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Full Length Article

Increased platelet activation occurs in cystic fibrosis patients and correlates to clinical status

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ABSTRACT

Cystic fibrosis (CF) is an inflammatory lung disease. Platelets have an emerging role in inflammation, however previous studies of platelet activation in CF have generated conflicting results. In this study, we determined platelet function in CF patients and correlated platelet activation to establish clinical and laboratory parameters. Twenty-two patients, aged 20.7 to 54.4 (mean 34.0, SD 9.45) years and with a mean FEV1%pred (forced expiratory volume in one second, % of predicted) of 72 (SD 21.4, range 32–110) were recruited. A combination of platelet assays was used: platelet aggregation, platelet activation and platelet-leukocyte complex formation. Platelets from CF patients exhibited significantly increased aggregation when stimulated ex-vivo, a tendency towards increased platelet upregulation of CD62P, but no increase of GPIIb/IIIa activation (PAC-1). Platelet-monocyte complex (PMC) formation was significantly increased in CF patients compared to controls, while platelet-neutrophil complex formation was not. In the CF group, platelet aggregation correlates with levels of anti-neutrophil cytoplasmic antibodies (ANCA) with specificity for bactericidal/permeability-increasing protein (BPI), BPI-ANCA (r = 0.56). The formation of PMCs correlates with lung function decline (1-FEV1%), CRP and BPI-ANCA (r = 0.61, 0.55, 0.5). We therefore confirm the presence of increased platelet activation in CF patients, and determine that further evaluation of platelet activation in relation to prognostic factors in CF is warranted.

1. Introduction

The primary role of platelets is to respond to vascular damage and maintain haemostasis. Circulating platelets become activated in response to vessel damage; adhere to the endothelium and form a stable platelet aggregate. Platelet activation also occurs during inflammation and activated platelets release pro-inflammatory mediators, such as lipid metabolites and chemokines [1]. Activated platelets bind to and modulate the function of immune cells, such as monocytes and neutrophils. Monitoring platelet activation during inflammatory conditions may provide diagnostic or prognostic information for the disease. Platelet activation has been reported to occur in some inflammatory diseases, including lung disease [2,3], atherosclerosis [4], and diabetes [5–7]. Increased platelet activation has also been reported in renal failure patients, psoriasis and Crohn's disease [8–10].

Dysregulated inflammatory processes cause early and sustained damage of lung tissue in cystic fibrosis (CF), eventually leading to pulmonary insufficiency and death. Great improvement in treatment has prolonged life expectancy in CF, but there is still no available treatment to cure this exaggerated inflammation, although there are treatments that influence the inflammatory process, such as dornase alfa, ibuprofen and azithromycin [11]. Some investigators have reported increased platelet activation in CF patients [12–15], while others have reported normal levels of platelet activation [16]. In the current study, we investigated platelet function in 22 CF patients and compared this with healthy controls using a combination of platelet activation assays; platelet aggregation, platelet activation, platelet-leukocyte complex formation, and leukocyte activation. Platelet function was correlated to clinical factors known to influence the health status and prognosis for CF patients, including anti-neutrophil cytoplasmic antibodies (ANCA) with bactericidal/permeability-increasing protein (BPI) specificity; BPI-ANCA. BPI-ANCA is a valuable prognostic factor, especially in *P. aeruginosa* colonized CF patients [17–20].

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2. Materials and methods

2.1. Patients cohort

Adult patients registered at the CF centre at Skåne University Hospital, Lund, Sweden, were eligible for the study. Patients performing their annual CF assessment during the period April to December 2015 were asked to participate in the study and 24 patients agreed to do so. None of the patients were inpatients or on continuous oral treatment with corticosteroids. Neither were any of the patients on regular treatment with ibuprofen. The study was approved by the local ethical committee, approval number 2011/434, and all patients signed the informed consent form. Patients were recruited according to the Declaration of Helsinki. Citrated blood samples were obtained for platelet analyses. Two patients who were initially included in the study were later excluded due to low platelet counts ($< 150 \times 10^9$/l). Seventeen patients left blood samples for the study and performed the annual CF assessment on the same day. Six patients who left blood for the annual assessment on a different day than recruitment to the platelet study were excluded from correlation analyses. A control group of 22 gender and age matched controls was recruited during the same time period.

2.2. Whole blood impedance aggregometry

Platelet aggregation in citrated whole blood was determined using an impedance aggregometer (Multiplate, Roche). 300 µl of citrated blood was added to an equal volume of 0.9% saline containing 3 mM $CaCl_2$ in a test well and warmed to 37 °C for 3 min. Collagen (3.2 µg/ml) or ADP (6.5 µM) was added to initiate platelet aggregation according to the standardised recommended assay procedure of the manufacturer. The change in electrical impedance between the two electrodes was monitored for six minutes. The Area under the curve (AUC) was calculated by the Multiplate software, reflecting the magnitude of platelet aggregation that occurred in response to the platelet agonists.

2.3. Flow cytometry of platelet-leukocyte complex formation and platelet/leukocyte activation

20 µl of citrated blood was added to 40 µl of HEPES Buffer pH 7.4 and incubated in the presence or absence of agonist the for 10 min at room temperature; platelet activation by thrombin (0.5 U/ml) or leukocyte activation by fMLF (1 µM). Fluorescent labelled antibodies (BD Biosciences) were added to investigate platelet activation; CD42-PerCP, PAC-1-FITC and CD62P-PE. In parallel samples a second panel of fluorescent labelled antibodies was added to investigate platelet-leukocyte complex formation and leukocyte activation; CD61-PE and CD11b-PE.Cy5. Antibody staining was stopped after 10 min by addition of red cell lysis reagent and fixative (Uti-Lyse, DAKO Cytomation). Samples were analysed on a BD Accuri flow cytometer using logarithmic settings to acquire platelet samples and linear settings to acquire leukocyte samples.

2.4. Clinical parameters of health status in CF patient cohort

A number of clinical parameters were assessed in the patient cohort as a part of their routine annual assessment. Platelet count, C reactive protein (CRP), and total Immunoglobulin (IgG) levels were analysed in the Laboratory of Clinical Chemistry at Skåne University Hospital, Lund, Sweden. BPI-ANCA was analysed by Eurodiagnostica in Malmö, Sweden as previously described [20]. Bacterial cultures from sputum samples were performed by the Dept. of Microbiology at Skåne University Hospital, Lund, Sweden and description of bacterial colonization was based on cultures from the inclusion date combined with earlier cultures, available in the Dept. of Microbiology database. *Pseudomonas aeruginosa* colonization was classified according to the Leeds criteria [21]. Lung function was measured at the Dept. of Clinical Physiology, Lund, and FEV1%pred calculated from the Swedish reference material used in Lund [22]. FEV1% is the most common lung function assay in CF and levels above 80% are regarded as normal lung function. Severely ill CF patients may have FEV1 as low as 20–30% of predicted values.

Table 1
Patients recruited to the study.

Patients (n)	22		
Sex (n)	f = 9	m = 13	
Age (years)	34.0 (mean)	SD: 9.45	Range: 20.7–54.4
Pancreatic function (n)	PI	PS	
	19	3	
Lung function FEV1%pred (n = 20)	72.0 (mean)	SD: 21.5	Range: 32–110
Bacterial colonization (n)	Chronic PA	Chronic SA	Other bacteria
	11	11	4
Platelet count	292 (mean)	SD: 99.7	Range: 179–620
CRP	6.1 (mean)	SD: 8.4	Range: 0.65–38
Azithromycin (n)	Yes	No	
	12	10	
IgG	13.4 (mean)	SD: 3.1	Range: 9.5–22
IgA BPI-ANCA	282.3 (mean)	SD: 603.8	Range: 2–2251
IgG BPI-ANCA	56.9 (mean)	SD: 92.6	Range 0–372
CF related diabetes	Yes	No	
	5	17	

3. Results

3.1. Health status of the CF patient cohort at inclusion in the study

The 22 patients, presented in Table 1, were aged from 20.7 to 54.4 (mean 34.1, SD 9.45) years at the time of inclusion. There were nine female and thirteen male patients and the majority were pancreatic insufficient (nineteen versus three), reflecting the number of severe CF mutations in the study group [23]. Mutations represented in the cohort were mainly F508del homozygous (fifteen patients). Other mutations were F508del/394delTT (two patients), F508del/I507del, F508del/S945L in the pancreatic insufficient group, and F508del/R117C, S549I/S549I and F508del/unknown in the pancreatic sufficient patients. Two patients were unable to perform spirometry, for psychological reasons in one patient, and physical in another. In one patient, no sputum cultures were available due to a very low sputum production. There were eleven patients chronically colonized with *P. aeruginosa* and two patients were intermittently colonized. Other bacteria found in these patients at time of the study were predominantly *Staphylococcus aureus* (n = 11), *Stenotrophomonas maltophilia* (n = 1), *Achromobacter xylosoxidans* (n = 1), *Hemophilus influenzae* (n = 1) and *Mycobacterium avium intracellulare* (n = 1).

The mean lung function was 72% of FEV1pred (n = 20, SD 21.5, range 32–110) which indicates that patients were in general doing fairly well, but there were also patients with severe disease and low lung function in the cohort. The mean platelet count was 292, (SD 99.7, range 179–620). Mean CRP level was 6.1 (SD 8.4, range 0.6–38). Azithromycin, an anti-inflammatory treatment in CF, was used three times per week by twelve patients. The BPI-ANCA levels were variable, ranging from almost undetectable levels to very high. Mean BPI-ANCA IgA level was 282 (SD 604, range 2–2251) and mean IgG level was 57 (SD 92.6, range 0–372). Five patients were treated for CF related diabetes.