Proteinase 3 mRNA expression is induced in monocytes but not in neutrophils of patients with cystic fibrosis

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Abstract Proteinase 3 (PR3), a serine proteinase which can degrade lung tissue, is present in the cystic fibrosis (CF) sputum. In the present study, PR3 protein and mRNA expression was determined in circulating neutrophils and monocytes. CF neutrophils contained similar PR3 concentrations as healthy controls and poorly expressed PR3 mRNA. In contrast, CF monocytes showed significantly higher PR3 concentrations than controls, together with an upregulation of PR3 mRNA expression especially during pulmonary exacerbation. Interestingly, antibiotic treatment fully abrogated PR3 mRNA expression and decreased PR3 protein in monocytes. Our findings highlight a potential role of monocyte-derived PR3 in CF-associated airway inflammation.

Key words: Proteinase 3; Inflammation; Lung disease; Serine proteinase; Myeloblastin; Cystic fibrosis

1. Introduction

The hallmark of the pathophysiology of cystic fibrosis (CF) is the neutrophil-dominated inflammation which occurs early in the course of the disease and which is potentiated by chronic colonization with bacteria and especially *Pseudomonas aeruginosa* during pulmonary exacerbation. Indeed, the prognosis in CF is largely dependent upon the airway inflammation and the incidence, severity and duration of pulmonary infections in the CF lung disease. For most of the patients, this disease is lethal between the age of 20–30 due to respiratory insufficiency [1].

At the site of infection, when activated neutrophils accumulate and die, they release large amounts of various serine proteinases, myeloperoxidase (MPO) and metalloproteinases which have a potential to produce tissue injury [2,3]. Because of its potent matrix degradation capacity, neutrophil elastase (NE) has been particularly investigated in the respiratory tract of CF patients. Indeed, NE is involved in epithelial damage of the lung at very early stages of CF and its pathophysiological action is aggravated by an insufficient protection by two antiproteinases, the α1-anti-trypsin and the secretory leucoprotease inhibitor (SLPI) [4]. Proteinase 3 (PR3) [5,6], also called myeloblastin [7,8], is a serine proteinase homolog to NE and cathepsin G [5]. PR3 can degrade a variety of extracellular matrix proteins including elastin, fibronectin, type IV collagen and laminin [9]. Both NE and PR3 can process interleukin-8 [10], a major neutrophil chemoattractant in the lung, which can stimulate the respiratory burst and release of lysosomal enzymes. PR3 has also been shown to process tumor necrosis factor-α [11]. Those properties could explain that tracheal instillation of PR3 induces emphysema to the hamster [6]. In contrast to NE, PR3 is not inhibited by the SLPI and can enzymatically process it [12]. We have recently shown that PR3 is present at significantly higher levels than NE in sputum of patients with CF, though circulating neutrophils contained five times less PR3 than NE [13]. This difference was even more pronounced in CF patients during pulmonary exacerbation with *P. aeruginosa*. Moreover, PR3 enzymatic activity which was resistant to SLPI was highly correlated with the clinical status and the respiratory scores of CF patients [13].

In the present study, we investigated the cellular origin of PR3 in patients with CF. We therefore analyzed PR3 intracellular levels and the respective capacity of circulating neutrophils and monocytes to upregulate PR3 mRNA expression in CF patients in relation to pulmonary exacerbation and the infection state.

2. Materials and methods

2.1. Patients

30 Patients with CF (19 males and 11 females), 12.2 ± 5.5 years of age (range 0.4–20.3 years), were evaluated. Seven patients had negative sputum cultures and 23 patients had chronic airway infection with one or several of the following pathogens: *P. aeruginosa* ($n = 13$), *Pseudomonas cepacia* ($n = 2$), *Pseudomonas maltophilia* ($n = 3$), *Staphylococcus aureus* ($n = 9$) and *Haemophilus influenzae* ($n = 8$). 16 Patients presented pulmonary exacerbation defined by the presence of five or more of the following parameters: increased cough and sputum production, increased respiratory rate or dyspnea, new lung infiltrates, fatigue and decreased exercise tolerance, deterioration of pulmonary function tests, decreased appetite associated with loss of weight and/or fever [14]. 14 Patients without pulmonary exacerbation were defined as being in stable condition of the disease. 10 Patients were investigated before and after 2 weeks of antibiotic treatment. The control group consisted of 13 healthy donors of the Necker Hospital blood donation center. The protocol was approved by the Ethics Committee of the Paris Saint-Antoine University and informed consent was obtained from parents before the study.

2.2. Preparation of monocytes and neutrophils

Peripheral blood (8 ml) was collected on EDTA and processed for monocytes purification using a Nycoprep gradient [15]. Neutrophils were isolated using density centrifugation on Ficoll-Hypaque (Phar-

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Abbreviations: PR3, proteinase 3; CF, cystic fibrosis; NE, neutrophil elastase; SLPI, secretory leucoprotease inhibitor; MPO, myeloperoxidase; RT-PCR, reverse transcription-polymerase chain reaction

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macia LKB Biotechnology, Piscataway, NJ, USA). Cytocentrifuged preparations were obtained and stained with May Grunwald Giemsa. No morphological modification was detected in monocytes prepared from patients before and after antibiotic therapy.

2.3. Measurement of PR3 by enzyme-linked immunosorbent assay (ELISA)

PR3 ELISA was performed using the anti-PR3 monoclonal antibody, WGM2 (kind gift of Dr Csernok, Borstel, Germany) and the biotinylated PR3 monoclonal antibody (Clone CLB 12.8) as previously described [13].

2.4. Immunoblotting

Total monocyte lysates from 10^4 cells were electrophoresed through a 12.5% sodium dodecyl sulfate-polyacrylamide gel. Immunoblotting was performed using polyclonal anti-peptide PR3 antibodies [7], revealed by a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.5. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from both monocytes and neutrophils using a RNeasy Kit (Qiagen GmbH, Hilden, Germany). After denaturation at 70°C, RT was performed at 42°C for 55 min using Moloney Murine Leukemia Virus Reverse Transcriptase (Superscript II, Gibco BRL, Life Technologies, Paisley, UK). Expression of PR3, MPO and control β-actin mRNA in monocytes and neutrophils was analyzed by semi-quantitative PCR using a GeneAmp PCR System 9600 (Perkin Elmer, Forster City, CA, USA) with the following primers corresponding to distinct exon sequences: β-actin, sense: 5′-CGGCATCGTCACCAACTG-3′ and antisense: 5′-TGTCACGCACGATTTCCC-3′; PR3, sense: 5′-CACTGCCTGCGGGACATA-3′ and antisense: 5′-GTCAGGGAAAAGGCGGGT-3′; MPO, sense: 5′-GACCCCCGCATCAAGAAC-3′ and antisense: 5′-CTCTCCCCATCCCACCTA-3′. After denaturation, PCR cycles were conducted as follows. (i) For β-actin: denaturation 30 s at 94°C, annealing 20 s at 62°C and extension 25 s at 72°C. (ii) PR3 and MPO: denaturation 30 s at 94°C, annealing 25 s at 60°C and extension 30 s at 72°C. The number of PCR cycles was determined for PR3 or MPO (45 cycles) and β-actin (25 cycles) to insure that quantification of the PCR products was conducted in a linear range of amplification. The β-actin PCR reaction was performed using the cDNA preparation from each patient in order to determine those dilutions providing equal amounts of RT products for all samples and analyzed using a PhosphorImager (Storm, Molecular Dynamics, Sunnyvale, CA, USA). As a negative control, a parallel PCR was conducted without addition of cDNA. As a positive control, the PCR reaction was conducted with cDNA samples obtained from U937 cells. Aliquots of each PCR reaction were electrophoresed, transferred onto nylon filters and hybridized with the following P^{32}-labelled probes: β-actin, 5′-ACCCCGTGCTGCTGACCGAGG-3′; MPO, 5′-CTTTGACAACCTGCACGATGACCCCTGTCT-3′ and PR3, full length cDNA [8].

3. Results

3.1. Neutrophil and monocyte PR3 intracellular contents in CF patients as compared to controls

PR3 concentrations were compared in neutrophil and monocyte lysates (adjusted to 10^7 cells/ml) of CF patients ($n=10$) and healthy controls ($n=13$) (Fig. 1A). PR3 concentrations in neutrophil lysates did not differ between CF patients and controls (2659 ± 179 versus 2418 ± 148 μg/ml; mean ± S.E.M.). Likewise, no difference could be detected between CF patients without ($n=5$) or with ($n=5$) pulmonary exacerbation (2880 ± 629 versus 2523 ± 476 μg/ml).

PR3 concentrations were much higher in neutrophils than in monocytes and both in healthy controls (2418 ± 148 versus 96.2 ± 13 μg/ml, $P < 0.0001$) and in CF patients (2659 ± 179 versus 271 ± 22 μg/ml, $P < 0.0001$) (Fig. 1). However, in contrast to neutrophils (Fig. 1A), monocytes of CF patients in a stable condition showed significantly higher concentrations of PR3 than controls (271 ± 22 versus 96 ± 13 μg/ml, $P < 0.01$) (Fig. 1B). Interestingly, the PR3 intracellular content was further enhanced in monocytes from CF patients with pulmonary exacerbation as compared to CF patients with stable conditions (385 ± 68 versus 195 ± 30 μg/ml, $P < 0.05$) (Fig. 1B).

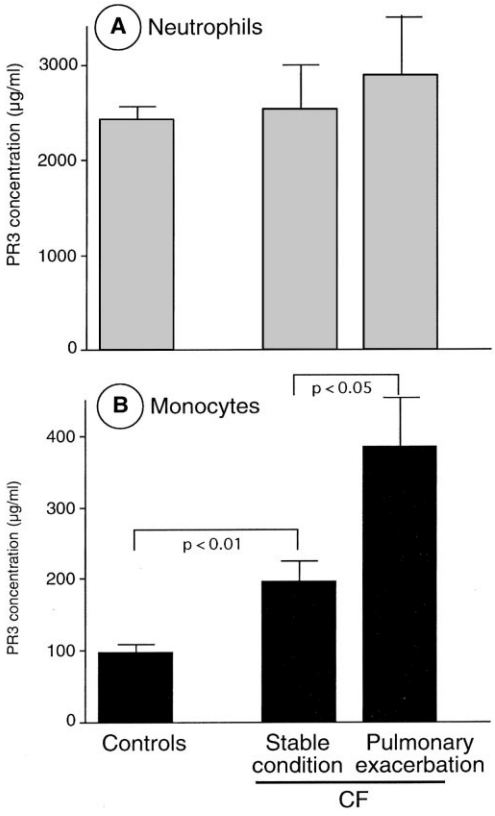

Fig. 1. Measurement of neutrophil- and monocyte-derived PR3 in CF patients as compared to controls. The PR3 concentration was measured in neutrophils (A) and in monocytes (B). Cells (10×10^6 cells/ml) were lysed in PBS containing 1% Triton and PR3 was measured by ELISA in 13 healthy controls and in 10 CF patients being either in stable condition ($n=5$) or having pulmonary exacerbation ($n=5$). Statistical analysis to compare PR3 concentrations in different conditions was performed using the non-parametric Mann-Whitney test.

3.2. Influence of the stage of the lung disease on PR3 mRNA expression in neutrophils and monocytes in CF patients

20 Patients were investigated for PR3 mRNA expression in CF neutrophils purified as exemplified in Fig. 2A. PR3 mRNA expression was either undetectable or poorly expressed in neutrophils, independently of the stage of their disease (stable condition, $n=8$ and pulmonary exacerbation, $n=12$) without any influence of antibiotic treatment as exemplified for three patients in Fig. 2B.

Monocytes of CF patients in stable condition ($n=8$), purified as exemplified in Fig. 3A, did not show significant PR3 mRNA expression measured by semi-quantitative RT-PCR (data not shown). In contrast, PR3 mRNA was heavily expressed in monocytes of all the patients with pulmonary exacerbation ($n=12$) as exemplified in four patients (Fig. 3B, lanes 1, 3, 5 and 7). Interestingly, PR3 mRNA was no longer detectable in nine out of 10 patients tested at the time of

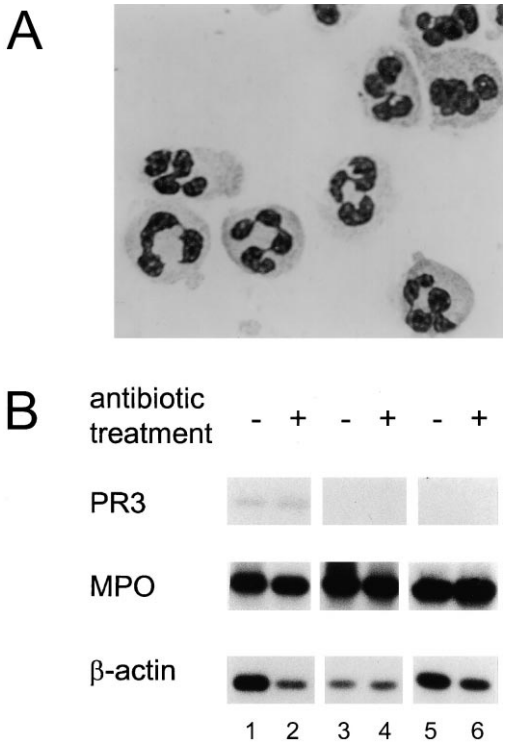

Fig. 2. PR3 in neutrophils. (A) A representative May Grunwald Giemsa stain of cytocentrifuged purified neutrophils. (B) Southern blot analysis of RT-PCR products from neutrophils of three exemplified CF patients. 1, 3, 5 and 2, 4, 6 are three individual CF patients before (−) and after (+) antibiotic treatment, respectively. MPO and β-actin were used as another phagocytic cell granular enzyme and an assessment of cDNA concentrations in each lane, respectively.

complete remission after a 2 week antibiotic treatment as exemplified in Fig. 3B, lanes 4, 6 and 8 versus 3, 5 and 7. In one case, in which no significant change in leukocyte count and no improvement of pulmonary function was found, PR3 mRNA was still expressed after antibiotic treatment (Fig. 3B, lane 2 versus 1). These results demonstrate that mPR3 expression in monocytes is highly correlated with the inflammation/infection state. At the protein level, at the time of pulmonary exacerbation, PR3 protein could also be detected by immunoblot analysis of monocyte lysates in the four patients explored and significantly decreased following successful antibiotic therapy (Fig. 3C, lanes 4, 6 and 8 versus 3, 5 and 7), except for the patient who did not respond to antibiotic treatment (Fig. 3C, lane 2 versus 1). We then investigated whether the expression of MPO, another azurophil granule-stored enzyme, was also regulated in patients with CF. In contrast to PR3 mRNA, MPO mRNA could be detected both in neutrophils and in monocytes and remained at the same level whether CF patients were under stable condition or pulmonary exacerbation (Fig. 2B and 3B).

4. Discussion

CF represents a model of neutrophil-dominated airway inflammation and chronic bacterial colonization involving an imbalance in the ratio of proteases to anti-proteases [4]. Since PR3 is the only neutrophil serine protease not inhibited by SLPI which is present at very high levels in sputum, we investigated the in vivo regulation of PR3 expression in both neutrophils and monocytes of CF patients.

In the present study, we first showed that circulating neutrophils contain much higher concentrations of PR3 than monocytes. To our knowledge, such a comparative analysis has not yet been reported. This important difference (approximately 20 times) could easily be explained by the well-documented observation of a much higher number of both azurophil and secondary granules in mature neutrophils than in monocytes [16]. A more important finding was that the PR3 mRNA of monocytes could be significantly enhanced at the time of CF pulmonary exacerbation. This was not the case with neutrophils, although these cells have a protein synthesis capacity, since recent studies have stressed their full capacity to synthesize cytokines [17]. The potential significance of such an upregulation of PR3 in monocytes both at the mRNA and the protein level should be considered along the two following aspects.

4.1. Fundamental implications of inducibility of PR3 expression in circulating monocytes

PR3, together with other phagocytic cell granular enzymes such as NE and MPO, is considered to be stored in intracellular compartments and eventually released upon degranulation. So far, neutrophils have been considered as the major source of these granular enzymes and there was no evidence, in these cells, of de novo biosynthesis and modulation of PR3 during inflammatory/infectious processes. The expression and

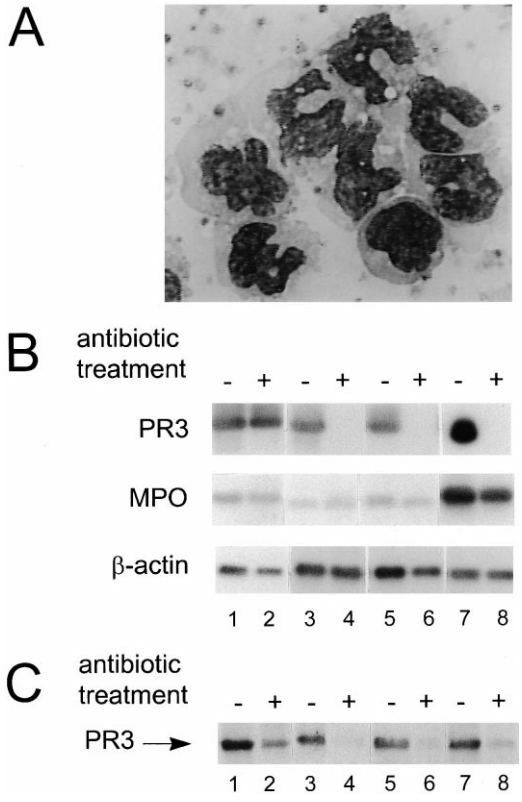

Fig. 3. PR3 in monocytes. (A) A representative May Grunwald Giemsa stain of cytocentrifuged purified monocytes. (B) Southern blot analysis of RT-PCR products from monocytes. (C) Immunoblot analysis with anti-peptide PR3 antibody. In B and C, 1, 3, 5, 7 and 2, 4, 6, 8 are four individual CF patients before (−) and after (+) antibiotic treatment, respectively.

regulation of PR3 in monocytes clearly modifies this classical picture. Our observation that PR3 can be upregulated in vivo during the CF inflammatory process may suggest a potential regulatory role for PR3 in monocyte activation and especially in cytokine processing, as previously described [10,11]. Thus, besides its direct deleterious effect on connective tissues largely mediated by neutrophils, PR3 could be a mediator of inflammation through its expression and regulation in monocytes [18].

4.2. Clinical relevance of PR3 inducibility in CF pathophysiology

The release of serine proteinases plays a role in the degradation of the lung in patients with CF [19,20]. Our present results suggest that the high concentrations of PR3 measured in sputum [13] could be the result of both the neutrophils and monocytes-derived alveolar macrophage producing PR3. While numerous studies have shown the deleterious role of neutrophils on lung tissues at very early stages of the disease [21], the role of the monocytes has rarely been investigated. However, monocytes were consistently found in blood vessels and the adjacent interstitial space in areas with overt evidence of ongoing inflammation in patients with CF [22].

We have now shown that PR3 mRNA expression in peripheral blood cells of patients with pulmonary exacerbation is restricted to monocytes, suggesting that the PR3 gene is newly expressed during the inflammatory process. The fact that in contrast to PR3, MPO was not upregulated renders the specific role of PR3 in the CF lung inflammatory process more accurate.

Although the ongoing inflammation is restricted to the airways, previous studies have shown that circulating neutrophils are in a priming state in the very early phase of the disease [23,24]. Likewise, we have now shown that circulating monocytes may play a significant role in the inflammatory process since PR3 mRNA in these cells was highly correlated with the inflammatory status of the patient. Our present findings stressed out that the PR3 content in circulating monocytes is clearly affected by, and could serve as a peripheral marker of, pulmonary exacerbation and ongoing airway infection in patients with CF.

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