Pathogenetic and clinical aspects of ANCA-associated vasculitis
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CHAPTER 6

C5a primes neutrophils for ANCA-mediated activation: role of P38 mitogen-activated protein kinase

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(Submitted)
Abstract

[Background] The pathogenesis of antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV) has not been fully elucidated. Recent studies have suggested that complement activation via the alternative pathway is one of the important contributing factors in the development of AAV. Complement component 5a (C5a) can dose-dependently prime neutrophils for ANCA-induced respiratory burst; C5a and the neutrophil C5a receptor may compose an amplification loop for ANCA-mediated neutrophil activation. The current study further investigates the signal transduction pathway of C5a-dependent ANCA-mediated neutrophil activation.

[Methods] Neutrophils were isolated from healthy donors and incubated with C5a and membrane expression of ANCA target antigens was investigated. The effects of C5a priming on ANCA-induced production of reactive oxygen species were assessed using oxidation of dihydrorhodamine (DHR) to rhodamine. The effect of the p38-MAPK inhibitor, AR447, was tested on the respiratory burst of C5a-primed neutrophils activated with ANCA, as well as on C5a-induced increase in membrane expression of PR3 (mPR3) on neutrophils.

[Results] C5a dose-dependently increased membrane expression of PR3 on neutrophils. Priming of neutrophils with C5a enhanced both proteinase 3 (PR3)-ANCA and myeloperoxidase (MPO)-ANCA-induced oxygen radical production. Preincubation with AR447 decreased the respiratory burst in C5a-primed neutrophils stimulated by patient derived PR3-ANCA and MPO-ANCA. Inhibition rates were 85.6±14.9% and 76.5±17.9%, respectively. AR447 inhibited C5a-induced increase in mPR3 on neutrophils. The inhibition rate was 71.6±25.7%.

[Conclusions] C5a-C5aR interaction plays an important role in ANCA-mediated neutrophil activation. This effect is partially mediated by activation of the p38-MAPK signal transduction pathway.
Introduction

Antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (AAV) comprise a group of autoimmune disorders, including Wegener’s granulomatosis (WG), microscopic polyangiitis (MPA), Churg-Strauss syndrome (CSS) and renal-limited vasculitis (RLV). These diseases are characterized by necrotizing small-vessel vasculitis. ANCA are the serological hallmarks for the above mentioned primary small vessel vasculitides. ANCA are predominantly IgG class autoantibodies directed against neutrophil cytoplasmic constituents, in particular proteinase 3 (PR3) and myeloperoxidase (MPO).

The pathogenesis of AAV has not been fully elucidated. Many in vitro and in vivo experimental data suggest that ANCA-induced neutrophil activation and degranulation play an important role in the pathogenesis of AAV. In vitro, ANCA activate primed neutrophils to undergo a respiratory burst and release various proteases, which may play a direct pathogenic role in vasculitic lesion development [1-5]. In an anti-MPO antibody-induced mouse vasculitis model, ANCA and neutrophils are necessary for the initiation of glomerulonephritis [6, 7].

Recent studies in this mouse model of anti-MPO IgG-mediated glomerulonephritis suggest that complement activation via the alternative pathway is one of the important contributing factors in disease development [8, 9]. Schreiber et al. further found that recombinant C5a dose-dependently primes neutrophils for the ANCA-induced respiratory burst. As such, C5a and the neutrophil C5a receptor (C5aR) may compose an amplification loop for ANCA-mediated neutrophil recruitment and activation [10]. It has been reported that the p38-mitogen-activated protein kinases (p38-MAPK) pathway controls the translocation of ANCA antigens to the cell surface for TNFα-mediated priming of neutrophils enabling the subsequent ANCA-induced respiratory burst [11]. The current study investigates pathways involved in C5a-dependent ANCA-mediated neutrophil activation with special attention given to the role of the p38-MAPK pathway.
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Methods

Preparation of IgG

Normal IgG and ANCA-IgG were prepared from plasma of normal volunteers and patients with active MPO-ANCA- or active PR3-ANCA-positive vasculitis using a High-Trap-protein G column on an Akta-FPLC system (both from GE Healthcare) [12].

Neutrophil isolation

Neutrophils from 10 healthy individuals were isolated from heparinized blood by density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway) as previously described [13] with minor modification. Briefly, erythrocytes were removed twice by hypotonic lysis with ice-cold ammonium chloride. Thereafter, cells were washed with ice-cold Hanks balanced salt solution (HBSS) without Ca$^{2+}$ and Mg$^{2+}$ (Life Technologies, Breda, The Netherlands), and resuspended in HBSS with Ca$^{2+}$ and Mg$^{2+}$ (2.5×10$^6$/ml).

Membrane expression of ANCA target antigens on neutrophils after priming

Membrane expression of PR3 and MPO were assessed using flow cytometry as previously described [14], with minor modifications. All steps were performed on ice. Briefly, samples containing 2.5×10$^6$ neutrophils/ml HBSS were incubated with 2ng/mL tumor necrosis factor α (TNFα) or different concentrations of C5a (1, 10, 100 and 1000ng/ml) for 15 minutes at 37°C, then washed with HBSS/1% BSA by centrifugation at 1800 g, 4°C for 5 min, and incubated with 0.5mg/ml heat-aggregated goat IgG (Sigma) for 15 min to saturate Fcγ receptors. Next, cells were stained with a saturating dose of mouse monoclonal IgG1 antibody directed against human PR3 or MPO (IQProducts, Groningen, The Netherlands) [15] or with an irrelevant IgG1 control antibody (MCG1; IQProducts, Groningen, The Netherlands) for 30 min. Next, non-bound antibodies were washed off with PBS/1% BSA. This step was followed by 30 min incubation with phycoerythrin (PE)-conjugated goat anti-mouse antibody (Southern Biotechnology Associates, Birmingham, AL USA) in the presence of 0.5-mg/ml heat-aggregated goat IgG and a subsequent washing step. Fluorescence intensity was analysed on a Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). The level of PR3- or MPO-expression was calculated as mean fluorescence intensity (MFI) of specific binding.
corrected for non-specific binding of the isotype control antibody [14]. For the inhibition test, neutrophils were incubated with AR447, a p38-MAPK inhibitor, at a concentration of 10μM or its vehicle, DMSO, as control, for 30 min at 37°C before TNFα or C5a was added. The inhibition rate was calculated according to the following formula:

\[
\text{Inhibition rate} = \frac{(\text{MFI}_{\text{DMSO}} - \text{MFI}_{\text{blank control}}) - (\text{MFI}_{\text{AR447}} - \text{MFI}_{\text{blank control}})}{(\text{MFI}_{\text{DMSO}} - \text{MFI}_{\text{blank control}})}.
\]

**Measurement of respiratory burst by oxidation of dihydrorhodamine (DHR) to rhodamine**

We assessed the generation of reactive oxygen radicals using DHR as described previously [11], with minor modifications. This method is based on the fact that reactive oxygen radicals cause oxidation of the nonfluorescent DHR to the green fluorescent rhodamine. In brief, neutrophils (2.5×10^6/ml HBSS) were incubated with cytochalasin B (5μg/ml, Sigma) for 5 min at 37°C to enhance the oxygen radical production. Then, neutrophils were loaded with 0.05 mM DHR (Eugene, OR) and 2 mM sodium azide (NaN3) at 37°C. Next, neutrophils were primed with TNFα (2ng/ml) or C5a (100ng/ml) for 15 min at 37°C. ANCA (IgG from patients or monoclonal IgG1 antibody, the final concentration was 0.2mg/ml) were added, and the reaction was stopped after another 60 min by addition of 3ml of ice-cold HBSS/1% BSA. We analyzed samples using a Calibur flow cytometer (Becton Dickinson). Data were collected from 10,000 cells per sample. The shift of green fluorescence in the FL-1 mode was determined.

For each condition, the MFI, representing the amount of generated oxygen radicals, was reported. For the inhibition test, neutrophils were incubated with AR447 (at a concentration of 10μM), or its vehicle, DMSO, as control, for 30 min at 37°C before TNFα or C5a was added. The inhibition rate was calculated according to the following formula:

\[
\text{Inhibition rate} = \frac{(\text{MFI}_{\text{DMSO}} - \text{MFI}_{\text{blank control}}) - (\text{MFI}_{\text{AR447}} - \text{MFI}_{\text{blank control}})}{(\text{MFI}_{\text{DMSO}} - \text{MFI}_{\text{blank control}})}.
\]

**Statistics**

Differences of quantitative parameters between groups were assessed using the t test (for data that were normally distributed) or non-parametric test (for data that were not normally distributed). A P value less than 0.05 was considered significant. Analysis was performed with SPSS statistical software package (version 11.0, Chicago, Ill, USA).
Results
C5a increases membrane expression of PR3 (mPR3) on neutrophils

We analysed mPR3 expression on neutrophils of healthy donors (n=10) before and after incubation with different concentrations of C5a (1, 10, 100 and 1000ng/ml). TNFα (2 ng/ml) was employed as the positive control. Before incubation with C5a or TNFα, the level of mPR3 expression (MFI) was 262.6±100.9. Upon incubation with C5a, the level of mPR3 expression on neutrophils increased dose dependently (mPR3 expression on neutrophils (MFI): 287.4±117.7, 280.9±19.9, 368.9±146.3 and 464.5±180.2 for 1, 10, 100 and 1000ng/ml C5a, respectively) (Figure 1 and 2). After incubation with TNFα, the level of mPR3 expression on neutrophils (MFI) was 516.7±183.1. Compared to non-primed neutrophils, the level of mPR3 expression was significantly higher on neutrophils primed with C5a at concentrations of 100 and 1000ng/ml as well as with TNFα (P<0.01, P<0.01 and P<0.001, respectively). However, no significant increase of membrane expression of MPO was observed on neutrophils after incubation with C5a irrespective of the dose (data not shown).

![Figure 1. mPR3 expression on neutrophils incubated with different concentrations of C5a (1, 10, 100 and 1000ng/ml). *P<0.01, compared to “before priming”](image-url)
Figure 2. A typical histogram of mPR3 expression on neutrophils primed with C5a
The dashed curve represents mPR3 expression on neutrophils incubated with isotype control antibody and without priming. The thin solid curve represents mPR3 expression on neutrophils incubated with anti-PR3 MoAbs and without priming. The bold solid curve represents mPR3 expression on neutrophils after priming with TNFα (2ng/ml). The shadowed curve represents mPR3 expression after priming with C5a (100ng/ml)

C5a primes neutrophils for ANCA-induced respiratory burst

ANCA-IgG were prepared from 5 patients with active MPO-ANCA-positive vasculitis and 5 patients with active PR3-ANCA-positive vasculitis, respectively. We next tested whether C5a primes neutrophils for ANCA-induced respiratory burst. Based on the observation described above that C5a at a concentration of 100ng/ml significantly increased mPR3 expression on neutrophils, this concentration of C5a was employed for testing the ANCA-induced respiratory burst. After incubation with C5a, PR3-ANCA-IgG from patients induced a respiratory burst as evidenced by a significant increase in MFI compared to the non-primed neutrophils incubated with PR3-ANCA-IgG (200.5±29.8 vs. 86.3±27.8, P<0.001) (Figure 3a). Similarly, after incubation with C5a, MPO-ANCA-IgG from patients induced a respiratory burst as demonstrated by a significantly higher MFI compared to neutrophils incubated with MPO-ANCA IgG alone (219.6±130.0 vs. 82.3±43.4, P<0.05) (Figure 3b).
Figure 3. C5a primes neutrophils for ANCA-induced respiratory burst
ANCA-IgG were prepared from 5 patients with MPO-ANCA-positive vasculitis with active disease and 5 patients with PR3-ANCA-positive vasculitis with active disease, respectively. The bars represent mean±SD

A. C5a primes neutrophils for PR3-ANCA-induced respiratory burst
- C5a-PR3-ANCA: neutrophils incubated with PR3-ANCA-IgG (0.2mg/ml) from patients after priming with C5a
- C5a-IgG: neutrophils incubated with IgG (0.2mg/ml) from normal donors after priming with C5a
- Blank-PR3-ANCA: neutrophils incubated with PR3-ANCA-IgG (0.2mg/ml) from patients without C5a priming
- Blank-IgG: neutrophils incubated with IgG (0.2mg/ml) from normal donors without C5a priming

B. C5a primes neutrophils for MPO-ANCA-induced respiratory burst
- C5a-MPO-ANCA: neutrophils incubated with MPO-ANCA-IgG (0.2mg/ml) from patients after priming with C5a
- C5a-IgG: neutrophils incubated with IgG (0.2mg/ml) from normal donors after priming with C5a
- Blank-MPO-ANCA: neutrophils incubated with MPO-ANCA-IgG (0.2mg/ml) from patients without C5a priming
- Blank-IgG: neutrophils incubated with IgG (0.2mg/ml) from normal donors without C5a priming
P38-MAPK inhibition blocks C5a-primed neutrophils for ANCA-induced respiratory burst

ANCA-IgG were prepared from 5 patients with active MPO-ANCA-positive vasculitis and 5 patients with active PR3-ANCA-positive vasculitis, respectively. We next tested whether C5a-priming of neutrophils for ANCA-induced respiratory burst was dependent on activation of the p38-MAPK pathway. Using the DHR test, the effect of AR447, a p38-MAPK inhibitor, on the respiratory burst of C5a-primed neutrophils activated with monoclonal antibodies (Moab) directed against MPO or PR3 and ANCA IgG from patients, was assessed in parallel. Preincubation of neutrophils with AR447 (at a concentration of 10 μM) decreased oxygen radical production in C5a-primed neutrophils induced by anti-PR3 and anti-MPO Moabs and ANCA IgG from patients. For PR3-ANCA IgG, the MFI value was 200.5±29.8 in C5a-primed neutrophils, which decreased to 81.7±14.2 upon preincubation with AR447 (P<0.01, inhibition 85.6±14.9%). A typical experiment is shown in Figure 4a. For anti-PR3 Moab, the MFI value decreased from 196.6±103.4 to 88.3±45.6 (P<0.05) upon preincubation with AR447 (inhibition rate of 84.4±20.3%). For MPO-ANCA IgG, the MFI value was 219.6±130.0 in C5a-primed neutrophils and decreased to 81.2±37.8 upon preincubation with AR447 (P<0.05, inhibition 76.5±17.9%). A typical experiment is shown in Figure 4b. For the anti-MPO Moab, the MFI value decreased from 160.6±80.0 to 96.5±58.1 (P<0.05) upon preincubation with AR447 (inhibition rate of 60.0±22.6%).
Figure 4. P38-MAPK inhibitor (AR447) inhibits ANCA-induced oxygen radical production (ROS) in C5a primed neutrophils.
A. Example of AR447 mediated inhibition of PR3-ANCA-IgG-induced respiratory burst in C5a primed neutrophils
B. Example of AR447 mediated inhibition of MPO-ANCA-IgG-induced respiratory burst in C5a primed neutrophils
The shadowed curve represents ANCA induced ROS production without the p38-MAPK inhibitor (AR447). The solid curve represents ANCA induced ROS production after preincubation with the p38-MAPK inhibitor (AR447). The dashed curve represents baseline ROS production.

P38-MAPK inhibition blocks C5a-increased mPR3 on neutrophils

Finally, we investigated a possible mechanism by which the p38-MAPK pathway may control ANCA-stimulated respiratory burst in C5a-
C5a and p38MAPK in AAV primed neutrophils. Since we previously found no significant increase of membrane expression of MPO on neutrophils after incubation with C5a, we only explored the hypothesis that p38-MAPK controls the C5a-mediated translocation of PR3 from the intracellular granules to the cell surface. Using flow cytometry, we observed that inhibiting p38-MAPK with AR447 (at a concentration of 10 µM) resulted in a decreased C5a-induced translocation of PR3. Untreated cells showed a mean MFI for mPR3 of 98.3 ± 21.8, which increased to 622.3 ± 587.1 upon C5a (100 ng/ml) priming. This C5a induced translocation of PR3 was partially inhibited by pretreating the cells with AR447 (mean MFI 444.6±416.1, P<0.05). The inhibition rate was 71.6±25.7%. A typical experiment is shown in Figure 5.

Figure 5. AR447 inhibits the increase in mPR3 expression on neutrophils induced by C5a (100ng/ml)
The shadowed curve represents mPR3 expression on neutrophils after priming with C5a and without preincubation of AR447.
The bold solid curve represents mPR3 expression on neutrophils after priming with C5a and with preincubation of AR447.
The thin solid curve represents mPR3 expression on neutrophils incubated with isotype control antibody and without priming.
The dashed curve represents mPR3 expression on neutrophils incubated with anti-PR3 Moabs antibody and without priming.
Chapter 6

Discussion

Recent observations in the anti-MPO IgG-induced crescentic glomerulonephritis mouse model suggest a critical role for complement activation in disease development. Xiao et al. found that induction of glomerulonephritis with anti-MPO-IgG or anti-MPO splenocytes required activation of the alternative complement pathway. The authors also hypothesized that activation of neutrophils by ANCA-IgG causes the release of factors that activate complement [8]. Huugen et al. further demonstrated that inhibition of C5 activation attenuated disease development in the mouse model of anti-MPO-IgG-induced glomerulonephritis [9]. Two recent studies on renal biopsies also suggest that the complement system, especially the alternative pathway, is involved in renal damage of human AAV [16, 17].

Among the complement activation products, C5a is one of the most potent inflammatory peptides, with a broad spectrum of functions. C5a is a strong chemoattractant for neutrophils and also has chemotactic activity for monocytes and macrophages (reviewed in Guo et al. [18]). C5a exerts its effects through the high-affinity C5a receptor. Recent investigations by Schreiber et al. demonstrated that ANCA-stimulated neutrophils activate complement and generate C5a. In turn, C5a was found to prime neutrophils dose-dependently for ANCA-induced respiratory burst [10] indicating a pivotal role of C5a and its receptor on neutrophils in disease induction. The current study confirms and extends these observations.

Schreiber et al. reported that C5a-conditioned serum could increase neutrophil PR3 membrane expression [10]. Our study confirmed this observation by demonstrating that purified recombinant C5a dose-dependently increases neutrophil PR3 membrane expression. Interestingly, however, both studies demonstrate that after incubation with C5a (or C5a-conditioned serum), increases in membrane-MPO expression are much lower than membrane PR3 expression. Similar results have been reported previously by Hess et al [19]. These authors suggest that in AAV, soluble MPO released by activated neutrophils may bind to unstimulated neutrophils, thereby rendering them susceptible for MPO-ANCA mediated activation.

We found that ANCA-mediated oxygen radical production is enhanced after neutrophils are incubated with C5a, whereas C5a itself did
not induce ROS production significantly. The most important finding in the current study is that p38-MAPK inhibition blocked C5a-dependent ANCA-mediated neutrophil activation. The p38-MAPK inhibitor blocked C5a-primed neutrophils for PR3-ANCA-induced respiratory burst probably via inhibition of PR3 translocation as the inhibitor decreased C5a-induced membrane expression of PR3. Since in the current study, no significant increase of membrane expression of MPO was observed on neutrophils after incubation with C5a, we did not further investigate whether the p38-MAPK inhibitor has effect on membrane expression of MPO. The mechanism of p38-MAPK inhibition on C5a-dependent MPO-ANCA-mediated neutrophil respiratory burst requires further study. It has been reported that several signal transduction pathways are involved in C5a induced priming of neutrophils and monocytes, including p38-MAPK, ERK1/2 and PKC signaling pathways [20]. Whether other signaling pathways also participate in C5a-priming of neutrophils for ANCA-induced respiratory burst requires further investigation.

In conclusion, C5a primes neutrophils for an enhanced ANCA-mediated neutrophil activation. This effect is to a large extent dependent on activation of the p38-MAPK pathway. Together with the observations in animal studies, these results indicate a prominent role for C5a-C5aR interactions in the pathogenesis of AAV.

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