CONCISE REPORT

 β Thromboglobulin and platelet factor 4 in bronchoalveolar lavage fluid of patients with systemic sclerosis

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Objective: To evaluate concentrations of the platelet activation markers β thromboglobulin (BTG) and platelet factor 4 (PF4) in bronchoalveolar lavage fluid (BALF) from patients with systemic sclerosis with and without scleroderma interstitial lung disease (SLD).

Methods: BTG and PF-4 were measured by enzyme immunoassay in BALF from 37 patients with systemic sclerosis. Controls were 10 healthy subjects. BALF was collected during routine bronchoscopy from the right middle lobe. SLD was diagnosed by high resolution computed tomography of the lungs.

Results: BTG was detected in 11 of the patients with systemic sclerosis (29.7%) and PF4 was found in eight (21.6%). Mean (SD) concentrations of BTG and PF4 in BALF from patients with detectable levels of these platelet activation markers were 106.9 (69.8) and 35.2 (17.4) IU/ml, respectively. The BTG:PF4 ratio was more than 2:1, indicating *in vivo* release. Both markers were found exclusively in patients with SLD. SLD patients with detectable platelet activation markers had a significantly shorter disease duration than those with undetectable BTG/PF4.

Conclusions: The study provides evidence that activation of blood platelets takes place within the lungs of patients with SLD and may contribute to the development of lung fibrosis.

Scleroderma interstitial lung disease (SLD) is one of the main causes of death in patients with systemic sclerosis.^{1,2} Inflammation and excessive fibrosis of the lungs are key features of SLD, although the exact mechanisms involved in the development of this condition are not clear.¹

Platelet granules contain a broad spectrum of biologically active mediators including chemotactic and growth factors, which are immediately released at sites of platelet activation.^{3,4} β Thromboglobulin (BTG) and platelet factor 4 (PF4) are platelet specific α granule proteins which are released from activated platelets and are considered markers of platelet activation.⁴

Microvascular injury, which may lead to chronic platelet activation, is an early and consistent finding in systemic sclerosis.⁵ Indeed, several studies have shown that activation of platelets occurs within the circulation in patients with this disorder.⁵ However, there are no available data on the activation of blood platelets within the lungs of patients with scleroderma.

Our aim in this study was to evaluate concentrations of BTG and PF4 in BALF from patients with systemic sclerosis with and without SLD.

METHODS

Patients

We recruited 37 patients fulfilling the American College of Rheumatology (ACR) classification criteria for systemic

sclerosis.⁶ SLD was diagnosed using high resolution computed tomography (HRCT) of the lungs, as described previously.⁷ Lung function tests were carried out using MasterScreen (Erich Jaeger, Hoechst, Germany). The HRCT scans and the pulmonary tests were subject to blinded evaluation. Patients selected for this study had not received immunosuppressive or corticosteroid treatment for at least five years, and non-steroidal anti-inflammatory drugs (NSAIDs), anticoagulants, or antiplatelet drugs for at least three months before the study. All other drugs were stopped seven days before the study. Patients with evidence of respiratory infections and current smokers were not included into the study.

Clinical analysis of the patients with systemic sclerosis included age, disease duration, disease subset (diffuse systemic sclerosis versus limited systemic sclerosis), lung function tests, erythrocyte sedimentation rate (ESR), blood platelet count, and cytological analysis of BALF.

Controls were 10 healthy non-smoking individuals who had no evidence of lung or systemic disease. This group consisted of healthy volunteers and subjects who underwent diagnostic bronchoscopy but in whom no evidence of systemic or lung disease was found.

The clinical characteristics of patients with systemic sclerosis and the control group are presented in table 1. The protocol of the study was approved by the local ethics committee and informed consent was obtained from all subjects studied.

Bronchoscopy and bronchoalveolar lavage

Bronchoscopy was carried out as previously described.⁷ Differential cell counts on the BALF samples were made from the cytospin samples after staining with May-Grunwald-Giemsa. BALF samples with evidence of bleeding were not included in the study.

Measurement of β thromboglobulin and platelet factor 4

BTG and PF4 were measured in BALF using commercially available Asserachrom enzyme immunoassay kits (Diagnostica Stago, New Jersey, USA). Detection limits of BTG and PF4 were 12.5 and 6.0 IU/ml, respectively.

Statistical analysis

Statistical analyses employed the Mann–Whitney U test, the Kruskal–Wallis test, and Fisher's exact test. Probability (p) values less than 0.05 were considered significant.

Abbreviations: BALF, bronchoalveolar lavage fluid; BTG, β thromboglobulin; FVC, forced vital capacity; HRCT, high resolution computed tomography; NSAID, non-steroidal anti-inflammatory drug; PF4, platelet factor 4; SLD, scleroderma interstitial lung disease

Table 1 Clinical characteristics and results of BALF investigation and measurements of BTG/PF4 in BALF from the patients with systemic sclerosis and the control group

Variable	Systemic sclerosis (n = 37)					
	Total (n = 37)	Without SLD (n = 13)	With SLD (n = 24)			Controls (n = 10)
			Total (n = 24)	Detectable BTG/PF4 (n = 11)	Undetectable BTG/PF4 (n = 13)	
Sex (F/M)	35/2	12/1	23/1	11/0	12/1	9/1
Age (years)	50.0 (11.7)	44.0 (13.8)	53.2 (9.2)	51.6 (10.7)	54.5 (7.8)	41.4 (13.7)
Disease subset diffuse/limited	17/20	2/11	15/9	9/2	6/7	
Disease duration* (years)	11.3 (9.8)	7.8 (5.0)	12.7 (11.4)	7.2 (6.5)§	17.2 (12.7)	
FVC (% of predicted)	76 (15)†	93 (7)	68 (16)†‡	78 (18)†	65 (14)†‡	102 (10)
HRCT findings consistent with SLD (n (%))	24/37 (64.9%)	0	24/24 (100%)	11/11 (100%)	13/13 (100%)	0
Recovery of BALF (%)	52.5 (7.2)	53.9 (5.1)	51.7 (8.0)	53.0 (5.0)	52.0 (6.0)	54.5 (5.0)
Total cells (cell/ml × 10 ⁴)	32.1 (21.5)†	21.0 (11.2)	38.1 (23.4)†‡	41.8 (30.1)†	35.0 (16.6)†	14.9 (8.7)
Neutrophils (% total)	4.2 (4.3)†	1.9 (1.6)	5.4 (4.8)†‡	6.0 (5.7)†	4.9 (4.1)†	1.4 (0.7)
Lymphocytes (% total)	20.5 (12.0)†	16.7 (6.9)	22.6 (13.7)†	24.4 (15.5)†	21.1 (12.4)†	11.3 (4.6)
Macrophages (% total)	74.1 (13.9)†	81.4 (7.1)†	70.2 (15.1)†‡	68.2 (19.8)†	71.9 (10.3)†	87.3 (4.7)
Patients with detectable BTG (n (%))	11 (29.7%)	0	11 (45.8%)	11 (100%)	0	0
Patients with detectable PF4 (n (%))	8 (21.6%)	0	8 (33.3%)	8 (72.7%)	0	0

Values are n or mean (SD) unless stated otherwise.

*The duration of systemic sclerosis was defined as the time from the first signs attributable to the disease.

†p<0.05 v controls; ‡p<0.05 v patients without SLD; §p<0.05 v patients with undetectable BTG/PF4.

BALF, bronchoalveolar lavage fluid; BTG, β thromboglobulin; FVC, forced vital capacity; HRCT, high resolution computed tomography; PF4, platelet factor 4; SLD, scleroderma lung disease.

RESULTS

Clinical data and analysis of BALF

SLD was diagnosed in 24 of the 37 patients with systemic sclerosis (64.9%). In the remaining 13 patients (35.1%) there were neither signs of SLD on HRCT nor any impairment of lung function tests.

Forced vital capacity (FVC) of patients with SLD was significantly lower than in the patients without SLD or the controls (table 1). There was no significant difference in FVC values between patients without SLD and the controls.

The mean total cell number per ml of BALF was greater in patients with SLD than in those without SLD or the controls (p<0.05). Patients with SLD had a greater percentage of neutrophils in BALF than patients without SLD or the controls (p<0.05). The mean percentage of lymphocytes in BALF of patients with SLD was greater than in the controls (p<0.05). The results of cytological analysis of BALF are presented in table 1.

Concentration of BTG and PF4 in BALF

BTG was detected in BALF from 11 of the 37 patients with systemic sclerosis (29.7%). The BTG levels in BALF ranged from 15.2 to 229.0 IU/ml (n = 11; mean (SD), 106.9 (69.8) IU/ml). All patients in whom BTG was found had features of SLD on HRCT. BTG was undetectable in all BALF samples from patients without SLD and in all those from the controls.

PF4 was detected in eight of the 37 patients with systemic sclerosis (21.6%) and its levels ranged from 7.0 to 53.0 IU/ml (n = 8; mean (SD), 35.2 (17.4) IU/ml). PF4 was detectable exclusively in BALF samples in which BTG was also found.

In all patients in whom BTG and PF4 were found, the BTG:PF4 ratio exceeded 2 (n = 8; range, 2.4 to 8.9). Concentrations of BTG and PF4 from individual patients are presented in fig 1.

Patients with detectable BTG/PF4 had a significantly shorter disease duration than SLD patients with undetectable platelet activation markers (table 1). Disease duration in patients with detectable BTG/PF4 ranged from 1 to 20 years; however in eight of these 11 patients the disease duration was less than 6 years (range, 1.0 to 5.5). The remaining three patients with SLD and detectable BTG/PF4, who had suffered from systemic sclerosis for more than 6 years, had a flare of their disease at the time of study, which was defined by

Figure 1 Concentrations of β thromboglobulin (BTG, panel A) and platelet factor 4 (PF4, panel B) in bronchoalveolar lavage fluid from patients with and without scleroderma lung disease (SLD) and in the control group. Horizontal bars are mean values.

increased dyspnoea, skin thickening, and an increase in ESR. The SLD patients with detectable platelet activation markers did not differ from those with undetectable markers with respect to age, disease subset, FVC, cytological analysis of BALF (table 1), ESR, or blood platelet count (data not shown).

DISCUSSION

We found increased levels of BTG and PF4 in BALF of SLD patients, with the BTG:PF4 ratio greater than 2, indicating in

vivo release.⁸ Our results suggest that platelet activation takes place within the lungs and are in agreement with those of Idell *et al*, who found increased levels of BTG and PF4 in BALF from patients with adult respiratory distress syndrome.⁹ Involvement of platelets in the development of interstitial lung disease has been demonstrated in an experimental model of bleomycin induced lung fibrosis¹⁰; in that model, accumulation of blood platelets correlated with collagen deposition in the lungs.

Increased concentration of platelet activation markers in BALF of our patients with a shorter disease duration suggests that activation of platelets occurs mainly in the early phase of lung disease. Indeed, Piguet *et al* showed in their bleomycin induced model that platelets accumulate in the lungs as early as seven days after injection of bleomycin.¹⁰ Interestingly, it has also been shown that the greatest loss of lung volume in patients with SLD occurs within the first few years of the disease.² This association of platelet activation and impairment of lung function suggests that platelets may play a role in remodelling the lungs in SLD patients.

Factors that may cause platelet activation within the injured lungs include collagen or subendothelium of injured cells, as injury to small blood vessels and endothelial cell damage are early and constant features of interstitial lung diseases.¹¹ Other platelet activators, thrombin and immunological complexes, have been shown to be raised in BALF from patients with systemic sclerosis.¹

When activated, platelets may take part in inflammation within the lungs through release of chemotactic factors for granulocytes and monocytes, such as PF4, platelet activating factor (PAF), and RANTES ("regulated on activation, normal T cell expressed and secreted").^{3,4} Activated platelets produce proinflammatory eicosanoids, particularly thromboxane A₂, and may contribute to the production of leukotrienes.^{3,12} We have recently shown that leukotriene B₄ and E₄ concentrations are significantly increased in BALF from patients with SLD.⁷ In addition, platelet granules contain several growth factors that may contribute directly to the development of fibrosis.^{3,4} Moreover, PF4 and BTG are chemotactic for fibroblasts.¹³

We did not find any correlation between the presence of blood platelet activation markers and other cellular markers of inflammation within the lungs, as estimated by cytological analysis of BALF. It cannot be ruled out that this reflects the low concentration of BTG/PF4 in the majority of BALF samples studied, which makes detailed comparison impossible. On the other hand, recent observations indicate that the presence of lung inflammation is not necessary for progression of the fibrosis.¹⁴ Therefore, activation of platelets may represent an additional mechanism contributing to the development of pulmonary fibrosis.

Platelet activation during the early course of systemic sclerosis, as demonstrated in this paper, suggests a possible role of antiplatelet drugs in the prevention of SLD. Unfortunately, NSAIDs are not the best choice in systemic sclerosis because they may, by inhibiting cyclooxygenases, further contribute to the development of pulmonary fibrosis.¹⁵ The newer antiplatelet drugs, which are in widespread use in the prevention of coronary disease, seem to be good

candidates for treating systemic sclerosis and may offer clinical benefit in these patients.

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