INTRODUCTION

Proteinase-3 (PR3) is the main antigen for cytoplasmic antineutrophil cytoplasmic antibodies (c-ANCA) detectable in serum of patients with granulomatosis with polyangiitis (GPA). PR3 is a serine protease stored mainly in neutrophil intracellular granules but some amount is also bound to the neutrophil’s external surface. Surface expression of PR3 can be increased by pre-stimulation of neutrophils with low concentration of certain pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α) (1). High membrane expression of PR3 is also a found in GPA patients (2). c-ANCA mediated neutrophil activation leads to production of reactive oxygen species, cytokines and leukotrienes (3-6). Activated neutrophils also degranulate releasing their storage proteins like neutrophil elastase, cathepsins and PR3, which can initiate apoptosis of endothelial cells (7, 8). Recent studies have shown that activation of neutrophil by anti-PR3 IgG antibodies requires involvement of the intact antibody molecule. Anti-PR3 antigen-binding region docks to the membrane PR3, whereas constant fragment of the immunoglobulin interacts with Fcγ receptors (9-11). Several studies described mechanism of neutrophil activation following binding of Fc fragment of c-ANCA to its receptor (9, 11), however, the knowledge on a signalling cascade activated by interaction of anti-PR3 F(ab)2 to the membrane PR3 is still limited. In a study by Yang et al. some differences in leukocytes gene expression of leukocytes stimulated with native anti-PR3 IgG were suggested (12). In our previous study, we analysed gene expression profile of neutrophils stimulated with anti-PR3 IgG F(ab)2. We observed a consistent up-regulation of 17 genes (CYSLTR1, HPGD, IL1R1, IL1R1I, MAPK1, MAPK8, NR3C1, PLA2G7, PTGDR, CD302, Dnajb1, F2R, F2RL1, IER3, RAC1, RPL41, PTGER3), whereas other 9 genes were up-regulated only in some donors. No reactive oxygen species production was observed in neutrophils stimulated with anti-PR3 F(ab)2. Stimulation of neutrophils with F(ab)2 of anti-PR3 autoantibodies activated cells to a lesser extent than intact IgG. However, several cellular pathways were up-regulated, involving calcium and phosphatidylinositol 3-kinase AKT, nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK) signaling. Interestingly, binding of F(ab)2 to the PR-3 present on the surface of neutrophil is sufficient for lipid mediators and G-protein pathways activation. Specific F(ab)2 antibodies against PR-3 seems not a good candidate for decoy therapy of granulomatosis with polyangiitis.

Key words: neutrophils, cANCA, anti-PR3 IgG F(ab)2, gene expression, real-time PCR

MATERIALS AND METHODS

IgG purification and F(ab)2 fragment preparation

The study received ethical approval from the Bioethical Committee of Jagiellonian University.
For enrolment of healthy blood donors, informed consent was obtained, and this non-interventional phase 1 *in vitro* study was performed in respect of Declaration of Helsinki considering confidentiality and lack of interest conflicts. Total IgG fraction containing native anti-PR3 IgG antibodies were extracted from pooled sera of patients with granulomatosis with polyangiitis (anti-PR3 IgG > 200 mU/L; anti-MPO < 20 mU/L). Control IgG were purified from the pooled serum of seven healthy volunteers (anti-PR3 IgG < 20 mU/L; anti-MPO < 20 mU/L). Total IgG fraction was purified by a sequential ammonium sulfate precipitation followed by removal of other serum proteins using a negative affinity adsorption chromatography (Melon Gel IgG Purification kits, Thermo Scientific, Rockford, USA). Purity of IgG samples was assessed by SDS-PAGE electrophoresis and ability of antigen binding was tested with the use of immunofluorescence method (Fig. 1). Concentration of total IgG following extraction and purification was determined by immunonephelometry (Siemens Dade Behring BN II Nephelometer, Munnich, Germany) and specific anti-PR3 IgG level was assessed by indirect immunofluorescence and ELISA with the use of commercial available kits according to manufacturer’s protocols (anti-PR3 ELISA kit and single slides for indirect immunofluorescence with ethanol-fixed granulocytes, EUROIMMUN Medizinische Labordiagnostika, Luebeck, Germany). Total IgG F(ab)2 containing either anti-PR3 IgG F(ab)2 or control IgG F(ab)2, were prepared by enzymatic digestion of purified IgG with pepsin using a commercially available reagents kit and according to the manufacturer procedure (Pierce F(ab)2, Preparation Kit, ThermoScientific, Rockford, USA). Remaining undigested IgG and Fc fragments were removed using NAb Protein A and Microcon - 50 K MWCO (EMD Millipore, Billerica, USA) columns. To remove possible endotoxin contamination, samples were cleaned up using AffinityPak Endotoxin Removal Column (Pierce, Rockford, USA). Integrity and purity of F(ab)2 preparations was assessed by SDS-PAGE electrophoresis (Fig. 1). Total protein concentration, measured by spectrophotometry (NanoDrop 2000, Wilmington, USA) was used to quantify F(ab)2 as there was no selective method for a measurement available. Anti-PR3 IgG F(ab)2 and control F(ab)2 samples were aliquoted and stored for further experiments at −20°C.

**Neutrophil-enriched granulocyte isolation**

Granulocytes were isolated from citrated blood of healthy donors (n = 12, average age 30 years, 2 males and 10 females) using dextran sedimentation and Histopaque-1077 centrifugation (Sigma-Aldrich Chemical Co, St Louis, USA) followed by hypotonic lysis of remaining erythrocytes. Purity (> 98%) of the neutrophil fraction was determined by flow cytometry and cells viability (> 95%) was verified by trypan blue exclusion staining. Immediately after isolation, granulocytes were resuspended in Hanks balanced salts solution (HBSS) with calcium and magnesium containing 5% fetal bovine serum.

**Fig. 1.** Panel A - gel electrophoresis of purified total IgG fraction containing anti-PR3 IgG (1) and control IgG (2) antibodies. Panel B - gel electrophoresis of anti-PR3 IgG F(ab)2 (1) and control IgG F(ab)2 (2) antibodies. Panel C - detection of PR3 antibodies on ethanol-fixed neutrophils (c-ANCA pattern) - serum sample before anti-PR3 IgG isolation. Panel D - verification of isolated anti-PR3 IgG antibodies on ethanol-fixed neutrophils. Presence of anti-PR3 IgG was confirmed by indirect immunofluorescence. Briefly 10 µl of diluted serum (1:10 in PBS) used for anti-PR3 antibody isolation or fraction of isolated total IgG was overlaid on the reaction plate containing ethanol-fixed granulocytes and incubated for 30 min. After incubation the reaction plate was washed in Tween-PBS solution. Next, 10 µl of FITC conjugated goat anti-human IgG was added and incubated. After 30 minutes plate was washed again and analysed using fluorescence microscopy.
Reactive oxygen species production by stimulated neutrophil-

enriched granulocytes

Generation of reactive oxygen species was analysed with the use of rhodamine 123 and flow cytometry (14). At the first step isolated granulocytes (5 × 10⁵/tube in HBSS with Ca²⁺ and Mg²⁺) were pre-stained with dihydrorhodamine 123 (5 µg/ml) and incubated for 5 min in 37°C with shaking (150 rpm). Next granulocytes were primed with 2 ng/mL recombinant TNF-α (R&D Systems, Minneapolis, MN, USA) for 15 minute at 37°C and then stimulated with phorbol myristate acetate (positive control, 50 ng/mL), anti-PR3 IgG control IgG, anti-PR3 F(ab)²; or control IgG F(ab)² at concentration of 200 µg/mL total protein (30 minute at 37°C). After stimulation neutrophils were washed with cold phosphate-buffered saline (PBS) and centrifuged (1300 rpm / 5 min / 4°C). Obtained granulocytes pellet was resuspended in PBS and analysed immediately for the rhodamine fluorescence.

Genes expression in stimulated neutrophil-enriched

granulocytes

Before stimulation experiments, neutrophils were primed with 2 ng/mL recombinant TNF-α (R&D Systems, Minneapolis, MN, USA) for 15 minute at 37°C. Primed neutrophils (3.5 × 10⁶/well) were incubated with purified anti-PR3 IgG F(ab)², or control IgG F(ab)², at concentration of 200 µg/mL total protein for 4 hours at 37°C. To evaluate gene expression in neutrophils after stimulation, six independent experiments were performed. In each experiment, neutrophils simulations were made in duplicates for two donors in parallel.

RNA isolation, reverse transcription and genes expression

Total cellular RNA was isolated using Total RNA kit (A&A Biotechnology, Gdynia, Poland) as recommended by the manufacturer. Reverse transcription was done using high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Relative expression of specific mRNA for 151 genes studied was quantified using a set of two low density expression arrays by 5' nuclease assay (TaqMan low-density array inflammation panel and custom made panel - Applied Biosystems) on 7900HT fast real-time PCR System (Applied Biosystems) (full list of analysed genes was published previously (13)). Quantification cycle data were normalized to ribosomal 18S rRNA used as the endogenous control. Relative quantities were calculated using 2⁻ΔΔCt formula. Results were
presented using TNF-α primed but not F(ab)2 stimulated granulocytes as the comparator.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 package (GraphPad Software Inc, San Diego, CA). Due to a small size of studied groups and distribution of variables departing from the normal one, all comparisons were done using Wilcoxon Signed Rank Test Mann-Whitney-U test or Kruskal Wallis ANOVA with Dunn's post hoc test. Descriptive statistics was presented as a median ± interquartile range of the fold change of mRNA abundance. Bioinformatics pathway analyses were performed with the use of DAVID Bioinformatics Resources 6.7 (15, 16).

RESULTS

Reactive oxygen species production

We observed that ROS were generated only by cells stimulated with PMA (positive control, MFI = 732.3 ± 85) or intact native anti-PR3 IgG (MFI = 500.8 ± 32.7). In the case of neutrophils stimulated by control IgG, anti-PR3 IgG F(ab)2 or control IgG F(ab)2, ROS levels were similar to those observed in unstimulated cells (Fig. 2).

Genes expression

Out of 151 measured transcripts, 129 were expressed at detectable levels of mRNA, while only 22 were not detected or showed very low abundance in some donors. By paired comparison of the expression between anti-PR3 IgG F(ab)2 and unstimulated cells, we observed biologically significant up-regulation (> 2 fold change in mRNA abundance, P < 0.05) of 17 transcripts (CYSLTR1, HPGD, IL1R1, IL1RL1, MAPK1, MAPK8, NR3C1, PLA2G7, PTGDR, CD302, DNAJB1, F2R, F2RL1, IER3, RAC1, RPL41, PTGER3), further 9 genes were up-regulated only in some donors (CANCN4, CD40, CD40LG, IL2RA-B, LTBR4, HMGB1, LPGAT1, CCL3, CCL4). Yet two other transcripts (IL-13, CACNA1D) were up-regulated in a few samples and down-regulated in others. Statistically significant differences were observed for all 17 up-regulated genes in

![Fig. 2. Production of reactive oxygen species in stimulated neutrophils. Neutrophils isolated from healthy donors were stimulated with PMA (positive control), anti-PR3 IgG control IgG or anti-PR3 F(ab)2 and control F(ab)2 IgG as described in Material and Methods section. Each bar represents the median ± interquartile range of MFI from twelve independent experiments performed in duplicates.](image)

![Fig. 4. Processes activated in neutrophils stimulated with native anti-PR3 IgG and anti-PR3 IgG F(ab)2. Genes activated by native anti-PR3 IgG are marked with red (13) and genes activated by anti-PR3 IgG F(ab)2 are marked with blue. Genes upregulated by complete anti-PR3 IgG and F(ab)2 are marked with pink. Bioinformatics pathway analyses were performed with the use of DAVID Bioinformatics Resources 6.7 (15, 16).](image)
upregulated by anti-PR3 IgG F(ab)2. Analyses showed that stimulation of neutrophils with anti-PR3 stimulation (Fig. 3), whereas there was no difference in genes expression profile between cells stimulated with control IgG F(ab), and unstimulated cells. An expression profile of the genes upregulated by anti-PR3 IgG F(ab)2 fragments was compared with the profile obtained with a complete anti-PR3 IgG stimulation (Fig. 4) and reported previously (13). Bioinformatics analyses showed that stimulation of neutrophils with anti-PR3 IgG F(ab)2 caused activation of several cellular processes, like immune response, calcium signalling, cell migration, apoptosis, membrane receptor signal transduction (Table 1). It was also surprising, that anti-PR3 IgG F(ab)2 stimulation was sufficient to up-regulate transcripts encoding the enzymes involved in eicosanoid metabolism, like phospholipase A2, microsomal synthase of prostaglandin E2, and receptors for prostaglandin E2, leukotriene B4, and cysteinyl leukotrienes.

**DISCUSSION**

The classical paradigm of ANCA-associated vasculitis syndromes assumes that neutrophil activation by ANCA requires a direct binding of PR3 via Fab region of the antibody and a simultaneous interaction of the Fc part of the antibody with FcγRs on the neutrophil surface. Following this, neutrophil activation occurs with degranulation, generation of reactive oxygen intermediates and ultimately in trans-migration through the endothelial cell layer. Neutrophils express on their surface mostly two types of FcγRs: RIIa (CD32) and RIIb (CD16b). Both these receptors can participate in c-ANCA mediated neutrophils activation (9, 10). PR3 is a protein without a cell surface receptor linked signal transduction (Table 1). It was also surprising, that anti-PR3 IgG F(ab)2 stimulation was sufficient to up-regulate transcripts encoding the enzymes involved in eicosanoid metabolism, like phospholipase A2, microsomal synthase of prostaglandin E2, and receptors for prostaglandin E2, leukotriene B4, and cysteinyl leukotrienes.

**Table 1. Bioinformatics analysis of processes activated in neutrophils stimulated with anti-PR3 IgG F(ab)2.**

<table>
<thead>
<tr>
<th>Process (pathway)</th>
<th>Gene involved</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defence response</td>
<td>PTGER3, CD302, F2R, IL1R1, PLA2G7, RAC1, IL1RL1, CYSLTR1</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>PTGER3, CD302, F2R, PLA2G7, RAC1, CYSLTR1</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>MAPK1, MAPK8, NR3C1, F2R, RAC1, IER3</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Cell motility/migration</td>
<td>MAPK1, F2RL1, F2R, RAC1</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Calcium signalling</td>
<td>PTGER3, F2RL1, F2R, CYSLTR1</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>cell surface receptor linked signal transduction</td>
<td>MAPK1, PTGER3, HPGD, F2RL1, PTGDR, F2R, IL1R1, CYSLTR1</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>Eicosanoid metabolism</td>
<td>PTGER3, HPGD, PTGDR</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>NFκB signaling</td>
<td>ILR1, IL1RL1, MAPK1, MAPK8, RAC1</td>
<td>P &lt; 0.005</td>
</tr>
</tbody>
</table>

Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anion radical or hydroxyl radical, play an important role in inflammation processes observed in several diseases (20-22). ROS can also modify synthesis of prostanooids from arachidonic acid (23).

In the present study we have showed that a full activation of neutrophil and ROS production is possible only when the cell is stimulated with native form of anti-PR3 IgG. However we observed that anti-PR3 IgG F(ab), stimulated neutrophils overexpressed mRNA of the key enzymes for the major pathways of arachidonic acid-derived prostaglandins and leukotrienes (PTGDR, PTGER3, HPGD, CYSLTR1, PLA2G7). Early works of Simpkins and Ney showed that prostaglandin D2 can modulate calcium influx and ROS production in neutrophil (24, 25). More recent studies showed that neutrophils are scarcely responsive to stimulation by prostaglandin D2; however, animal studies demonstrated that prostaglandin D2 receptor may play a role in neutrophil migration (27). Ottolino et al. described that prostaglandin E2 and prostaglandin E2 receptors could be responsible for delayed neutrophil apoptosis (28). Our genes expression panel did not include ones usually involved in apoptosis, however, bioinformatics analysis suggested that up-regulated after F(ab)2 neutrophils stimulation transcripts (MAPK1, MAPK8, NR3C1, F2R, RAC1, IER3) were participating in apoptotic pathways of neutrophil. There were no evidences available that PR3 could directly modulate neutrophil apoptosis, however, in contrast with myeloperoxidase or elastase, PR3 is expressed on the neutrophil surface at the early stage of the programmed death (29). Located in the cell membrane raft within a complex with phospholipid scramblase-1, PR3 can act as a "don't eat me signal" delaying apoptotic neutrophil clearance by macrophages (30-31). Some clinical studies, indeed suggested delayed neutrophil apoptosis in GPA (32, 33).

We also observed that binding of anti-PR3 IgG F(ab)2 to the membrane PR3 resulted in a concerted transcription of protease-activated receptors genes (F2R encoding PAR1 and F2RL1 encoding PAR2). PAR1 and PAR2 are G protein coupled receptors causing calcium signalling and activation of kinases PI3K/Akt (34-36). Stimulation of protease-activated receptors leads to secretion of interleukin-6 (IL-6), interleukin-8 (IL-8) and prostaglandin E2 by endothelial cells of the lung (37). PR3-PAR interactions are also involved in the regulation of endothelial leukocyte transmigration (38). Moreover, recent studies showed that neutrophil elastase and PR3 can modulate neutrophils activity via PAR2 (39). Thus, our results indicate that PR3
released by activated neutrophils can participate in modulation of immune cells activity in an autocrine or paracrine manner.

Increased mRNA expression for ILR1, IL1RL1, MAPK1, MAPK8 and RAC1 in neutrophils stimulated with anti-PR3 IgG F(ab)2, is highly specific for activation of NF-kB pathway responding to IL-1 stimulation of the cell (34). Up-regulation of CD302 gene suggest also that this lectin receptor is involved in activation of neutrophils stimulated by anti-PR3 IgG F(ab)2 (40).

In our previous work we described genes expression profile of neutrophils stimulated with native anti-PR3 IgG (13). When we compared previous results with a profile of genes activated in cells stimulated with F(ab)2 only, we confirmed in both experimental setups activation of the same pathways (calcium signalling, cytokines production, eicosanoids metabolism or NF-κB activation). It led to activation of several cellular pathways like immune cells activity in an autocrine or paracrine manner.

Effects were highly variable across healthy donors. There was, however, a consistent pattern of genetic expression, which partially overlapped with the whole anti-PR3 IgG effects. Thus, the one observed with c-ANCA anti-PR3 IgG, the activation arms of neutrophils stimulated by anti-PR3 IgG F(ab)2 fragments could mask PR3 antigen on the neutrophil surface of GPA patients, they might be considered as an alternate therapy modality in this difficult to treat disease. However, our results suggest, that binding of anti-PR3 F(ab)2 to neutrophils also incites the cell activation. Although lessened than the one observed with c-ANCA anti-PR3 IgG, the activation arms the cell for inflammatory response.

Conclusion

In conclusion, the current results demonstrated that c-ANCA F(ab)2 stimulation of neutrophil is not sufficient for the full cell activation. It led to activation of several cellular pathways like phosphatidylinositol 3-kinase AKT, MAPK, NF-kB signalling, clearly involved in inflammation. Also other molecular responses enrolling G-protein signalling, lipid and prostaglandin metabolism were upregulated. We observed that some anti-PR3 effects were highly variable across healthy donors. There was, however, a consistent pattern of genetic expression, which partially overlapped with the whole anti-PR3 IgG effects. Thus, PR3 membrane docking and transduction mechanism is highly integrated with an immune response signal transduction and only partially depends on FcγRs co-stimulation.

Acknowledgements: The work was supported by National Centre of Science in Poland, grant number: DEC-2011/03/N/NZ6/01578

Conflict of interests: None declared.

REFERENCES


Received: December 19, 2014
Accepted: July 17, 2015

Author's address: Marek Sanak, M.D, Ph.D., Department of Internal Medicine, Jagiellonian University Medical College, 8 Skawinska Street, 31-066 Cracow, Poland.

E-mail: nfsanak@cyf-kr.edu.pl