Journal of Autoimmunity 61 (2015) 17–28

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

The molecular basis for development of proinflammatory autoantibodies to progranulin

Lorenz Thurner [a, *], Natalie Fadle [a], Evi Regitz [a], Maria Kemele [a], Philipp Klemm [a], Marina Zaks [a], Elisabeth Stöger [a], Birgit Bette [a], Gabi Carbon [a], Vincent Zimmer [b], Gunter Assmann [a], Niels Murawski [a], Boris Kubuschok [a], Gerhard Held [a], Klaus-Dieter Preuss [a], Michael Pfreundschuh [a]

[a] Saarland University Medical School, José Carreras Center for Immuno- and Gene Therapy, Internal Medicine I, Homburg, Saar, Germany
[b] Department of Internal Medicine II, Saarland University Medical Center, Homburg, Saar, Germany

ARTICLE INFO

Article history:
Received 7 November 2014
Received in revised form
27 April 2015
Accepted 3 May 2015
Available online 23 May 2015

Keywords:
Progranulin
TNFR1&2
DR3
Hyperphosphorylation of Ser81
Alternative conversion
Neoantigen
Proinflammatory autoantibody
PP1
PKCβ1

ABSTRACT

Recently we identified in a wide spectrum of autoimmune diseases frequently occurring proinflammatory autoantibodies directed against progranulin, a direct inhibitor of TNFR1 & 2 and of DR3. In the present study we investigated the mechanisms for the breakdown of self-tolerance against progranulin. Isoelectric focusing identified a second, differentially electrically charged progranulin isoform exclusively present in progranulin-antibody-positive patients. Alkaline phosphatase treatment revealed this additional progranulin isoform to be hyperphosphorylated. Subsequently Ser81, which is located within the epitope region of progranulin-antibodies, was identified as hyperphosphorylated serine residue by site directed mutagenesis of candidate phosphorylation sites. Hyperphosphorylated progranulin was detected exclusively in progranulin-antibody-positive patients during the courses of their diseases. The occurrence of hyperphosphorylated progranulin preceded seroconversions of progranulin-antibodies, indicating adaptive immune response. Utilizing panels of kinase and phosphatase inhibitors, PKCβ1 was identified as the relevant kinase and PP1 as the relevant phosphatase for phosphorylation and dephosphorylation of Ser81. In contrast to normal progranulin, hyperphosphorylated progranulin interacted exclusively with inactivated (pThr320) PP1, suggesting inactivated PP1 to cause the detectable occurrence of phosphorylated Ser81 PGRN. Investigation of possible functional alterations of PGRN due to Ser81 phosphorylation revealed, that hyperphosphorylation prevents the interaction and thus direct inhibition of TNFR1, TNFR2 and DR3, representing an additional direct proinflammatory effect. Finally phosphorylation of Ser81 PGRN alters the conversion pattern of PGRN. In conclusion, inactivated PP1 induces hyperphosphorylation of progranulin in a wide spectrum of autoimmune diseases. This hyperphosphorylation prevents direct inhibition of TNFR1, TNFR2 and DR3 by PGRN, alters the conversion of PGRN, and is strongly associated with the occurrence of neutralizing, proinflammatory PGRN-antibodies, indicating immunogenicity of this alternative secondary modification.

1. Introduction

Recently we reported frequently occurring progranulin-antibodies (PGRN-Ab) in a wide spectrum of autoimmune diseases including different primary vasculitides, autoimmune connective tissue disorders, inflammatory arthritis as rheumatoid arthritis and psoriatic arthritis, inflammatory bowel diseases as Crohn's disease and ulcerative colitis, but not or very infrequently in controls [1–3]. PGRN is a secreted glycoprotein of 88 kDa expressed in neurons, neuroglia, chondrocytes, epithelial cells and leukocytes [4]. The gene (GRN) encoding PGRN is conserved and located on chromosome 17q21.32. Several synonyms for PGRN such as granulin epithelin precursor (GEP), PC-cell derived growth factor (PCDGF), acrogranin, proepithelin (PEPI) or CLN11 exist. PGRN is a precursor protein with an N-terminal signal peptide and seven granulin motifs, each containing 12 cysteins responsible for 6

* Corresponding author. Dept. of Internal Medicine I, University Hospital Homburg/Saar, Kirrberger Strasse, D-66424 Homburg, Saar, Germany. Tel.: +49 68411623097; fax: +49 68411623092.
E-mail addresses: lorenz.thurner@uks.eu (L. Thurner), michael.pfreundschuh@uks.eu (M. Pfreundschuh).

http://dx.doi.org/10.1016/j.jaut.2015.05.002
0896-8411/

disulfide bridges in every motif [5]. In addition of a central role in wound healing [6], PGRN is a key neuronal survival factor [7] and allelic haploinsufficiency due to loss-of-function mutations [8] or gene promoter methylation [9] is associated with TDP43 positive frontotemporal lobe dementia. Moreover, PGRN has been suggested to play a pro-oncogenic role in several tumor entities [10–14]. With regard to autoimmune diseases, the most interesting characteristic of PGRN is its strong anti-inflammatory effect [15,16], which is mediated by high affinity binding and direct inhibition of TNF-α receptors (TNFR) 1&2 [17] and death receptor 3 (DR3) [18], antagonizing TNF-α and TL1A [17,18]. In collagen-induced arthritis and collagen antibody-induced arthritis very strong anti-inflammatory effects of recombinant PGRN and Atsttrin, a synthetic derivate of PGRN, were noted, comparable to the effects of TNFR/Fc fusion protein Etanercept [17,19]. Likewise strong antiinflammatory properties of PGRN have been reported in LPS-induced acute respiratory distress syndrome [20], oxazolone-induced dermatitis [21] and in a cerebral ischemia–reperfusion (I/R) injury by middle cerebral artery occlusion [22]. Hence PGRN and Atsttrin have been proposed as possible next generation TNF-blockers [23,24]. Paying regard to this anti-inflammatory effect of secreted PGRN, PGRN-Abs might not only be an innocent bystander in a wide spectrum of autoimmune diseases, but might play an active role in the inflammatory process by neutralizing the physiologic TNF-α- and TL1A-blocker PGRN. This was supported by the fact, that the presence of PGRN-Abs was significantly associated with active disease states in patients with granulomatosis with polyangiitis [1]. Moreover, the proinflammatory activity of PGRN-Abs was confirmed in TNF-α induced cytotoxicity assays [2,3] and by suppressive effects on Tregs [3]. Because of the widespread occurrence of PGRN-antibodies in autoimmune diseases and their proinflammatory effects, we intended to further investigate the mechanisms underlying the breakdown of self-tolerance against PGRN in the present study.

2. Methods

This study was approved by the local ethical review board ("Ethikkommission der Ärztekammer des Saarlandes" N242/11) and conducted according to the Declaration of Helsinki.

2.1. PGRN antibody ELISA

Plasma samples were screened for PGRN-Abs as described previously [1].

2.2. TNF-α induced cytotoxicity assay

A non-radioactive cytotoxicity assay (EZ4U, Biomedica) was performed according to the manufacturer's instructions as reported before [2,3].

2.3. Lymphoblastoid cell lines

Lymphoblastoid cell lines (LCLs) were established by infection of PBMCs with EBV as described before [25].

2.4. Isoelectric focusing and treatment with alkaline phosphatase

Isoelectric focusing was performed as described before [26]. Treatment of whole blood cell lysates or LCL lysates with alkaline phosphatase was conducted as previously described [26] using FastAP thermo-sensitive alkaline phosphatase (Fermentas/VWR, Darmstadt, Germany).

2.5. Cloning of PGRN fragments and expression in HEK293 cells and LCLs

Recombinant full-length PGRN and fragments thereof were constructed based on the coding sequence of full-length PGRN, and expressed with C-terminal FLAG-tag and N-terminal HIS-tag in HEK293 cells under the control of a CMV promoter (pSFI) or in LCLs in pRTS.

2.6. Site-directed mutagenesis

Using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, US) and a PGRN DNA fragment coding for FLAG-tagged 1 AA to 112 AA, two mutants were constructed of the two amino acids with the highest predicted probability as phospho-sites (http://www.cbs.dtu.dk/services/NetPhos/), each with an exchange of a serine to an alanine, i.e., Ser38Ala and Ser81Ala. Both FLAG-tagged fragments were cloned into pRTS and transfected and expressed in LCLs.

2.7. Selection of phosphorylation-site specific Fabs

The selection of Fabs with specific reactivity against either the hyperphosphorylated progranulin (pSer81 PGRN) or the non-hyperphosphorylated progranulin (PGRN) isoform, was performed as described before [27,28]. As phagemid library, a non-immune, semi-synthetic human Fab repertoire containing $3.7*10^{10}$ different possible antibody fragments was used [29]. For differential selection N-terminally biotinylated PGRN AA70-91 peptides were used either phosphorylated or non-phosphorylated at Ser81 (peptides are listed in the supplementary). To obtain Fabs discriminating between pPGRN and non-phosphorylated PGRN five rounds of selection were necessary. The specificities of these Fabs for the non-phosphorylated or the phosphorylated (Ser81) PGRN isoform were verified by IEF (SI 2).

2.8. ELISA for pSer81 PGRN plasma levels and of pSer81 PGRN in whole blood cell lysate

Nunc maxisorb plates were coated overnight at 4°C with rabbit anti human PGRN directed against the C-Terminus at a dilution of 1:2500 (v/v; LsBio, Seattle, WA, USA). Blocking was performed with 1.5% (w/v) gelatin in TBS and washing steps were conducted with TBS-Tx [TBS, 0.1% (v/v) Tx100]. Individual sera were utilized at a dilution of 1:2, individual whole blood cell lysates were utilized at a dilution of 1:100. ELISA was performed according to standard protocols. For the detection of the hyperphosphorylated PGRN, the previously selected phospho-Ser81 specific Fab was utilized at a concentration of 10 μg/l. Following this, correspondent biotinylated anti-human Fab secondary antibodies and subsequently peroxidase-labeled streptavidin (Roche) were used.

2.9. Staining and flow cytometry of hyperphosphorylated PGRN

Blood smears were fixed for 10 min in 4% PFA, followed by 0.5% Tx100 for 10 min at room temperature. For inhibition of endogenous peroxidase, blood smears were pretreated with 3% hydrogen peroxide diluted in methanol for 10 min at room temperature. Blocking with 5% BSA was performed for 30 min at room temperature. Myc-tagged Fabs specific either for phosphorylated 81 PGRN or non-phosphorylated PGRN were used at a concentration of 10 μg/ml followed by incubation with mouse anti-myc antibody (1 μg/ml), biotinylated anti-mouse IgG antibody (1:500) and streptavidin-Pox (1:10.000). For phospho-unspecific staining of PGRN a commercially available goat anti-human PGRN antibody