatic diseases. Here we conducted a study to evaluate the prevalence of PGRN-abs in seropositive and seronegative rheumatoid arthritis (RA).

Methods

PGRN-abs were determined in the sera from 257 RA patients being seropositive for RF-IgM and/or ACPA-IgG and from 224 seronegative RA patients who were prospectively included in this study (total RA cohort n=481). All serum samples from the included participants were tested for RF-IgM as well as for ACPA-IgG, and PGRN-abs were determined using a previously described ELISA. Statistics was performed using the χ^2 test for evaluating differences in clinical data; to evaluate independent statistical effects on the frequency of PGRN-abs status a logistic regression model with Wald-test was performed.

Results

PGRN-abs were detected in 25.3% from seropositive RA and in 21.0% from RF- and ACPA-negative RA resulting in a prevalence of 23.7% for both cohorts together. Comparing mean DAS28 values in the PGRN-abs positive cohort with the PGRN-abs negative cohort, the DAS28 value was significantly higher in PGRN-abs positive RA patients (3.81 vs. 3.50, p=0.038). A trend for higher frequencies of PGRN-abs in sera of RA patients with unfavourable characteristics such as erosive disease or requiring TNFi medication was observed.

Conclusion

These data suggest that the determination of PGRN-abs in seronegative RA patients may reduce their seronegative status. Further studies are required to evaluate PGRN-abs as a potential diagnostic marker in RA.

Key words
progranulin, autoantibodies, rheumatoid arthritis, ACPA, seronegative

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Introduction

Progranulin (PGRN) has been identified as being abundantly expressed in epithelial cells, leukocytes, neuronal cells and chondrocytes. Besides its function as a growth factor and neurotropic factor, PGRN has been demonstrated to function as an anti-inflammatory protein; via direct antagonism of TNF-α and TNF-like antigen-1 (TLA1/TNFSF15) by high affinity binding of PGRN to tumour necrosis factor (TNF) receptors 1 and 2 and death receptor 3 (DR3/TNFRSF25) in multiple inflammatory conditions including arthritis (1, 2). This anti-inflammatory effect has been confirmed in several mouse models of autoimmune diseases *in vivo*; the described physiologic TNF antagonism led to the question whether PGRN or a recombinant derivate thereof could be introduced as a candidate for a therapeutic TNF inhibition (3, 4). PGRN can be detected in the synovial tissue derived from patients suffering from rheumatoid arthritis (RA); furthermore, the PGRN levels determined in synovial fluid and in blood serum were higher in RA than in non-inflammatory arthropathy (5). However, further cohorts of small sample sizes have not yet clearly demonstrated that serum PGRN concentrations can be used as a prognostic biomarker for RA (6, 7). We had previously shown that in autoimmune diseases PGRN undergoes hyperphosphorylation of serin81 (P-PGRN) preceding the development of autoantibodies against PGRN (PGRN-abs) (8). Furthermore, PGRN-abs can be detected in a wide spectrum of autoimmune diseases with a prevalence ranging from 20 to 30% (9-11). *In vitro* experiments can clearly demonstrate that PGRN-abs reduce PGRN, leading to a pro-inflammatory environment via reduced PGRN-mediated TNF receptor inhibition. In addition, the pro-inflammatory effects in autoimmune diseases are reinforced by the presence of P-PGRN, which in contrast to PGRN prevents the direct interaction with TNF receptor 1 & 2 and DR3 (4, 12). Against this background, the occurrence of PGRN-abs in sera of RA patients seems to reflect not only autoimmune processes in RA, but the determination of serum PGRN-abs may improve the diagnostic RA serology, particularly for differentiating between autoinflammatory and non-autoinflammatory aetiology of arthritis; in this context, the determination of rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPA) has been established as diagnostic criteria for RA according to the currently valid ACR/EULAR classification criteria (13).

Here we conducted an observational study to evaluate the prevalence of PGRN-abs in patients with seropositive and seronegative RA in a prospective trial. Moreover, we prospectively investigated whether the proportion of seronegative (RF-negative, ACPA-negative) RA patients can be reduced by the proportion of PGRN-abs positive RA patients.

Material and methods

PGRN-abs were determined in the sera from 257 RA patients seropositive for RF-IgM and/or ACPA-IgG and from 224 seronegative RA patients, who were prospectively included in this study between January 2013 and December 2015. Sera from healthy donors (n=97) served as control cohort, of which only one serum was tested positive for PGRN-abs (1.03%). The study was conducted according to the Declaration of Helsinki and was approved by our regional ethical review committee (Ethikkommission der Aerztekammer Saarland, approval number: N242/11). All study participants gave their written informed consent. The study population was recruited from the Outpatient department of Rheumatology of Saarland University Medical School and from the Outpatient Rheumatology Center Berlin-Lichtenberg, Berlin, Germany.

All RA patients fulfilled the revised diagnostic ACR/EULAR criteria for RA from 2010 (13). For the seronegative RA cohort the presence of psoriatic manifestations was excluded by physical examination. All RA patients were under anti-rheumatic medication including conventional synthetic disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate, leflunomide, hydroxy-/chloroquine, and/or sulfasalazine with or without glucocorticosteroids; only tumour necro-

sis factor alpha inhibitors (TNFi) were permitted as further treatment option (Table I). An active tumour disease was excluded in the included study patients. Specifically, no serum samples were taken from patients who had previously been treated with rituximab, tocilizumab, abatacept or tofacitinib. TNFi failures were defined as any treatment failure due to a lack of TNFi efficacy. TNFi failures were defined as patients with an activity score of DAS28 >5.1 or DAS28>3.2 together with a steroid requirement as long-term therapy in the dose >10mg prednisolone equivalent, as well as patients with progressive radiographically measurable erosions.

All serum samples of the included participants were tested for RF-IgM and for ACPA-IgG using a commercial ELISA kit (Euroimmune, Germany) according to standard procedures, values higher than 14 IU/ml (for RF-IgM) and higher than 5 U/ml (for ACPA-IgG) were considered as seropositive. For the determination of PGRN-abs we used an ELISA as previously described (9); for investigating the serum levels of P-PGRN, the analysis was performed according to the protocol as outlined in (9). PGRN-abs in serum samples were tested twice to confirm the values: two samples were excluded from the study because of non-corresponding values (one with a positive test followed by a negative test and one *vice versa*). Clinical data documenting the duration of disease, disease activity, the presence of erosive joint disease, and the present treatment strategy were available for the majority of the RA patients. To investigate the development of PGRN-abs depending on disease duration the RA population was stratified into two subgroups determining disease duration of RA shorter *versus* longer than two years.

The statistics were performed (by IBM SPSS Statistics, software v. 20) using a χ^2 test for evaluating differences in clinical data between the seronegative and seropositive RA cohort; furthermore, a χ^2 test was also applied to detect the prevalence of PGRN-abs in the RA population including their clinical subgroups and treatment strategies. To compare the age of RA patients and DAS28-CRP values, evaluated at the day of venous blood collection, in different cohorts, we applied an analysis of variance. To evaluate independent statistical effects on the frequency of PGRN-abs positivity, multivariate analysis applying a logistic regression model with a Wald-test was performed. A *p*-value of <0.05 was considered as statistically significant.

Table I. RA population (n=481) on treatment with biological and non-biological DMARDs stratified into subgroups according to the absence/presence of PGRN-abs

DMARDs	PGRN - n=369 (%)	PGRN + n=112 (%)
Methotrexate (without TNFi)	221 (59.9)	70 (62.5%)
Leflunomide	51 (13.2)	13 (11.6)
Hydroxychloroquine	25 (6.8)	9 (8.1)
Sulfasalazine	14 (3.8)	8 (7.1)
Methothrexate combinations	38* (10.3)	21* (26.6)
Prednisolone <10mg	77 (20.9)	23 (20.5)
Prednisolone >10mg	0	0
TNFi mono	36 (16.3)	16 (14.3)
TNFi + methotrexate	60 (9.8)	17 (15.2)
Prednisolone <10mg	14 (3.8)	8 (7.1)
Prednisolone >10mg	2 (0.5)	0

DMARDs: disease-modifying anti-rheumatic drugs; PGRN-/+: progranulin antibodies negative/positive; TNFi: tumour necrosis factor alpha inhibitor.
*p<0.05 (χ^2 test: PGRN- *vs*. PGRN+).

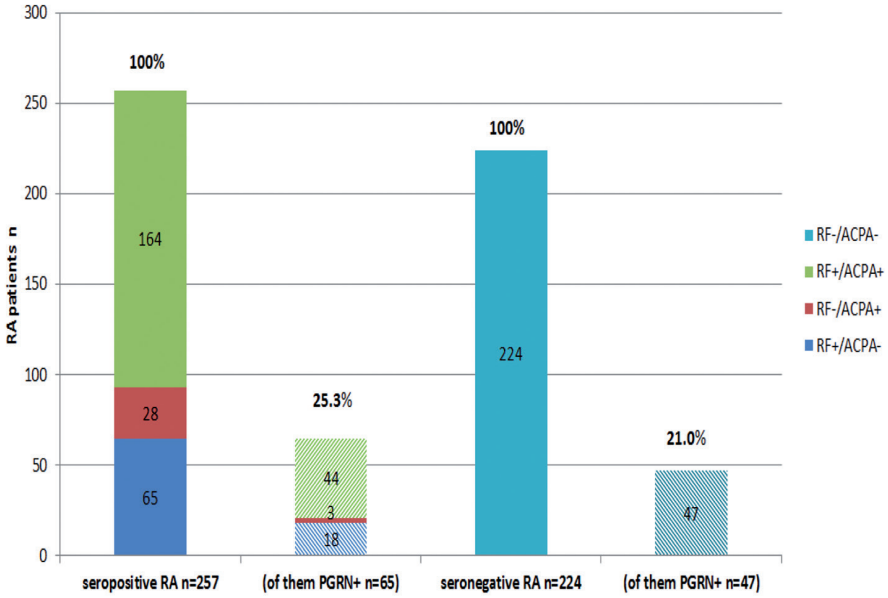

Fig. 1. Prevalence of PGRN-abs in sera of seropositive (RF+ and/or ACPA+) RA patients and seronegative (RF-/ACPA-) RA patients.
RF: rheumatoid factor IgM; ACPA: anti-citrullinated peptide antibodies; DAS28: disease activity score of 28 joints; PGRN-abs: progranulin antibodies.

Results

PGRN-abs were detected in 25.3% of seropositive RA and in 21.0% in RF- and ACPA-negative RA (Fig. 1), resulting in a prevalence of 23.3% for both cohorts together. Both cohorts did not differ in gender distribution (female 73.5% in seropositive cohort *vs*. 78.6% in seronegative) or age (median 63 *vs*. 59 years). In both cohorts the subgroup of patients with RA with less than 2 years of duration was comparable (27.4% *vs*. 29.0%). However, the seropositive and seronegative RA cohort showed significant differences in the frequency of erosive joint disease (57.6 *vs*. 29.0%, p=0.019) and TNFi treatment (38.5 *vs*. 13.4, p=0.023), but not in PGRN-abs status (25.3% *vs*. 21.0%, p=0.265).

Regarding both seropositive and seronegative RA cohorts together (Table II; n=481) PRGN-abs positive

Table II. RA population (n=481) stratified into subgroups according to the absence/presence of PGRN-abs.

RA subgroups	unit	PGRN-	PGRN+	odds ratio / CI*	*p*-value
Seronegative RA *vs*.	n (%)	177 (79.0)	47 (21.0)		
seropositive RA	n (%)	192 (74.7)	65 (25.3)	1.205 / 0.867-1.675	0.265#
No erosive disease *vs*.	n (%)	206 (82.1)	45 (17.9)		
erosive disease	n (%)	154 (73.0)	57 (27.0)	1.694 / 1.088-2.639	0.019#
RA disease duration <2 years *vs*.	n (%)	90 (81.8)	20 (18.2)		
RA disease duration >2 years	n (%)	200 (76.3)	62 (23.7)	1.395 / 0.795-2.447	0.244
DAS28 on treatment <3.2 *vs*.	n (%)	146 (82.5)	31 (17.5)		
DAS28>3.2	n (%)	150 (75.4)	49 (24.6)	1.407 / 0.941-2.101	0.093#
RA patients without TNFi *vs*.	n (%)	273 (77.6)	79 (22.4)		
RA patients onTNFi	n (%)	96 (74.4)	33 (25.6)	1.188 / 0.744-1.897	0.471
TNFi treatment effective *vs*.	n (%)	72 (77.4)	21 (22.6)		
TNF failure**	n (%)	24 (66.7)	12 (25.6)	1.714 /0.735-3.997	0.209
DAS28 value	mean (± SE)	3.50 (0.1)	3.81 (0.2)		0.038
Age (years)	median (range)	62 (19-93)	64 (37-87)		0.089

*OR/CI PGRNabs+ *vs*. PGRNbas- **n=129 RA patients on TNFi.
the values RF, ACPA, DAS28, erosive diseases were additionally analysed applying logistic regression model (wald-test): RF: 1.322 (Exp), 0.608–2.875 (CI), 0.481 (*p*-value. ACPA: 1.001, 0.999–1.002, 0.420.DAS28: 1.248, 0.967–1.612, 0.89. erosive disease: 1.260, 0.650–2.445, 0.494.
PGRN-abs: progranulin antibodies; DAS28: disease activity score of 28 joints; TNFi: tumour necrosis factor alpha inhibitors.

patients showed significantly more frequently joint erosions (27.0%, OR 1.694, p=0.019) and a higher disease activity evaluated by mean value of DAS28-CRP (mean 3.81 *vs*. 3.50, *t*-test, p=0.038). No significant differences could be observed for the occurrence of PGRN-abs subgroups of RA patients, which were on TNFi or developed TNFi failure (Table II). The median age of patients in the PGRN-abs positive cohort (64, range 37–87 years) did not differ from the PGRN-abs negative cohort (62, 19–90, p=0.119).

For a multivariate analysis a logistic regression model was applied to screen for independent statistic effects on the frequencies of PGRN-abs status; in the statistical analysis 205 RA patients were included and stratified into following parameters: erosive disease (+ *vs*. -), RF positivity (±), ACPA positivity (±, representing seropositive disease respectively), and DAS28>3.2 (*vs*. DAS28<3.2). In conclusion, RA patients with a DAS28>3.2 tend to be more frequently PGRN-abs positive than patients <DAS28, but without significance level (OR 1.248 (Wald-test), CI 0.967-1.612, p=0.089; Table II).

The sera of 194 RA patients were additionally tested for the presence of phosphorylated progranulin (pSer81 PGRN = P-PGRN), 158 PGRN-abs negative sera were all also negative for P-PGRN, 36 PGRN-abs positive sera tested positive for P-PGRN in 33 cases (91.7%, only three sera showed P-PGRN without the presence of PGRN-abs).

Discussion

In the present study we demonstrate in a RA cohort of 481 patients that the prevalence of serum PGRN-abs is 23.3%. Interestingly, the RF- and ACPA-seronegative RA cohort was found to be positive for PGRN-abs in 21% of cases. Based on these data, we could observe that the determination of serum PGRN-abs in so-called seronegative RA patients (defined as RF negative and ACPA negative) would reduce significantly the portion of seronegative RA patients.

In general, PGRN-abs have been clearly shown to be negative in healthy serum donors as well in disease entities other than various autoimmune diseases (9). In light of this specificity of serum PGRN-abs for autoimmune diseases the determination of serum PGRN-abs should be discussed for use as a novel diagnostic marker in the standard serological diagnostic for RA, next to RF and ACPA. However, the strength of the PGRN-abs testing does not lie in specificity for RA but for unambiguous correlation of autoimmune aetiology of arthritis. The diagnostic benefit of testing PGRN-abs might be considered, in particular, for RF negative and ACPA negative RA; in this context, so defined seronegative arthritis patients represent a challenge for the correct diagnosis, particularly, if patients present in very early stages of the disease.

Recent studies have investigated seronegative arthritis patients which have to be defined to the entity of so-called "very early" and/or "undifferentiated arthritis" as previously published (14, 15). In these studies the patients were not only classified into "early arthritis" representing "not-established RA" according to the ACR/EULAR diagnostic criteria, they were also exposed to anti-rheumatic treatment (preferentially methotrexate) with the goal to reach an optimized treatment effect, based on the assumption of early immunomodulation effects and the subsequent goal of "healing RA". In this context, the determination of PGRN-abs may be a helpful diagnostic tool indicating autoimmune aetiology of arthritis.

Although this approach is promising, based on our data, significant PGRN-abs positivity has only been evaluated for a part of established RA. This co-

hort consisted of the vast majority of patients with a disease duration of more than 2 years (n=262; 70.4%); patients with a course of disease less than 2 years were significantly less represented, but patients who met the definition of "early RA" were present in an unrepresentative number. This limitation is further supported by the consideration that measurable PGRN-abs only develop after formation of immunogenic P-PGRN, which is why PGRN-abs and even P-PGRN may still test negative in very early stage of autoimmune arthritis. Our study included "only" patients with established RA. Due to the link between P-PGRN and PGRN-abs all PRGN-abs negative samples tested also negative for P-PGRN; and of 36 PGRN-abs positive samples 33 cases were also P-PGRN positive (n=33, 91.7% of PGRN-abs positive).

With respect to the clinical introduction of PGRN-abs as a diagnostic tool additional limitations have to be mentioned: first, based on the presented data for RA, there is a relatively low rate of positive cases for PRGN-abs (25.3% for seropositive and 21% for seronegative RA), indicating low sensitivity. For comparison, RF and ACPA are commonly detectable in more than 70% and 50%, respectively, of patients with established RA depending on multiple RA studies (16). Second, in addition to the observed relatively low frequencies of PGRN-abs positive sera, the positive PGRN-abs finding is not specific for RA, but rather for various autoimmune arthritis, which also includes Crohn`s associated arthritis or psoriatic arthritis (10, 11).

Nevertheless, to the best of our knowledge the positive PGRN-abs findings in RA have to be viewed as a clear indication for autoimmune aetiology of arthritis. Moreover, for its diagnostic categorisation the determination of PGRN-abs should be regarded as an additional helpful tool, particularly in the situation of seronegativity for RF and ACPA.

The statistical evaluation of the RA subgroups showed a higher DAS28 values in PGRN-abs positive RA patients than in PGRN-abs negative patients; in addition, there is a clear trend that RA patients with erosive joint disease were tested more frequently PGRN-abs positive than patients without joint erosions. However, in which way the antiprogranulin effect of PGRN abs reflects the erosive course of RA must be evaluated in further studies. For this purpose, follow-up of RA patients would be better suited than the present study design. Especially against the background of significant differences in univariate analysis of PGRN prevalence in individual RA subgroups but not after multivariate analysis studies with larger case numbers are required to answer the question in what way the determination of serum PGRN-abs could be applied to RA as prognostic biomarker for unfavourable characteristics.

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