Membrane proteinase 3 expression and ANCA-induced neutrophil activation

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Membrane proteinase 3 expression and ANCA-induced neutrophil activation.

Background. Proteinase 3 is the major autoantigen in Wegener’s granulomatosis (WG). Membrane PR3 expression is bimodal; low expressing cells (mPR3low) can be distinguished from cells with high expression (mPR3high) within a given individual. High mPR3 expression is a WG risk factor and is associated with relapse. However, no mechanisms for this important clinical observation have been provided. We tested the hypothesis that mPR3 expression, rather than the expression of other membrane molecules implicated in anti-neutrophil cytoplasmic autoantibodies (ANCA) activation, determines the robustness of the PR3-ANCA-mediated response.

Methods. mPR3low and mPR3high neutrophils from a given individual were separated by magnetic cell sorting. Superoxide was measured by the ferricytochrome assay, and Akt phosphorylation by Western blotting. Double staining and flow cytometry were used to assay Fcγ-receptor and β2-integrin expression with respect to the mPR3 phenotype. Degranulation was measured via β-glucuronidase activity, migration with fibronectin-coated transwells, and cell quantification by the myeloperoxidase (MPO) assay.

Results. PR3-ANCA-treated mPR3high versus mPR3low neutrophils showed more superoxide generation (33.7 ± 15.2 nmol O2− to 14.6 ± 8.4, P < 0.01), more degranulation (29% ± 5 to 22% ± 3, P < 0.05), and more PI3-K/Akt activation. In contrast, all responses in both mPR3 subsets were similar after other stimuli. We observed no differences in the β2-integrin, FcγR IIa, and III expression with respect to the mPR3 subtype. Furthermore, we found no differences in the mobilization of PR3-containing granules and no differences in migration through fibronectin.

Conclusion. The degree of neutrophil mPR3 expression has definitive functional consequences.

Key words: neutrophils, ANCA, superoxide, signal transduction, PR3.

Wegener’s granulomatosis (WG) is characterized by anti-neutrophil cytoplasmic autoantibodies (ANCA) [1] with specificity for proteinase 3 (PR3) [2]. A pathogenic role for ANCA is suggested by several in vitro studies. The emerging picture is that ANCA interact with their target antigens on cytokine-primed neutrophils and on monocytes activating intracellular signal transduction pathways, such as PI3-K/Akt [3–6]. ANCA-activated neutrophils and monocytes generate reactive oxygen species (ROS), release toxic granule components, and up-regulate surface adhesion molecules, ultimately causing vascular damage [7–12]. Recently, the pathogenic role of ANCA was elegantly demonstrated in an animal model [13]. The major target antigen in WG is PR3. This serine protease is localized in azurophilic (primary) and specific (secondary) granules, and in secretory vesicles [14–16]. In addition to an intracellular pool, PR3 can be detected on the surface of isolated resting neutrophils [16, 17]. Membrane PR3 (mPR3) expression is bimodal. Within a given donor an mPR3high and mPR3low subset neutrophil population can be distinguished [18, 19]. We found recently that the mPR3 phenotype is under strong genetic influences [20]. A large percentage of mPR3high neutrophils provides a risk factor for vasculitis and is associated with relapse [19–21]. Possibly, neutrophils with high mPR3 expression can more easily interact with PR3-ANCA, resulting in stronger activation. We investigated whether or not the mPR3 phenotype affects ANCA-induced respiratory burst, intracellular signaling, and release of granule proteins. Because additional surface molecules, including β2-integrins and Fcγ receptors IIa and IIb, were reported to be important for neutrophil activation by ANCA, we also studied the expression of these molecules in the mPR3high and mPR3low neutrophil subsets. Finally, mPR3 is enzymatically active and is resistant to the naturally occurring inhibitors alpha1-antitrypsin and elafin [22]. Because PR3 degrades extracellular matrix components such as fibronectin [22–24], we examined the possibility that high mPR3 expression favors chemotactic migration.

METHODS

Materials

Recombinant tumor necrosis factor (TNF)-α was obtained from Genzyme (Rüsselsheim, FRG). The
monoclonal mouse antibody to PR3 was obtained from CLB (Amsterdam, The Netherlands), and fluorescein isothiocyanate (FITC)-conjugated F(ab)2-fragment of goat antimouse immunoglobulin (IgG) was from Dako (Hamburg, FRG). Dextran was purchased from Amer- sham Pharmacia (Amsterdam, The Netherlands). Hank’s balanced salt solution (HBSS), phosphate-buffered saline (PBS), and Trypan blue were from Seromed (Berlin, FRG). Endotoxin-free reagents and plastic disposables were used in all experiments. Histopaque 1083 was obtained from Sigma-Aldrich (Deisenhofen, FRG). The following antibodies were used: from Immunotech (Krefeld, FRG), CD11a (FITC), CD11b (FITC), CD11c (PE), and CD18 (FITC). CD16 and CD35 (FITC) were from Cymbus Biotech (Hants, UK), and CD32 (FITC) from Dako. CD63 (PE) was from CLB.

Isolation of human neutrophils

Neutrophils from healthy volunteers were isolated from heparinized whole blood by red blood cell sedimentation with dextran 1%, followed by Ficoll-Hypaque density gradient centrifugation, and hypotonic erythrocyte lysis. Neutrophils were centrifuged (10 minutes at 1050 rpm) and resuspended in HBSS with calcium and magnesium (HBSS ++ ). The cell viability was detected in every cell preparation by Trypan blue exclusion and exceeded 99%. The neutrophil percentage in the suspension was >95% by Wright-Giemsa staining.

Preparation of immunoglobulins

Human immunoglobulin G (IgG) was prepared from patients with biopsy-proven WG (two PR3-ANCA) and microscopic polyangiitis (two MPO-ANCA) as well as from two healthy control subjects as described [25].

Separation of mPR3 high and mPR3 low neutrophils by magnetic beads

mPR3 high and mPR3 low neutrophils were separated with magnetic cell sorting (MACS) separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany) as described in the manufacturer’s manual. All steps were carried out on ice. Freshly isolated neutrophils were stained with a monoclonal antibody (mab) to PR3 (CLB 12.8.IgG1x%). MACS rat antismouse IgG1 were added, and cells were pipetted onto a MACS LD Column (Miltenyi Biotec). The flow-through containing the nonlabeled mPR3 low neutrophils was collected. Columns were removed from the magnet and labeled mPR3 high neutrophils were collected. The cell 99% viability was detected in every cell preparation by Trypan blue exclusion.

Assessment of neutrophil adhesion

Well plates (96-well) coated with (10 μg/cm²) fibronectin were used for the adhesion assay. Polymor-phonuclear cells (PMN) (1 × 10⁵) in 100 μL HBSS ++ were either left untreated or were treated with 2 ng/mL TNF-α. Plates were incubated at 37°C in 5% CO₂ for the indicated time period. Wells were flicked dry, washed three times with PBS, and adherent cells were estimated using the MPO assay. Briefly, adherent cells were lysed in 100 μL of 0.5% Triton-X-100 for 10 minutes. Substrate (100 μL; 2.2’-Azino-bis (3-Ethylbenzthiazoline-6-sulfonicacid; Sigma, Deisenhofen, FRG) was added and optical density (OD) was read after 10 minutes at 450 nm with a microtiter plate reader. OD of the experimental sample was compared with a standard curve that showed an excellent correlation between OD and cell number (R² = 0.96).

Superoxide generation assay

Superoxide was measured using the assay of SOD-inhibitable reduction of ferricytochrome C as described by Pick and Mizel [26]. Neutrophils were pretreated with 5 μg/mL cytochalasin B for 15 minutes. Cells (0.75 × 10⁶) were primed with 2 ng/mL TNF-α for 15 minutes before human ANCA preparations were added. No priming was performed for phorbol 12-myristate 13-acetate (PMA) or N-formyl-methionyl-leucyl-phenyalanine (fMLP) stimulation. The final concentrations were 125 μg/mL for purified IgG preparations, 25 ng/mL for PMA, and 10⁻⁶ mol/L for fMLP. Experiments were done in duplicate. Samples were incubated in 96-well plates at 37°C for up to 60 minutes, and the absorption of samples with and without 300 U/mL SOD was scanned repetitively at 550 nm using a Microplate Autoreader (Molecular Devices, Munich, Germany). The final ferricytochrome C concentration was 50 μmol/L, and the final cell concentration was 3.75 × 10⁵/mL.

Degranulation assay

Freshly isolated neutrophils (5 × 10⁵) were preincubated with 5 μg/mL cytochalasin B and primed in a 96-well microtiter plate for 15 minutes with 2ng/mL TNF-α. Stimulation was performed with PR3-ANCA or MPO-ANCA or human control IgG or fMLP 10⁻⁶ mol/L for up to 120 minutes. At 30-minute intervals the cell-free supernatants were collected by centrifugation (7 min, 250g), and β-glucuronidase activity was assessed by the cleavage of phenolphthalein glucuronic acid (Sigma-Aldrich). Each tube contained 50 μL of a 0.01 mol/L solution phenolphthalein-glucuronic acid in 0.1 mol/L acetic buffer, pH 4.6, with 0.04% Triton X-100, and was mixed with 50 μL of the cell-free supernatant. After a 14-hour incubation in the dark at 37°C, the reaction was stopped by adding 1 mL of a 0.02 mol/L solution of glycine buffer, pH 10.4. OD values were measured at 405 nm. Nonstimulated neutrophils served as baseline, whereas the total neutrophil β-glucuronidase content was obtained by incubation 5 × 10⁵ neutrophils with 1% Triton X-100.
Determination of surface antigen expression by flow cytometry

Fluorescence-activated cell sorting (FACS) was used as described previously to evaluate the PR3 expression on neutrophils [25]. For double labeling, cells were incubated after stimulation with conjugated antibodies against CD11a, CD11b, CD11c, CD16, CD32, CD35, and CD63. Flow cytometry was performed using a FACSJUNctor (Becton Dickinson, Heidelberg, FRG), and 10,000 events per sample were collected.

Transmigration assay

The ability of neutrophils to migrate through extracellular matrix components was tested with 20 µg/mL fibronectin-coated transwells (3.0 µm, 6.5 mm from Corning, NY, USA). Neutrophils (1.5 × 10⁶) in HBSS ++ were stimulated at 37°C with 50 ng/mL interleukin-8 to transmigrate to the lower well. For time-course studies, the upper chamber of the transwell device was gently moved to a new well in 30-minute intervals. Transmigrated cells were quantified by MPO assay as described previously [27], and are given as the percentage of neutrophils that migrated from the apical to the basal aspect (Fig. 1A-C). After sorting, neutrophils were primed with TNF-α and subsequently activated with PR3-ANCA, MPO-ANCA, control immunoglobulins, respectively, or unprimed cells were stimulated with PMA. Stimulation of mPR3high neutrophils (Fig. 2) resulted in significantly higher amounts of superoxide generation, compared to mPR3low cells (33.7 ± 15.2 nmol O₂⁻/10⁶ neutrophils vs. 14.6 ± 8.4, P < 0.001, N = 5). In contrast, no difference in the response to human control immunoglobulins, MPO-ANCA, or PMA was observed. We performed experiments to ensure that the sorting algorithm did not influence neutrophil responsiveness, and that the separated mPR3low and mPR3high subgroups were not characterized by different activation status. We preincubated neutrophils with buffer control, mab to PR3, and mab to PR3 together with beads, respectively. After this preincubation samples were either challenged with TNF-α/human PR3-ANCA or with buffer control for 45 minutes. Superoxide generation was similar in the three preincubation groups after stimulation with TNF-α/human PR3-ANCA. The results were 16.5 ± 0.7 nmol/0.75 × 10⁶ cells/45 minutes in samples preincubated with mab to PR3 together with beads, and 14.5 ± 2.2 nmol in samples with the mab to PR3 alone. In contrast, buffer control did not result in superoxide generation in any of the three preincubation groups. The results were 2.9 ± 1.4 nmol/0.75 × 10⁶ cells/45 minutes in the buffer preincubation group versus 1.5 ± 2.2 nmol in samples preincubated with mab to PR3 alone, and 2.5 ± 2.1 nmol in samples with the mab to PR3 together with beads. We determined that the sorting procedure did not affect basal or TNF-α-induced phosphorylation of extracellular signal-regulated kinase (ERK) (Fig. 1D). ERK is a sensitive indicator of neutrophil activation. We have shown previously that ERK controls neutrophil priming [25]. Together, these experiments exclude the possibility that the sorting technique used in this study affected neutrophil responsiveness. These data also provide evidence that mPR3high neutrophils do not represent a more activated subgroup of cells. When we assessed the percentage of mPR3high cells in isolated neutrophils aged over 24 hours in culture, we observed no change in this percentage in two independent experiments (0 h: 64% ± 16.9 mPR3high neutrophils; 2 h: 65% ± 16.3; 4 h: 65% ± 16.3; 8 h: 65% ± 16.9; 12 h: 66% ± 16.3; 24 h: 67% ± 18.4). These experiments exclude both age and activation status as important factors affecting the percentage of mPR3 expression. Next, we titrated a dose response curve using neutrophils from a given individual. We chose this approach because interindividual comparisons are of limited help in neutrophil investigations because of the large degree of variation between different

Western blot analysis for phosphorylated Akt and ERK

Neutrophils were incubated after separation of mPR3high and mPR3low at a concentration of 2 × 10⁸/mL in the presence of buffer control or with the indicated stimuli. Western blotting was performed as previously described using phosphospecific antibodies [5]. Densitometry was performed with scanned x-ray films and the NIH image program.

Statistical analysis

Results are given as mean ± SEM. Comparisons were made by t test or analysis of variance (ANOVA) as appropriate.

RESULTS

ANCA-induced superoxide generation in mPR3high and mPR3low neutrophils

We tested the hypothesis that low and high mPR3 neutrophils differ in the magnitude of PR3-ANCA-induced activation. To exclude interaction between both subsets, we physically separated mPR3high and mPR3low neutrophils by magnetic cell sorting. We yielded two pop-
Fig. 1. Membrane proteinase 3 (mPR3) staining of neutrophils separated by magnetic cell sorting was assessed by flow cytometry (A–C) and extracellular signal-regulated kinase (ERK) phosphorylation by immunoblotting (D). The isotype control staining is depicted in each panel as a dotted line. Before magnetic cell sorting, 56% of the neutrophil population expressed PR3 on their plasma membrane (A). Sorting yielded two populations containing either 94% mPR3low cells (B) or 95% mPR3high cells (C). After the sorting procedure, mPR3low and mPR3high cells were treated with either buffer (CTRL) or 2 ng/mL TNF-α (TNF) for 10 minutes, and ERK activation was estimated by Western blot experiments. The data indicate that ERK was not phosphorylated by cell sorting, per se, and that TNF-α priming resulted in a similar phosphorylation increase. Reprobing for actin demonstrates equal protein loading. A typical of two independent experiments is shown.

Donors. Neutrophils were isolated, and the mPR3high and mPR3low subset were separated and remixed to achieve defined samples containing less than 10%, approximately 25%, 50%, 75%, and >90% mPR3high cells. Samples were primed with TNF-α, stimulated with PR3-ANCA, and superoxide generation was assayed. We observed a superoxide response that increased dose dependently with increasing the percentage of mPR3high-neutrophils (Fig. 3). A progressive increase in burst activity occurred from <10% up to 75% mPR3high-cells and a plateau thereafter.

**Phosphorylation of Akt in mPR3high and mPR3low neutrophils**

We studied whether or not the differences in superoxide generation after PR3-ANCA stimulation of mPR3high and mPR3low neutrophils were related to differences in PI3-K/Akt activation. After separation of both mPR3 subsets, samples were primed with TNF-α for 15 minutes and stimulated with PR3-ANCA, MPO-ANCA, or human control immunoglobulins for another 15 minutes. We had previously observed that TNF-α–induced Akt phosphorylation peaked after 10 minutes, declining thereafter, and that ANCA resulted in a second phosphorylation peak [5]. The simulation with control immunoglobulins induced very little Akt phosphorylation (Fig. 4), whereas PR3-ANCA and MPO-ANCA resulted in a significant activation of Akt. However, Akt phosphorylation was significantly higher in PR3-ANCA–stimulated mPR3high neutrophils compared with the mPR3low subset. In contrast, no difference was observed after stimulation with MPO-ANCA. Corresponding optical density (OD) measures are given in Figure 4B.

**Degranulation in mPR3high and mPR3low neutrophils**

ANCA were shown to stimulate neutrophil degranulation. Thus, we explored whether or not PR3-ANCA–induced release of granule proteins was higher in the mPR3high subset compared to the PR3low cells.
separation of both populations, TNF-α–primed samples were challenged with human PR3-ANCA, MPO-ANCA, and human control immunoglobulins, respectively. Because PMA did not trigger degranulation (data not shown), we used fMLP as an additional stimulus in this assay (Fig. 5). We found significant higher degranulation after stimulation with PR3-ANCA (29% ± 5 of total cellular β-glucuronidase vs. 22% ± 3, \( P < 0.05 \), \( N = 4 \)). In contrast, no difference was observed after stimulation with human control immunoglobulins, MPO-ANCA, or fMLP.

**β2-integrins, FcγRIIa, and FcγRIIIb expression on mPR3\(^{\text{high}}\) and mPR3\(^{\text{low}}\) neutrophils**

We investigated the expression of β2-integrins, FcγRIIa, and FcγRIIIb because these receptors were implicated in ANCA-induced neutrophil activation. We used double staining and flow cytometry to assess these molecules in both mPR3\(^{\text{high}}\) and mPR3\(^{\text{low}}\) subsets. The experiments were performed in unstimulated cells, in TNF-α–primed cells, in TNF-α–primed cells challenged with a mab to MPO or to PR3, or with fMLP, respectively. First, the β2-integrins were analyzed. We observed no difference in CD11a (LFA-1), CD11b (Mac-1; CR3), CD11c (p150,95; CR4), or CD18 expressions in resting mPR3\(^{\text{high}}\) and mPR3\(^{\text{low}}\) neutrophils (Fig. 6). We confirmed the increase in the amount of membrane-expressed CD11b, CD11c, and CD18 after incubating with the aforementioned activators, whereas CD11a was not up-regulated upon stimulation. However, we found that the up-regulation of these integrin molecules was similar in PR3\(^{\text{high}}\) and PR3\(^{\text{low}}\) cells (Table 1).

In addition to studying the expression of adhesion molecules, we assessed the ability of mPR3\(^{\text{low}}\) and mPR3\(^{\text{high}}\) neutrophils to adhere to extracellular matrix proteins. mPR3\(^{\text{low}}\) and mPR3\(^{\text{high}}\) neutrophils were separated by magnetic cell sorting and incubated on a fibronectin-coated surface in the absence and presence of 2 ng/mL TNF-α. Cells were harvested after 60 minutes, and adhesion was determined using the MPO-assay. We observed similar adhesion of mPR3\(^{\text{low}}\) and mPR3\(^{\text{high}}\) neutrophils after incubation in buffer control (3.4 ± 0.9 × 10\(^4\) and 4.0 ± 1.3 × 10\(^4\) neutrophils, respectively). Furthermore, both populations showed a similar increase in adherence after stimulation with TNF-α (7.0 ± 0.5 × 10\(^4\) and 7.9 ± 1.3 × 10\(^4\), respectively, \( N = 2 \)).
We then investigated the expression pattern of the Fcγ-receptor IIA (CD32) and FcγIIib (CD16), which were also reported to play a role in neutrophil activation by ANCA. The expression of both receptors was similar in mPR3\textsuperscript{high} and mPR3\textsuperscript{low} resting neutrophils (Fig. 7). Again, this finding did not change when cells were stimulated (Table 1). Because of the necessary fluorescence compensation for double-staining experiments comparisons between the stimuli are difficult. Our aim is merely a comparison between both mPR3\textsuperscript{high} and mPR3\textsuperscript{low} neutrophils for a given activator. Taken together, our experiments did not detect additional differentially expressed
molecules in the low and high mPR3 subsets of neutrophils.

**Mobilization of azurophilic, specific, and secretory granules in mPR3<sup>high</sup> and mPR3<sup>low</sup> neutrophils**

CD11b is mobilized from specific granules und our data have demonstrated no difference in the mobilization of these granule types. We therefore investigated degranulation of azurophilic granules (CD63) and secretory vesicles (CD35) to study if differences in mPR3 expression resulted from a more general difference in the ability to mobilize intracellular storages that contain PR3. However, we found similar expression of CD35 in resting mPR3<sup>high</sup> and mPR3<sup>low</sup> neutrophils as well as after stimulation (Fig. 8 and Table 1). CD63 increased only after the stimulation with fMLP, but not when cells were

### Table 1. Increased expression of different surface markers in mPR3<sup>high</sup> and mPR3<sup>low</sup> neutrophils was studied by double staining and flow cytometry

<table>
<thead>
<tr>
<th>Molecules</th>
<th>mPR3&lt;sup&gt;low&lt;/sup&gt;</th>
<th>mPR3&lt;sup&gt;high&lt;/sup&gt;</th>
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<tr>
<td>CD11a</td>
<td>121 ± 13</td>
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<td>104 ± 7</td>
<td>109 ± 3</td>
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<td>106 ± 5</td>
<td>99 ± 5</td>
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<td>CD11b</td>
<td>237 ± 37</td>
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<td>228 ± 27</td>
<td>215 ± 95</td>
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<td>244 ± 13</td>
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<td>280 ± 29</td>
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<td>CD11c</td>
<td>208 ± 45</td>
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<td>194 ± 25</td>
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<td>CD18</td>
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<td>CD35</td>
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The data represent membrane expression for the indicated molecules after stimulation with TNF-α, TNF-α + monoclonal anti-PR3, TNF-α + monoclonal anti-MPO, and IMLP, respectively, compared to unstimulated neutrophils (mean percentage ± SEM).
The expression of the Fc γ receptor IIA (CD32) and Fc γ IIIb (CD16) in mPR3<sub>high</sub> and mPR3<sub>low</sub> neutrophils was compared by double staining and flow cytometry. Unstimulated isolated neutrophils were stained with both anti-PR3 and anti-Fc γ receptor mab. The data demonstrate no difference in the expression of CD16 (A) or CD32 (B), with respect to the mPR3 expression.

These experiments do not support the contention that mPR3<sub>high</sub> and mPR3<sub>low</sub> subsets result from differences in the ability to mobilize granules or vesicles. Additional flow cytometry experiments were performed to assess the membrane expression pattern of other serine proteinases and MPO as another ANCA antigen. Our results indicate that only PR3, but not elastase, cathepsin G, or MPO, was characterized by a bimodal membrane distribution (Fig. 9).
Transmigration of separated neutrophils and surface-staining for mPR3 after migration

Finally, we investigated potential consequences of mPR3 expression in a more general setting—in the absence of ANCA. We tested the hypothesis that mPR3\textsuperscript{high} neutrophils would transmigrate faster through extracellular matrix toward an interleukin (II)-8 gradient. Transwell plates were coated with fibronectin, an extracellular matrix that is easily degraded by PR3. mPR3\textsuperscript{high} and mPR3\textsuperscript{low} were separated and subjected to the upper well. Cells that had migrated into the IL-8–containing lower well were assayed. Under these conditions no difference was observed (Fig. 10). To exclude that this result was influenced by the mab to PR3 that was used for cell sorting, and could still be bound to the neutrophil surface, we repeated these experiments using a different approach. Transmigration of unseparated neutrophils toward an IL-8 chemotactic gradient was monitored. We hypothesized that mPR3\textsuperscript{high} neutrophils would migrate faster through fibronectin, and therefore cells with the higher mPR3 expression would arrive at first in the lower well. Thus, we collected transmigrated cells at 30-minute intervals up to 120 minutes, and performed surface staining for membrane PR3. There was no migration advantage for mPR3\textsuperscript{high} neutrophils at any of the indicated time points.
points (Fig. 10). These data suggest that mPR3 phenotype is not the only determinant of neutrophil transmigration, and that additional proteolytic enzymes are at work, compensating for differences in mPR3 expression.

**DISCUSSION**

We explored the functional consequences of mPR3\textsuperscript{high} and mPR3\textsuperscript{low} neutrophil phenotypes in the setting of PR3-ANCA vasculitis. Our data are the first to show that PR3-ANCA–induced generation of reactive oxygen species and release of granule proteins are significantly higher in the mPR3\textsuperscript{high} neutrophil subset than in the mPR3\textsuperscript{low} subset. In addition, we found significant stronger activation of the PI3-K/Akt pathway in PR3-ANCA–stimulated mPR3\textsuperscript{high} neutrophils. Moreover, the mPR3\textsuperscript{high} and mPR3\textsuperscript{low} subsets differed neither in the ability to mobilize primary, specific, and secretory granules, nor in the expression of other molecules with putative implications in ANCA-induced neutrophil activation.

In WG, PR3 is the major autoantigen recognized by ANCA. This serine protease is stored intracellularly in azurophilic, specific, and secretory granules of neutrophils [14–16, 28], but some PR3 can also be detected on the cell surface of resting cells [17, 19]. mPR3 expression is bimodal and an mPR3\textsuperscript{high} and mPR3\textsuperscript{low} subset can be distinguished [18, 19].

ANCA can activate TNF-α–primed neutrophils, and activated neutrophils induce endothelial cell damage. Whether or not the mPR3 phenotype is of functional significance for the activation process is yet unknown; however, the PR3 phenotype could be one of the reasons for the clinical observation that WG patients with a larger percentage of mPR3\textsuperscript{high} neutrophils have higher relapse rates during the disease [21]. We tested the hypothesis that PR3-ANCA can more easily interact with mPR3\textsuperscript{high}...
neutrophils resulting in increased cell activation. Our data, obtained using physically separated mPR3\textsuperscript{high} and mPR3\textsuperscript{low} subsets of a given donor, demonstrate that PR3-ANCA indeed triggered a significantly stronger respiratory burst activity and degranulation in mPR3\textsuperscript{high} neutrophils compared with the mPR3\textsuperscript{low} counterparts of the same individual. Neutrophil responses show high donor variability. The advantage of our approach was to exclude this donor variability that may have influenced our results, had we merely compared different low and high mPR3 expressing donors. “Reintroducing” mPR3\textsuperscript{high} cells into mPR3\textsuperscript{low} samples of the same donor increased the ability of PR3-ANCA to trigger a higher degree of superoxide generation, demonstrating the dose-dependency of this effect. Finally, differences in the activation of the mPR3\textsuperscript{high} cells and mPR3\textsuperscript{low} subset occurred only when PR3-ANCA were used for stimulation, but not when other compounds, such as MPO-ANCA, control immunoglobulins, PMA, or fMLP, were employed.

In search for mechanisms explaining our functional observations, we studied intracellular signaling events with respect to the mPR3 expression. We and others demonstrated recently that the PI3-K/Akt pathway is activated by ANCA, but not by control immunoglobulins, and that this pathway controls ANCA-induced respiratory burst [4, 5]. In agreement with these observations, we found in this study that activation of PI3-K/Akt was significantly stronger in PR3-ANCA–treated mPR3\textsuperscript{high} cells in comparison to their mPR3\textsuperscript{low} counterparts from the same individual. These data underscore the importance of the antibody antigen interaction for ANCA-induced neutrophil activation. Our findings may, at least in part, explain the differences in the clinical course in WG patients dependent on the mPR3 phenotype.

However, additional surface molecules were implicated in ANCA-induced neutrophil activation, including Fcy receptors and β2-integrins. Therefore, we studied the expression of these molecules with respect to the mPR3 phenotype. Both cross-linking of ANCA target antigens on the surface of neutrophils and engagement of Fcy receptors may play a role in the activation process [4, 8–10, 29–31]. We observed that expression of the Fcy receptors IIa and IIb was similar on mPR3\textsuperscript{high} and mPR3\textsuperscript{low} neutrophils. In addition to Fcy-receptors, β2-integrins were implicated in ANCA-mediated neutrophil activation. This class of integrins consists of a common β chain (CD18) that is nonevitably linked to three unique but related α-chains (CD11a, CD11b, CD11c). In fact, CD11b/CD18 were up-regulated in granulocytes of patients with active WG [32], and ANCA-positive sera stimulated up-regulation of the same adhesion molecule in vitro [33]. Moreover, using blocking antibodies to CD18, Reumaux et al demonstrated that activation of the respiratory burst by ANCA was dependent on engagement of β2-integrins [10]. However, when we investigated membrane expression of CD11a, CD11b, CD11c, or CD18 with respect to the mPR3\textsuperscript{high} and mPR3\textsuperscript{low} subset, we found no differences. Low and high mPR3 expressing cells did also not differ in their ability to adhere to extracellular matrix proteins. These data indicate that differentially expressed mPR3 is not accompanied by differences in the expression of other surface molecules that are implicated in ANCA-mediated neutrophil activation. This finding holds true for constitutive expression, as well as for up-regulated expression during activation, and provides further support for the importance of ANCA-binding to the target antigens in the activation process because mPR3 expression was the only identifiable variable on both neutrophil subsets. We investigated a total of 12 membrane molecules, and we excluded bimodal expression also of other serine proteinases, such as elastase and cathepsin G and the second main ANCA target antigen, MPO. However, we cannot exclude additional yet unknown differences that may have influenced our findings and may play a role in neutrophil activation by ANCA.

We demonstrated recently that the intracellular content of PR3 was similar in mPR3\textsuperscript{high} and mPR3\textsuperscript{low} neutrophils [20]. Thus, conceivably low and high mPR3 expression may result from differences in the ability to mobilize intracellular granules. PR3 is found in azurophilic, specific, and secretory granules [14–16]. We used markers for all three of these granules and did not find differences in the translocation of azurophilic (CD63), specific (CD11a), or secretory (CD35) vesicles when comparing both mPR3 subsets. Further studies are needed to explore this issue. Potential explanations include modifications in the PR3 molecule itself resulting in more or less effective membrane insertion or differences in PR3 receptor status. In fact, a PR3 receptor was suggested in endothelial cells [34], but evidence for its existence in neutrophils is still lacking.

Finally, we investigated potential consequences of the mPR3 phenotype in a more general biological setting in the absence of ANCA. We explored neutrophil migration through extracellular matrix toward a chemotactic gradient and speculated that mPR3\textsuperscript{high} neutrophils had a migration advantage in that they would more easily degrade matrix substance such as fibronectin. Cepinskas et al demonstrated that membrane-bound elastase plays a role in transmigration [35, 36]. MembranePR3 is a bioactive enzyme and is, in contrast to soluble PR3, resistant to physiologic proteinase inhibitors [22]. Using two different approaches, we did not find accelerated transmigration of mPR3\textsuperscript{high} neutrophils through a fibronectin matrix.
CONCLUSION

Our data demonstrate functional consequences of the mPR3 expression phenotype in human neutrophils in the presence of PR3-ANCA. This study may shed some light in the clinical observation that a larger percentage of mPR3high subset is associated with both the occurrence and a higher relapse rate in WG patients.

References