Evaluation of Proteinase 3 Expression on Membrane of Papillon-Lefèvre Syndrome Neutrophils

N’Guessan Koffi¹,², *, Dasse Sery Romuald²

¹Inserm U-1100, Faculty of Medicine, University of Tours, Tours, France
²Department of Immunology-Allergology, Faculty of Medical Sciences, Felix Houphouët Boigny University, Abidjan, Ivory Coast

Email address: koffi.nguessan@live.fr (N. Koffi)
*Corresponding author

To cite this article:

Received: August 8, 2019; Accepted: September 6, 2019; Published: December 7, 2019

Abstract: Background: Proteinase 3, in its membrane form, is the main target antigen of anti-neutrophil autoantibodies in granulomatosis with polyangiitis (autoimmune disease). This neutrophil serine protease is synthesized as inactive zymogens at the early stage of neutrophil maturation and is activated by cathepsin C, the physiological activator of serine proteases. In neutrophils of Papillon-Lefèvre syndrome patients, a genetic form of cathepsin C deficiency, amounts of intracellular proteinase 3 detected are very low or even undetectable. The aim of our study was to evaluate membrane expression of proteinase 3 on cathepsin C-deficient neutrophils by using the Papillon-Lefèvre syndrome model in view of a therapeutic approach of granulomatosis with polyangiitis. Methods: We evaluated membrane expression of proteinase 3 on activated neutrophils of Papillon-Lefèvre syndrome patients by cytometry. Results: Proteinase 3 was detected at neutrophils surface of Papillon-Lefèvre syndrome patients, but significantly less than at healthy neutrophils surface. Conclusion: Pharmacological inhibition of cathepsin C may be an attractive therapeutic approach to eliminate the target autoantigen proteinase 3 of granulomatosis with polyangiitis patients.

Keywords: Cathepsin C, Granulomatosis with Polyangiitis, Neutrophils, Papillon-Lefèvre Syndrome, Proteinase 3

1. Introduction

Proteinase 3 (PR3) is a neutrophil serine protease (NSP). It is synthesized as inactive zymogens at the myeloblast/promyelocyte stage of neutrophil maturation in bone marrow [1, 2]. It matures in this very early developmental stage, induced by cathepsin C (CatC), also known as dipeptidyl peptidase I, the physiological activator of several immune cell associated serine proteases such as neutrophil serine proteases [3, 4]. The mature PR3 is stored in the intracellular granules of neutrophils. Unlike other NSPs (human neutrophil elastase (HNE), cathepsin G (CG), neutrophil serine protease 4 (NSP4)), PR3 is also expressed at the surface of resting, naïve blood neutrophils. The membrane exposure of PR3 is mediated by a hydrophobic patch at the protease surface, which is not conserved in other related NSPs [5]. This membrane-bound PR3 (PR3m) is the major target antigen of anti-neutrophil cytoplasmic autoantibodies (ANCA) in granulomatosis with polyangiitis (GPA) [6-8]. This inflammatory disease is a systemic autoimmune vasculitis of the small vessels most commonly affecting the upper and lower respiratory tract and the kidneys [9, 10]. Interaction of ANCA with PR3m on cytokine-primed neutrophils induces neutrophils activation and resulting in the release of microvesicles that export the PR3 autoantigen at their surface [11, 12], the production of extracellular neutrophil traps (NETs) and the release of granular proteins and reactive oxygen species [13, 14]. Secreted active proteases, including PR3 and related NSPs, exert proteolytic activity on endothelial cells, contributing to vascular necrosis [7, 15]. Secreted NETs also trap PR3 and related NSPs and are directly involved in ANCA induction as well as in endothelial lesions [16]. To date, there is no treatment for GPA based on disease-specific mechanisms. Current protocols are immunosuppressive and involve combined administration of steroids with cyclophosphamide or rituximab [17, 18]. A therapeutic strategy could be the reduction of the expression or even absence of membrane-bound PR3 at neutrophils surface by
pharmacological inhibition of cathepsin C. In humans, loss of function of CatC is responsible of Papillon-Lefèvre syndrome (PLS), a rare hereditary disorder with a prevalence of 1 to 4 cases per million, resulting from mutations in the CTSC gene that codes for cathepsin C (CatC) [19, 20]. Deficiency of CatC activity in PLS almost completely reduces both NSP proteolytic activity and level of their mature zymogen in neutrophils [4, 21]. However, expression of PR3 at the surface of these cathepsin C-deficient neutrophils is not known. The aim of our study was to evaluate PR3 expression at cathepsin C-deficient neutrophils in view of a therapeutic approach of granulomatosis with polyangiitis for the elimination of the target autoantigen proteinase 3 by pharmacological inhibition of CatC.

2. Material and Methods

2.1. Samples

2 to 15 mL peripheral venous blood samples were collected in EDTA K2 preservative tubes by peripheral venipuncture from 6 PLS patients and a healthy control subject. PLS patients and the healthy subject were Egyptian. Diagnosis of PLS had been firmly established by genetic testing. All material was acquired with informed consent. Erythrocyte lysis was performed with ammonium chloride solution (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA) at pH 7.4. Samples were gently inverted for 10 minutes at room temperature to allow red blood cells lysis. Then tubes were centrifuged at 400 x g for 5 minutes at (4°C). The supernatant was then decanted by careful aspiration and the white cells pelleted was recovered and washed 3 times in 2ml of phosphate buffered saline (PBS) at 400 x g for 5 minutes.

2.2. Purification of Neutrophils from Peripheral Blood

Neutrophils were isolated by manual Ficoll technique with two discontinuous gradients 1.079 and 1.098 and purification was continued by erythrocyte lysis with ammonium chloride solution (0.83% NH4Cl, 1% KHCO3, 0.04% EDTA and 0.25% BSA). Cells were then resuspended in PBS, 1 mM glucose with cations (1 mM MgCl2, 1.5 mM CaCl2). Cell viability exceeded 98%. It was confirmed by the exclusion of trypan blue in a 1:1 dilution. Cell purity was confirmed at Cytospin.

2.3. Measurement of Proteinase 3 Activity in Cell Lysates

Purified blood neutrophils were lysed in HEPES buffer (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) (50 mM, 750 mM NaCl, 0.05% NP-40, pH 7.4). We lysed the cells at 100,000 cells/µL lysis buffer. After vortexing the tubes for 1 min, soluble fractions were separated from cell debris by centrifugation at 10,000 x g for 10 min in the centrifuge at 4°C. Soluble fractions were collected and concentrated by ultrafiltration (Vivaspin (filtration threshold 10 KDa)). The proteins were assayed with a bicinchoninic acid assay (Thermo Fisher Scientific, Villebon sur Yvette, France). PR3 activity in cell lysates was measured by spectrofluorometry (Spectra Max Gemini EM) at 420 nm with ABZ-VAD(nor)VADQY-EDDnp (20 μM final, Genecust, Dudelange, Luxembourg) as a substrate in a buffer HEPES (50 mM, 750 mM NaCl, 0.05% NP40, pH 7.4 at 37°C).

2.4. Flow Cytometry

Neutrophils of PLS patients and control subject were resuspended in PBS and a blocking step was performed with 5% BSA, 2.5 mM EDTA in PBS for 15 min at 4°C; or were fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton-X 100 in PBS and non-specific binding sites were blocked with 5% BSA. Flow cytometry analyzes were performed using a MACSQuant analyzer (Miltenyi Biotec, Bergisch-Gladbach, Germany) and VenturiOne software (Applied Cytometry, Sheffield, UK). These assays were performed using the following antibodies: V450-conjugated CD14 (MoP9, 1:200), PE-conjugated CD3 (HIT3a, 1:200), PE-conjugated CD11 (HIT3a, 1:200), CD11b conjugated to PE-Cy7™ (M1/70, 1:100), APC-conjugated CD45 (3G8, 1:200), APC-H7 conjugated CD45 (2D1, 1:200) (BD Biosciences, Le pont de Claix, France), PerCP-Vio700 conjugated CD15 (VIMC6, 1:100) (Miltenyi Biotec, Bergisch-Gladbach, Germany), FITC-conjugated IgG1 (679.1Mc7, 1:20) (Dako, Hamburg, Germany), FITC-conjugated CD16 (DJ130c, 1:20) (Dako, Hamburg, Germany), FITC conjugated CD18 (7E4, 1:20) (Beckmann Coulter, Krefeld, Germany), FITC-conjugated CD66b (80H3, 1:20) (Beckmann Coulter, Krefeld, Germany). PR3 was labeled with CLB12.8 (1:50) primary mouse monoclonal antibody (Sanquin, Amsterdam, The Netherlands) and FITC-conjugated anti-mouse IgG secondary antibody (sc-2010, 1: 100) (Santa Cruz Biotechnology, Heidelberg, Germany) or the FITC conjugated secondary antibody Fab2 IgGl (DAK-GQ1, 5µg/mL) (Dako, Hamburg, Germany). The dead cells were stained with a Viobility 405/520 fixable dye (1:200) (Miltenyi Biotec, Bergisch-Gladbach, Germany). The compensation was performed using the VenturiOne software.

2.5. Statistical Analysis

Data were analyzed using the GraphPad Prism 6 software (GraphPad Software, La Jolla, USA). Comparison of fluorescence intensities was made using the Student's parametric t test at the 0.05 level of significance.

3. Results

3.1. Patients PLS Informations

<table>
<thead>
<tr>
<th>Patients</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Mutation CSTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLS 1</td>
<td>8</td>
<td>F</td>
<td>c.711G&gt;A (p.W237X) nonsense</td>
</tr>
<tr>
<td>PLS 2</td>
<td>13</td>
<td>F</td>
<td>Not identified</td>
</tr>
<tr>
<td>PLS 3</td>
<td>12</td>
<td>M</td>
<td>Not identified</td>
</tr>
<tr>
<td>PLS 4</td>
<td>8</td>
<td>M</td>
<td>a splice site mutation in intron 3</td>
</tr>
<tr>
<td>PLS 5</td>
<td>17</td>
<td>M</td>
<td>IVS3-1G→A</td>
</tr>
<tr>
<td>PLS 6</td>
<td>13</td>
<td>M</td>
<td>Not identified</td>
</tr>
</tbody>
</table>

Table 1. Characteristics of PLS patients.
3.2. Measurement of PR3 Activity in the Neutrophil Supernatant

Cells of PLS patients and control subject were activated with calcium ionophore A23187 and PR3 activity in supernatants of activated cell was measured using selective PR3 substrate: ABZ-VAD(nor)VADYQEDDnp. PR3 activity in cell supernatants of PLS subjects was very low and about 20-fold less than in control cells (Figure 1).

![Figure 1. Proteinase 3 activity in activated PLS neutrophil supernatant.](image)

3.3. PR3 at Active PLS Neutrophil Surface

Expression of CD16, CD66b and CD11b neutrophil surface markers by flow cytometry confirmed that the labeled cells were indeed neutrophils (Figure 2). 81 ± 5% neutrophils were viable in all samples. Analyzes were performed 30 min after the blood collection. There was a significant amount of PR3 at activated PLS cells surface. The pattern of PR3 on PLS cells was unimodal (Figure 3), whereas that of PR3 at the control cells surface was bimodal (Figure 3). There was a significant difference between amount of membrane-bound PR3 on PLS neutrophils and healthy neutrophils (p<0.01).

![Figure 2. Distribution of viable neutrophils in PLS subjects compared to healthy control.](image)
4. Discussion

Our study aimed to evaluate expression of proteinase 3 at membrane of cathepsin C-deficient neutrophils in Papillon-Lefèvre syndrome in view of a therapeutic strategy for elimination of membrane-bound PR3 in granulomatosis with polyangiitis by pharmacological inhibition of CatC.

For this purpose, we evaluated proteinase 3 proteolytical activity and its surface expression on cathepsin C-deficient neutrophils using PLS subjects as a study model. At the end of the study, PR3 activity tests in the PLS cell supernatant showed that proteolytically active PR3 subsisted after PLS subjects neutrophil activation. However, this activity was weak and about 20 times less than that of the control cells. CatC deficiency in PLS patients had altered the activity of PR3 in the activated neutrophil cells supernatant, most likely due to the small amount of PR3 in the intracellular granules that are mobilized after cell activation. Indeed, as reported by authors, only very small amounts of PR3 and other NSPs are detected in PLS subjects neutrophils [21-23]. Lack of CatC activity in these patients reduced amount of PR3 and other serine proteases in intracellular neutrophil granules [4, 21] and in addition this loss of CatC activity was associated with a strong reduction of proteolytic activity of NSPs [4]. Roberts et al. have identified a variety of neutrophil defects in PLS patients that occur subsequent to the failure of NSP activation by CatC. These functional defects included the inability to produce NETs, which trap PR3 to induce ANCA production, reduced chemotaxis, and over-releasing cytokines and reactive oxygen species [24].

PR3 in its membrane form represents the target antigen in GPA. It is the only NSP that is constitutively present on the surface of circulating neutrophils and remains partially bound to the surface of neutrophils even after cellular activation. The interaction of circulating PR3-ANCA with membrane-bound PR3 initiates the activation of circulating neutrophils and thereby triggers necrotic inflammation [8]. Flow cytometric analysis of PR3 expression at surface of activated neutrophils PLS subjects reported that there was a significant amount of PR3 at PLS cells surface. This observation suggests that PR3 expression at neutrophils surface would be independent of low levels of intracellular PR3 in PLS patients. However, the amount of PR3 in PLS subjects is much lower than that observed on control cells. Inactivation of CatC in PLS patients results in a dramatic decrease of PR3 in intracellular granules [4, 21], but does not interfere with PR3 expression at neutrophils surface. This suggests a different intracellular storage site and a different intracellular pathway for PR3 exported to the membrane surface. Direct insertion of the plasma membrane, distinct from the association with CD177, is the second possible way to present PR3 on the surface of neutrophils, as indicated by some authors [5, 25, 26]. Moreover unexpectedly and in contrast to control cells which had a bimodal expression profile of PR3, expression of PR3 at membrane activated cells in PLS subjects was unimodal. We have no obvious explanation for this finding at this time.
5. Conclusion

Neutrophils of PLS patients lacking CatC activity show significant amounts of PR3 at the surface of neutrophils, but less than healthy neutrophils. A total pharmacological inhibition of the activity of CatC could cause a blocking of NETs production and/or PR3 elimination on its intracellular, membrane and free form. However only clinical studies in GPA patients could prove if CatC could be a target on elimination of the target GPA antigen.

Acknowledgements

The authors thank Brice Korkmaz for planning the study, Vanderlynden Lise and Seda Seren (INSERM U-1100) for their contribution and assistance.

References


