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Interrelationship between serum and sputum inflammatory mediators in chronic obstructive pulmonary disease

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Little is known about airway inflammatory markers in chronic obstructive pulmonary disease (COPD). The objective of the present study was to identify and try to correlate pulmonary and peripheral blood inflammatory markers in COPD. In a cross-sectional study on patients with stable COPD, induced sputum and blood samples were collected for the determination of C-reactive protein, eosinophilic cationic protein, serum amyloid A protein, α-1 antitrypsin (α-1AT), and neutrophil elastase. Twenty-two patients were divided into two groups according to post-bronchodilator forced expiratory volume in the first second (%FEV_1): group 1 (N = 12, FEV_1 <40%) and group 2 (N = 10, $FEV_1 \geq 40\%$). An increase in serum elastase, eosinophilic cationic protein and α-1AT was observed in serum markers in both groups. Cytology revealed the same total number of cells in groups 1 and 2. There was a significantly higher number of neutrophils in group 1 compared to group 2 ($P < 0.05$). No difference in eosinophils or macrophages was observed between groups. Serum elastase was positively correlated with serum α-1AT (group 1, $r = 0.81$, $P < 0.002$ and group 2, $r = 0.83$, $P < 0.17$) and negatively correlated with FEV_1 ($r = -0.85$, $P < 0.03$ and -0.14, $P < 0.85$, respectively). The results indicate the presence of chronic and persistent pulmonary inflammation in stable patients with COPD. Induced sputum permitted the demonstration of the existence of a subpopulation of cells in which neutrophils predominated. The serum concentration of all inflammatory markers did not correlate with the pulmonary functional impairment.

Key words: Chronic obstructive pulmonary disease; C-reactive protein; Eosinophilic cationic protein; Serum amyloid A protein; α-1 antitrypsin; Neutrophil elastase

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Introduction

Chronic obstructive pulmonary disease (COPD) has recently been defined as a systemic pulmonary inflammatory disease whose physiopathology is poorly understood (1), with a growing interest in the understanding of the relationship between systemic inflammation and pulmonary events (2). The balance between pro-inflammatory and anti-inflammatory mediators determines the course of an inflammatory process and these mediators involved in COPD have been less studied than those involved in asthma (1).

Systemic inflammation has been recognized as an important risk factor for a diverse number of co-morbidities including atherosclerosis (3), cachexia (4), anorexia (5), and osteoporosis (6). Notably, all of these complications are commonly observed in patients with COPD (7). However, the extent of systemic inflammation in stable patients and the nature of the inflammatory markers present during the chronic phase of the disease have not been demonstrated.

Neutrophils are the predominant cells in this type of pulmonary inflammation (8). Elevated levels of mediators such as interleukin-6, -8, C-reactive protein, eosinophilic

cationic protein (ECP), elastase, α-1 antitrypsin (α-1AT), myeloperoxidase, and serum amyloid A protein (SAA) have been detected in the blood of patients with COPD, even during exacerbations (9,10). SAA and C-reactive protein are related to neutrophil chemotaxis (11,12). C-reactive protein is an acute-phase protein and a sensitive indicator of systemic inflammation.

Little information is available regarding the airway inflammatory markers present in patients with moderate or severe COPD (13), with this inflammation being characterized by elevated numbers of neutrophils and macrophages in the airways (14). Neutrophils are the main producers of proinflammatory mediators such as cytokines and proteases (15) and the induced sputum technique permits the investigation of these patients (16,17). Inflammation and oxidative stress can be evaluated not only in the airways and other pulmonary compartments but also in blood (18).

Is it possible to demonstrate in peripheral blood the repercussions of inflammatory alterations that occur in the airways and lungs? The purpose of the present study was to assess the relationships between serum inflammatory markers, induced sputum and lung function in these patients. A correlation between these findings might be useful for the medical management of these patients.

Patients and Methods

A cross-sectional study was conducted in which patients were recruited from the COPD outpatient clinic of the Pulmonary Division, Heart Institute (InCor), University of São Paulo Medical School, São Paulo, SP, Brazil. The Ethics Committee of the Institution approved the study, and all selected patients signed an informed consent form.

Inclusion and exclusion criteria

The criteria for inclusion in the study were: patients with COPD according to the criteria established by the American Thoracic Society (ATS) (19), patients of both genders ranging in age from 40 to 85 years, patients clinically stable for at least 8 weeks, and patients not taking antibiotics or anti-inflammatory drugs such as inhaled or oral corticosteroids. Exclusion criteria were patients with a past or current history of basal airway inflammation such as allergic exposure and/or respiratory tract infections that might interfere with the evaluation and patients with a history or diagnosis of severe heart disease, asthma, and bronchiectasis or tuberculosis sequelae.

A COPD exacerbation in the last year was defined in the protocol as "a sustained worsening of the patient's condition, from the stable state and beyond normal day-to-day variations, that is acute in onset and necessitates a change in regular medication in a patient with underlying COPD" (20).

Lung function and induced sputum

The patients were submitted on the same occasion to spirometry performed according to the criteria of the ATS (21) and the following parameters were determined: forced vital capacity (%FVC), forced expiratory volume in the first second in relation to the predicted values (%FEV_1), and FEV_1/FVC actual ratio, and the values are reported as percent predicted. The patients discontinued a short-acting bronchodilator at least 12 h and a long-acting bronchodilator 24 h before the spirometry. Spirometry was carried out before and 15-20 min after the administration of 400 µg salbutamol inhaled via a spacer device and groups were defined according to post-bronchodilator spirometry.

Sputum was collected and analyzed as described previously (22,23). Briefly, sputum was induced after three sequential inhalations of 3% hypertonic saline for 7 min each. The aerosol was generated with an ultrasonic nebulizer (Ultra-Neb 99, DeVilbiss, Somerset, PA, USA) with an output of 2.4 mL/min. The sputum sample was considered adequate if it met the following criteria: sputum induction tolerated for at least 14 min, sputum volume >2 mL, presence of squamous cells ≤80%, at least 200 inflammatory cells per slide (10 µL), and examination of sputum performed within 2 h. Sputum samples were separated from saliva with the help of an inverted microscope. At least 200 cells were counted and classified as eosinophils, lymphocytes, neutrophils, macrophages, squamous cells, goblet or ciliated cells based on their morphology.

Inflammatory mediators

After sputum induction, blood samples were collected for the determination of the following inflammatory markers in serum and plasma: C-reactive protein (Dako AIS, Glostrup, Denmark), ECP (fluoroenzyme immunoassay, Pharmacia and Upjohn Diagnostics, Uppsala, Sweden), SAA (enzyme immunoassay, Hemagen, São Paulo, SP, Brazil), α-1AT (Alpha Diagnostics, San Antonio, TX, USA), and neutrophil elastase (Merck, Darmstadt, Germany).

Statistical analysis

Data are reported as median and 95% confidence range or mean and standard deviation and were analyzed statistically using appropriate software (SPSS No. 10 software program, Chicago, IL, USA). The normal distribution of the variables was evaluated by the Kolmogorov-Smirnov test. Differences between groups were determined by the Student *t*-test and Kruskal-Wallis test. Correlations between variables were calculated using the Spearman cor-

relation test. A P value < 0.05 was considered to be significant. The patients were stratified by functional involvement (24) into group 1, FEV_1 <40% of predicted value, and group 2, FEV_1 ≥40% of predicted value, always post-bronchodilator. Since this was a pilot study, we did not calculate the sample size.

Results

Thirty-three subjects were selected and 22 who performed all procedures were included. Among the 11 patients who were excluded, eight did not fulfill the criteria for acceptance of the sputum sample and three had bronchospasm during induction, which prevented collection of the sputum sample. However, no clinical difference was observed between included and excluded patients. None of the patients had smoked actively for at least 6 months prior to the study; received inhaled or oral corticosteroids, or other systemic drugs including theophylline, antibiotics or nonsteroidal anti-inflammatory drugs, or reported any acute exacerbations for at least eight weeks prior to sputum induction.

The clinical and functional characteristics of the patients included in the study are shown in Table 1, with 12 patients in group 1 (FEV_1 <40%) and 10 patients in group 2 (FEV_1 ≥40%). There was a predominance of males in both groups and the mean age was 60 years. All patients were long-term heavy smokers (mean pack-years = 74). The mean time since the diagnosis of COPD was at least 8 years in both groups. The mean time since smoking cessation was 8 years for group 1 and 14 years for group 2. At least 4 patients in group 1 and 2 in group 2 had used oral corticosteroids (20 mg/day for 10 days) in the last year. Exacerbation in the last year was observed for 4 patients in group 1 and 2 in group 2. No significant difference in these variables was observed between groups.

The pulmonary function test revealed an important reduction of FEV_1 and FEV_1/FVC ratio in patients of group 1 (P < 0.001) compared to group 2, and FVC was also lower in group 1 but the difference was not significant. None of our patients had a bronchodilator response after 400 µg of inhaled salbutamol.

An increase was observed in the serum inflammatory markers of neutrophil (elastase) and eosinophil (ECP) activity and α-1AT in the two groups when compared to the reference values of the kits used. There was no difference in C-reactive protein or SAA levels compared to the reference values of the respective kits (Table 2).

The total number of cells in sputum of groups 1 and 2 was not significantly different. A significantly greater number of neutrophils was observed in group 1 (6.5 (1.6-37) x 10^6 cells/mL) compared to group 2 (3.2 (1.3-11.8) x 10^6 cells/mL) (P < 0.05). No difference in eosinophils, macrophages or lymphocytes was observed between groups (Figure 1).

Serum elastase was positively correlated with α-1AT in both group 1 and group 2 (r = 0.81, P < 0.002 (Figure 2) and r = 0.83, P < 0.17, respectively). A negative correlation was observed between FEV_1 and elastase in group 1 and group 2 (r = -0.85, P < 0.03 (Figure 3) and r = -0.14, P < 0.85, respectively).

Table 1. Clinical and functional characteristics of patients with chronic obstructive pulmonary disease (COPD).

	Group 1 (N = 12)	Group 2 (N = 10)
Male/female	10/2	9/1
Age (years)	65 (52-85)	55 (43-77)
Pack-years	78 ± 11	70 ± 13.5
Smoker/ex-smoker	1/11	4/6
Years since smoking cessation	8 (1-20)	14 (1-40)
On oral corticosteroids (yes/no[a])	4/8	2/8
Duration of COPD (years)	7.5 ± 1.1	8.5 ± 1.8
Exacerbations (yes/no[b])	4/8	2/8
FVC (% predicted)	64 ± 5.7	73 ± 3.3
FEV_1 (% predicted)	30 ± 1.8	50 ± 2.3*
FEV_1/FVC ratio (% predicted)	47 ± 3.1	68 ± 1.1*

Data are reported as mean ± SD or as otherwise indicated. Group 1 = FEV_1 <40% predicted; group 2 = FEV_1 ≥40% predicted; FVC = forced vital capacity; FEV_1 = forced expiratory volume in the first second. [a]No use of oral corticosteroids in the last year. [b]No exacerbations in the last year. Dose of oral corticosteroids: 20 mg/day for 10 days.
*P < 0.001 compared to group 1 (*t*-test).

Table 2. Serum markers of inflammatory activity in patients with chronic obstructive pulmonary disease.

	Group 1 (N = 12)	Group 2 (N = 10)	Reference value (lower limit of detection)
Elastase (µg/L)	58 ± 7.4	46 ± 9	12-32 (8)
ECP (µg/L)	13 ± 2	22 ± 6*	2-10 (0.5)
α-1AT (mg/dL)	205 ± 22	215 ± 20	83-199 (20)
CRP (mg/dL)	0.19 (0.13-0.87)	0.63 (0.16-1.4)*	≤0.5 (0.2)
SAA (µg/L)	3.5 (3-4.5)	5.2 (4-20)*	3-4 (2)

Data are reported as mean ± SEM or median and 95% confidence interval for C-reactive protein and SAA. Group 1 = FEV_1 <40% predicted; group 2 = FEV_1 ≥40% predicted; ECP = eosinophilic cationic protein; α-1AT = α-1 antitrypsin; CRP = C-reactive protein; SAA = serum amyloid A protein.
*P < 0.05 compared to group 1 (*t*-test).

Discussion

The results obtained for patients with moderate and severe COPD, indicated the presence of chronic and persistent pulmonary inflammation in stable patients. The use of induced sputum permitted the demonstration of the existence of a subgroup in which neutrophils predominated and of another in which these cells did not. Serum inflammatory markers did not correlate with the degree of pulmonary function impairment in this group of stable patients with COPD.

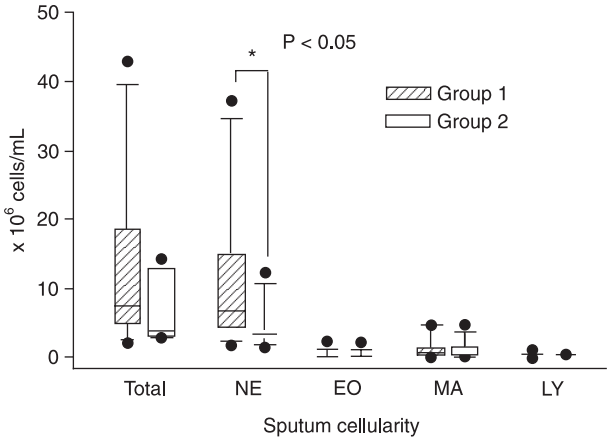

Figure 1. Range and distribution of sputum cellularity in patients with chronic obstructive pulmonary disease. Data are reported as box plots showing the median (line inside), the interquartile range (25th to 75th percentiles), the bars (10th and 90th percentiles), and outliners (closed circles) for sputum cellularity comparing group 1 with FEV_1 <40% and group 2 with FEV_1 ≥40%. NE = neutrophils; EO = eosinophils; MA = macrophages; LY = lymphocytes. For statistical analysis the Wilcoxon signed rank test was used.

An elevated number of neutrophils in sputum were observed in the two groups when compared with asthmatic patients or normal subjects (25), with the number of these cells being higher in the group presenting greater functional impairment (group 1). The predominance of neutrophils in sputum observed for group 1 is in agreement with data reported in the literature (15,25,26). Neutrophils possess granules that contain proteins such as myeloperoxidase and lipocalin. These neutrophil markers have been shown to be elevated in sputum of patients with COPD, indicating the degranulation of primary and secondary granules in neutrophils (25).

The extracellular matrix of the lung is a dynamic structure whose integrity requires a balance between the synthesis and degradation of its components. Neutrophils are the main source of elastase in the human lung (27), followed by macrophages. Elastase secreted by these cells destroys structural pulmonary components and thus contributes to obstruction (28). Several studies have suggested that the activity of proteolytic enzymes, particularly neutrophil elastase, plays an important pathogenic role in the development of emphysema (29-31). An increase in the degradation of elastin in the organism is observed during periods of COPD exacerbation (32).

Neutrophils and T lymphocytes are related to the degree of obstruction and airflow limitation observed in patients with COPD (14,33). In the present study, an increase in neutrophil elastase and an impairment in %FVC, $\%FEV_1$, and in FEV_1/FVC were observed in the two groups, with these findings being more expressive in group 1.

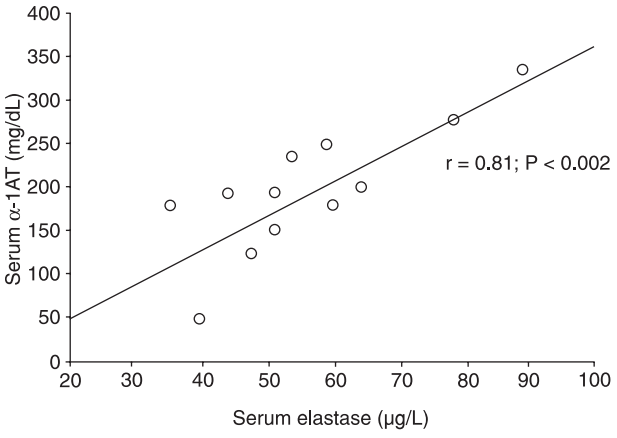

Figure 2. Correlation between serum elastase and serum α-1 antitrypsin (α-1AT) in patients of group 1 (FEV_1 <40%).

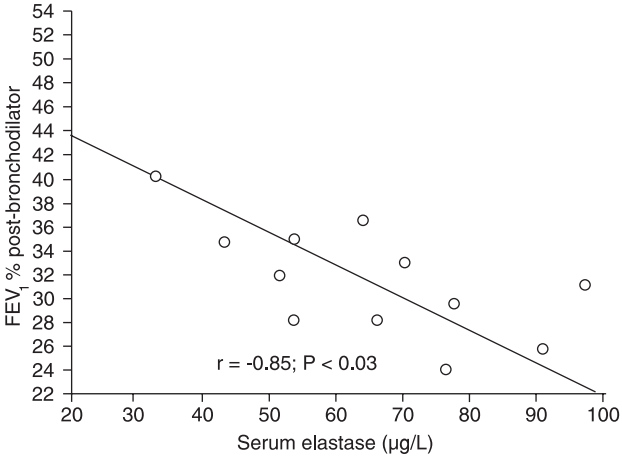

Figure 3. Correlation between serum elastase and forced expiratory volume in the first second (FEV_1) post-bronchodilator in group 1 (FEV_1 <40%).

The main inhibitor of elastase is α-1AT (34), a serum protein that functions as an endogenous inhibitor of serine proteinases. Serum α-1AT levels were also found to be higher than normal in the two groups of patients with COPD, with no significant difference between groups. In group 1, the elevated elastase levels were positively correlated with the increase in α-1AT and negatively correlated with FEV_1.

Our results support the thesis that pulmonary obstruction is a consequence of the presence of inflammatory stimuli (35-37). One hypothesis can be raised to explain the development of tissue damage associated with COPD and suggests an imbalance between proteases and antiproteases and the other proposes changes in the oxidant-antioxidant ratio (1).

We also investigated markers of systemic inflammation, including C-reactive protein and SAA, and correlated them with COPD. Some studies have demonstrated elevated C-reactive protein levels in COPD during exacerbations, indicating the possible presence of infection (10,38), and in stable COPD patients (39,40), and elevated SAA levels in asthma. No expressive increase in C-reactive protein was observed, a finding that might be explained by the fact that the population consisted of stable severe patients. Curiously, SAA and C-reactive protein, even within the reference values of the kit, were found to be more elevated in group 2, with the increase in SAA being significant when compared to group 1.

One limitation of our study was the fact that the study population presented an advanced degree of pulmonary function impairment. Thus, the present findings are limited to patients with a similar degree of pulmonary involvement secondary to smoking. Therefore, caution may be necessary when using the serum and sputum inflammatory mediator in COPD patients. Because the comparison between outcomes of the serum and sputum was performed in only a limited number of patients, further studies should focus on a large number of individuals. However, the power of our results was tested, and it was found to be >80%. Therefore, a type 1 statistical error is unlikely.

Despite these limitations, the analysis of inflammatory markers can be an important tool to better understand the physiopathological alterations that occur in COPD. Together with the analysis of pulmonary function, this approach may contribute to the understanding of clinical variables and permit a better treatment guidance of these patients.

These results may have implications for our understanding of the pathogenesis of the disease. The results indicate the presence of chronic and persistent pulmonary inflammation in stable patients with COPD. These results confirm the presence of inflammatory processes in the airways and circulation of patients with COPD. Knowledge of the relationship of these inflammatory markers allows us to design clinical trial of anti-inflammatory therapies that include appropriate outcomes.

In summary, therefore, we have assessed the interrelationship between serum inflammatory markers, induced sputum and lung function in patients with stable COPD with a wide range of bronchial disease. Although relationships can be demonstrated, it is clear that they are complex, and understanding the interplay of various mediators will require appropriately designed intervention studies. Further studies are needed to determine whether attenuation of the systemic inflammatory process can modify the risk of complications in COPD.

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