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Clara cell 16 protein in COPD sputum: A marker of small airways damage?☆

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Summary
Rationale: The development of chronic obstructive pulmonary disease (COPD) in smokers and their susceptibility to infections is not fully understood. Recent evidences suggest that Clara cells play a part in host defense, immunomodulatory response and airways remodelling through the production of specific factors such as Clara cell 16 (CC-16). This protein has never been related to patients' lung function tests, blood gases parameters and diseases severity.
Objectives: To evaluate a possible correlation between CC-16 expression in sputum, measured by a new methodological approach, and the degree of severity in patients with moderate and severe COPD. We also analyzed possible correlations between CC-16 and cytological sputum population, arterial blood gases and lung function.
Main Findings: We analyzed 20 patients, mean age 72.95, classified on the basis of the global initiative for chronic obstructive lung disease guidelines (GOLD 2006). The samples were processed for cytological analysis and CC-16 levels were assessed by Western blot. We found lower levels of CC-16 in severe COPD compared to moderate ones ($p<0.027$). No statistically significant differences were found between CC-16 expression and sputum cellularity (except for macrophages), arterial blood gases, and spirometric parameters. Multiple linear regression analysis of CC-16 versus functional and cytological parameters showed no significance.
Conclusions: We found a significantly different expression of CC-16 in COPD patients, according to their stage of severity, as defined by the GOLD 2006 guidelines. Considering CC-16 properties in innate immunity, a possible link between protein expression, innate

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immune system, and COPD infectious exacerbations may be hypothesized but further investigation are needed.

Introduction

Chronic obstructive pulmonary disease (COPD) is one of the major causes of morbidity and mortality among respiratory diseases and is characterized by an airflow limitation that is not fully reversible. Airflow limitation is usually both progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases. Many structural changes can be seen in COPD lung: increased mucus secretion, airway remodelling, and loss of elastic recoil with emphysema, the latter two constituting the main mechanisms of irreversible airway obstruction in severe COPD.[1,2]

Two kinds of risk factors are recognized in COPD pathogenesis. Host factors include genetic pattern, airway hyperresponsiveness, and lung growth, while environmental factors include exposure to tobacco smoke, occupational dusts and chemicals, infections, and socioeconomic status. Although cigarette smoking is considered the major risk factor for COPD, only a small rate (10–15%) of smokers develop the disease, suggesting that a relationship between environmental and host immunological factors might be critical in its pathogenesis.

At present there is no test available that predicts susceptibility to the disease, which is often diagnosed late in its course; moreover, even though neutrophilic and lymphocytic infiltration are associated with FEV_1 decline, the relationship between immune system and structural airway changes remains unclear.[3,4] However, The National Heart, Lung, and Blood Institute and the World Health Organization developed the global initiative for chronic obstructive lung disease (GOLD) which produced evidence-based guidelines describing four stages of COPD severity for worldwide use in its detection and treatment. These four stages are classified as mild (I), moderate (II), severe (III), and very severe (IV).[5]

COPD pathogenesis involves the peripheral airways, and pathologic changes seen in the lung samples of COPD patients include bronchial and bronchiolar obstruction, mucosal oedema, goblet cells hyperplasia, collagen deposition and injury of epithelial airway cells.[6]

Clara cells, namely located in the terminal bronchioles, are one the most multifunctional epithelial cell types in mammalian lungs. They appear to be devoted to the protection of the respiratory tract from inhaled toxic agents. Indeed, Clara cells repair damaged epithelium, detoxify xenobiotics, and secrete proteins with important biological activities such as leukocyte-protease inhibitors and the 16 kDa Clara cell protein (CC-16).[7,8]

CC-16 is a non glycoprotein with a molecular mass of 16 kDa, which is produced by Clara cells in the lung and mucosal epithelial cells in uterus and prostate. Structurally it is a homodimer of subunits of 70 amino acids covalently bound in an antiparallel manner[9] and one of the main secretory proteins of the lung. It can be found in high concentrations in the epithelial lining fluid where it appears to play an antioxidant/inflammatory role by modulating the production and/or activity of phospholipase-A_2, interferon-γ, and tumour necrosis factor α.[10] From a diagnostic point of view, CC-16 could be the fulcrum for a novel approach for assessing the integrity of respiratory epithelium. Bernard and colleagues showed a significant reduction of CC-16 in bronchoalveolar lavage of smokers and COPD patients compared with healthy subjects and hypothesized that this protein might serve as a sensitive indicator of nonciliated bronchial cell dysfunction.[11] The function of this protein, so far elucidated, is immunoregulatory and anti-inflammatory in innate immunity. It has been demonstrated that knockout mice of UG gene resulted in aggravation of inflammation triggered by allergic and hyperoxic stimuli, that could be represented by viral/bacterial infection or by exposure to noxious particles.[12] Recent evidences confirm and strengthen the hypothesis that CC-16 protects the respiratory tract against oxidative stress and inflammation.[13] A weaker airways immunity could be caused by reduced levels of this protein and could confer a lack of protection against viral and bacterial infection and environmental pollutants. This could eventually lead to the fact that COPD patients with lower levels of CC-16 are more prone to develop exacerbations, and consequently suffer from more severe forms of COPD and vice versa.

Lung samples or BAL from COPD patients show a decrease in CC-16,[14,15] but no spontaneous sputum study has been performed to investigate the potential correlation between the level of this protein and the patient's lung function tests, sputum cellularity and arterial blood gases.

Aim of the study

The aims of the present study were:

- to identify a possible different expression of CC-16 in spontaneous sputum in moderate and severe COPD patients,
- to investigate possible correlations between CC-16 levels and sputum cellularity, arterial blood gases and lung function.

Methods

Patients population

About 20 COPD patients were enrolled. All the patients were hospitalized for COPD exacerbation and classified according to GOLD 2006 guidelines. Blood gas analysis and spontaneous sputum collection were performed for each patient (Table 1).

None of them had undergone any systemic steroid therapy for at least 1-month prior or was suffering from any malignant neoplasm. Patients have been treated with

Table 1 Patients' demographic and clinical/investigational data.

Patient	Sex	Age	Stage	FEV_1	TI	PO_2	PCO_2	OD	%Neu	%Eos	%Macr	%Linf
R-A	M	64	Moderate	50	36	83	39	0.13	77	3	18	2
I-S	M	77	Moderate	59	52	75	37	0.49	70	2	25	3
P-A	M	61	Moderate	60	69	73	38	0.07	49	1	47	3
S-A	M	62	Moderate	70	65	75	40	0.11	71	1	28	0
C-L	M	79	Moderate	60	69	78	39	0.13	98	0	2	0
L-F	M	57	Moderate	64	64	69	37	0.10	90	2	7	1
G-A	M	69	Moderate	52	48	60	43	0.24	86	1	8	5
C-M	M	69	Moderate	67	62	67	35	0.10	92	2	2	4
P-F	M	72	Moderate	48	63	63	44	0.50	78	1	21	0
V-E	M	84	Moderate	60	46	73	45	0.22	92	1	6	1
M-M	M	86	Moderate	54	40	76	34	0.10	94	0	0	6
A-A	M	71	Severe	32	50	55	50	0.22	81	2	15	2
F-C	M	73	Severe	38	51	74	64	NA	NA	NA	NA	NA
B-F	M	68	Severe	48	45	55	47	0.10	94	0	6	0
P-O	M	80	Severe	40	50	41	37	NA	NA	NA	NA	NA
DN-A	M	54	Severe	35	43	56	50	0.17	97	0	3	0
F-G	M	81	Severe	43	56	56	46	0.09	92	0	6	2
R-F	F	85	Severe	38	40	58	54	0.07	96	0	4	0
A-S	M	86	Severe	45	68	55	36	0.07	63	0	10	27
C-C	M	81	Severe	45	48	52	53	0.09	NA	NA	NA	NA
G-S	M	76	Severe	24	50	73	33	0.06	92	0	8	0
M-A	M	70	Severe	35	60	50	60	0.10	93	0	6	1

Patients characteristics: *n* 10 stage III and *n* 11 stage IV, according to GOLD guidelines 2006.
TI = Tiffenau index (FEV_1/FVC); PO_2 arterial partial pressure (tension) of O_2 mmHg; CO_2 arterial partial pressure (tension) of CO_2 mmHg; NA = Not available.

inhaled long-acting β_2 agonists (formoterol or salmeterol) and theophylline.

Methods

Each sample of sputum was inserted in a centrifuge tube and overlaid with an equal volume of diluted Sputolysin reagent (Calbiochem, San Diego, CA), mixed on a vortex mixer for 30 s and allowed to stand at 37 °C for 15 min. After incubation the mixture was filtered and centrifuged at 1400 rpm for 15 min. About 300 μl of M-PER (PIERCE) and 1 μl of Protease Inhibitor Cocktail (SIGMA) were added to the supernatant. The suspension was shaken for 10 min and then centrifuged at 27,000*g* for 15 min. The supernatant was collected in an Eppendorf tube and stored at −20 °C.

Western blot analysis

Western blot analysis was performed using 15% SDS-PAGE (BIO-RAD protocol) and marker (BIO-RAD) as control: the gel was transferred electrophoretically to a nitrocellulose membrane sheet at 12 mA overnight at room temperature.

Western blot analysis was performed on all the sputum samples for CC-16 detection. A semiquantitative measure of CC-16 was obtained by means of optical density (OD) methodology value in all the moderate patients ($N = 10$) and in eight severe patients ($N = 8$). The nitrocellulose sheet was saturated with 5% milk and subsequently incubated with polyclonal rabbit anti-human urine protein 1 (Dako, 1:1000 dilution) at room temperature for 3 h with shaking. After washing with buffer, the membrane was incubated with alkaline phosphatase conjugated secondary antibody monoclonal anti-rabbit immunoglobulin (Sigma, 1:1000 dilution) for 90 min at room temperature with shaking. Finally, the nitrocellulose membrane was washed and then incubated with NBT/BCIP (Pierce) diluted in alkaline buffer for 10 min and the reaction was stopped with distilled water. Quantification of CC-16 levels was performed by computerized densitometry analysis. Protein concentrations are expressed as OD. OD is defined as the absorbance of an optical element for a given wavelength λ per unit distance and it is measured in ODU, which are equivalent to "Absorbance units"/cm (AU cm^{-1}).

Cytology analysis

Each sample of sputum was inserted in a centrifuge tube and overlaid with an equal volume of diluted sputolysin reagent (calbiochem, San Diego, ca), mixed on a vortex mixer for 30 s and allowed to stand at 37 °C for 15 min. After incubation the mixture was filtered. The slides were prepared using cytocentrifuge and standard technique(200 μl of fluids—800 rpm for 3 min).

Slides were stained with Diff Quik stain to differentiate between eosinophils, neutrophils, macrophages and lymphocytes and were viewed with optic microscope (Olympus U-SPT). The number of inflammatory cells was express as the percent of cell.[16,17]

Spirometric tests were conducted according to ATS guidelines.[18]

The volume of air forcibly exhaled from the point of maximal inspiration (forced vital capacity, FVC) and the volume of air exhaled during the first second of this maneuver (forced expiratory volume in 1 s, FEV_1) has been measured, and the ratio of these two measurements (FEV_1/FVC) has been calculated. Spirometry measurements are evaluated by comparison with reference values based on age, height, sex, and race.[19]

All statistical analyses were carried out with the SPSS12.0 statistical package. A statistically significant cut-off value was set at $p<0.05$.

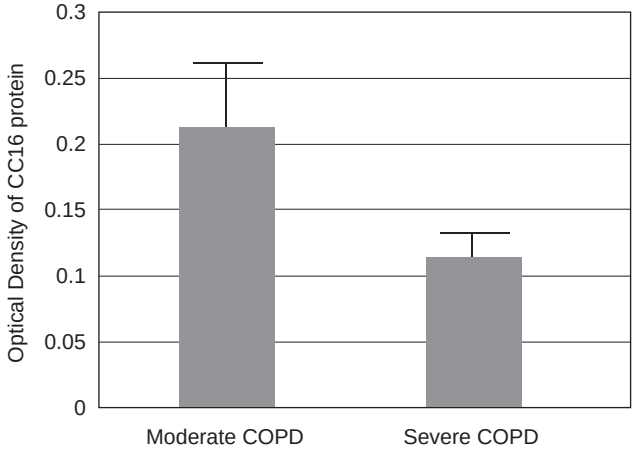

Figure 1 CC-16 optical density in spontaneous sputum in moderate and severe COPD ($p>0.05$).

Results

In our sample 10 patients were stage II and 10 stage III according to GOLD 2006, aged from 54 to 86 years (mean age 72.95 ± 9.44 years), 19 male and 1 female, all were former smokers.

We found significantly lower levels of CC-16 in severe COPD patients as compared to moderate ones ($p=0.027$ Mann Whitney test) (Fig. 1). The laboratory method used to identify and obtain a semi-quantitative value of CC-16 was reliable and reproducible. Cytological analysis showed no significant difference in cell populations between the two groups of patients, although there was an increasing trend in neutrophils in severe as compared to moderate COPD ($p=$ ns Mann Whitney test). The percentage of neutrophils out of 500 cells was 81.5% in moderate COPD while in severe COPD it was 88.2%.

In the entire sample no Pearson correlation was found between OD and age, FEV_1, IT, PO_2, PCO_2, neutrophils,

Table 2 Statistical Correlations among CC-16 optical density, age, lung function, arterial blood gases, sputum cytology.

	OD	Age	FEV_1	TI	PO_2	PO_2	%Neu	%Eos	%Macr
Age									
R	−0.038								
P	0.881								
FEV_1									
R	0.070	−0.123							
P	0.781	0.605							
TI									
R	0.043	−0.064	0.370						
P	0.866	0.787	0.108						
PO_2									
R	0.186	−0.114	0.637[b]	0.001					
P	0.459	0.633	0.003	0.997					
PCO_2									
R	−0.117	−0.048	−0.666[b]	−0.224	−0.302				
P	0.643	0.840	0.001	0.342	0.196				
%Neu									
R	−0.371	−0.027	−0.151	−0.278	−0.151	0.377			
P	0.142	0.919	0.564	0.280	0.564	0.136			
%Eos									
R	0.327	−0.389	0.352	−0.084	0.481	−0.368	−0.397		
P	0.200	0.123	0.166	0.750	0.051	0.146	0.114		
%Macr									
R	0.571[a]	−0.225	0.200	0.162	0.274	−0.146	−0.807[b]	0.503[a]	
P	0.017	0.386	0.443	0.535	0.287	0.575	0.000	0.040	
%Lym									
R	−0.183	0.402	−0.066	0.265	−0.184	−0.381	−0.546[a]	−0.154	−0.047
P	0.482	0.110	0.802	0.304	0.479	0.131	0.023	0.556	0.859

[a]Correlation is statistically significant at 0.05 (2-tailed).
[b]Correlation is statistically significant at 0.01 (2-tailed).

eosinophils and lymphocytes. A significant correlation was found between OD and macrophages (Table 2).

From the correlation results, some necessary preconditions emerged to deepen the research trying to give an interpretation and a causal sense to the variables investigated, by means of a multiple regression analysis. Our main specific purpose was to identify the parameter that could predict the OD variable. This is the reason why two multiple linear regressions (stepwise method) were made in which OD was considered as dependent variable. In the first regression FEV_1, PO_2 and PCO_2 represented the independent variables, but the result was not significant. In the second one, neutrophils, macrophages, eosinophils and lymphocytes represented the independent variables. Even in this case the results were not significant. As a consequence, FEV_1, PO_2, PCO_2, neutrophils, macrophages, eosinophils, and lymphocytes do not seem to be able to influence OD values.

Discussion and conclusions

Recent evidence in mice demonstrate that there is a marked decrease in the synthesis and secretion of CC-16 during LPS-induced acute lung inflammation and that CC-16 deficiency is associated with increased lung susceptibility to viral infections and oxidative stress.[20,21]

In a previous study on BAL of patients at risk for ARDS, CC-16 concentration was inversely correlated with the amount of elastase and a decrease in its concentration was associated with an increase in neutrophil-mediated lung damage, indicating the likelihood of its acting as a natural neutrophils inhibitor.[22] Thus, a decrease in CC-16 might contribute to inflammatory metaplastic transformation in the peripheral respiratory tract and could play a role, via loss of regulatory control, in the cascade of events which leads to remodelling and irreversible airway obstruction.

CC-16 has two important modulatory functions: (1) modulation of surfactant metabolism, in which SP-A determines secretion and reuptake,[23] and CC-16 potentially inhibits of PLA_2-mediated fatty acid release and lysophosphatidylcoline formation[24]; and (2) modulation of cyclooxygenase and lipooxygenase pathways with control over the production of lipid mediators such as platelet activating factor and leukotriene B_4 by the inhibition of PLA_2 activity.[25–28]

The present study was designed to evaluate CC-16 levels in the airways of moderate and severe COPD patients and evaluate possible correlations of this protein with cellular sputum population and arterial blood gases and lung function.

We found significantly lower levels of CC-16 in severe COPD patients as compared to moderate ones, demonstrating a significant different expression of protein between different GOLD stages of COPD. A decrease in CC-16 in the sputum of COPD patients could reflect inflammation of the lower airways and may contribute to progressive anatomic damage typical of the different COPD stages.[8] A previous study by Bernard found lower level of CC-16 in BAL in COPD patients.[29] Ekberg–Jansson demonstrated that healthy smokers had a relative high level of CC-16 in the airways and this was interpreted as the reason for not developing COPD.[30] We could therefore hypothesize that lower levels of CC-16 could contribute to fibroblast activation, airway remodelling and frequent viral and bacterial infections. The same study on neutrophil associated activation markers, using a multiple regression analysis, showed that CC-16, an indicator of Clara cell activity, was one of the factors influencing a decrease of lung diffusion CO capacity (DLCO) independently from smoking habit.[30]

Our results suggest that despite a different expression of CC-16 according to GOLD COPD classification of severity, the level of this protein seems to be independent from single functional (FEV_1, FVC, PO_2, PCO_2) and cytological (sputum neutrophils, macrophages, lymphocytes and eosinophils) parameters. Even the multiple regression analysis performed considering functional and cytological parameter altogether as independent variable showed lack of significance.

This observation could be relevant and some explanations could be suggested. In fact it is quite unlikely to think that the most abundant secretory protein of the bronchial district, with emergent physiological functions, is not involved and related directly or indirectly in the impairments caused by COPD in the lung.

The lack of correlation between CC-16 and the functional parameters could be explained by the fact that we only considered FEV_1 and TI values, and a correlation could be found with other values, such as hyperinflation index (TLC, RV), or with parameters reflecting a membrane impairment (DLCO).

Even a correlation with cellularity is missing, but maybe other markers of inflammation could be linked to the levels of CC-16 (LPS-binding peptide, MPO, Lysozyme, and so on).

Another intriguing possibility could be that CC-16 is totally independent from function and inflammation but related to the relationship between innate immunity and infective exacerbation of COPD. In other words, a deficit of this protein may be indirectly related to severity through a progressive worsening due to repeated infectious events.

Further investigations are needed to explore if CC-16 could be a valid biomarker for monitoring peripheral airway damage and if it could be potentially investigated as a predictor of early lung functional dysfunction and of predisposition to COPD infective exacerbations. Clinical and functional parameters, although simple and useful tools for diagnosis and treatment of COPD, are not good predictors of tissue damage and functioning, which so far has only been evaluated with invasive procedures such as BAL or lung biopsy.[26–28] The determination of CC-16 levels in spontaneous sputum of COPD patients, instead, is non invasive, easy to perform and reliable. In fact its dosage could indicate the degree of terminal bronchiolus damage and susceptibility to diseases worsening. Anyway, further investigations are needed to elucidate its clinical and pathogenetical significance and the existing correlations with COPD markers, as these aspects were not identified by our study, mainly due to the small sample size and the chosen parameters.

Conflict of interest

No conflict of interest exists.

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