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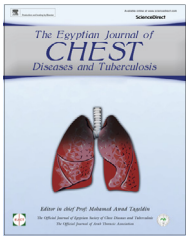

ORIGINAL ARTICLE

Study of serum Granzyme B in heavy cigarette smokers with and without chronic obstructive pulmonary disease

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KEYWORDS

COPD;
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Abstract *Background:* Inflammation of the airways is present in COPD with increased number of inflammatory cells including killer cells that lyse their target cells by two mechanisms; membranolysis in which secreted molecules such as granzymes form pores in the membrane of target cells; and apoptosis. Granzyme B has the strongest apoptotic activity of all granzymes.

Aim of this work: Aim of this work was to study the relation between Granzyme B, tobacco smoking and chronic obstructive pulmonary disease.

Methods: The study included 40 clinically stable COPD patients classified according to GOLD (2013) criteria into two groups; moderate (GOLD II) and severe (GOLD III) plus 40 apparently healthy control subjects (20 smokers and 20 nonsmokers). Pulmonary function results and serum levels of Granzyme B (measured by ELISA) were recorded.

Results: Granzyme B levels are elevated in COPD. Cigarette smoking appears to be a direct stimulus to Granzyme B production. Granzyme B could play a role in the pathogenesis of COPD. Aging seems to be a risk factor for Granzyme B production and pathogenesis of COPD.

Conclusion: Granzyme B levels are elevated in COPD. Cigarette smoking appears to be a direct stimulus to Granzyme B production. Granzyme B could play a role in the pathogenesis of COPD. Aging seems to be a risk factor for Granzyme B production and pathogenesis of COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is an incapacitating, highly prevalent airway disease that arises as a result of noxious injury to the lungs, most commonly due to cigarette smoking. The disease causes serious morbidity and mortality

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[11]. Cigarette smoking is by far the most important risk factor for COPD. However, only a susceptible minority (15–20%) of tobacco smokers develops clinically significant COPD, suggesting that genetic factors (such as the rare hereditary deficiency of α1-antitrypsin) must modify each individual's risk. Although the major environmental risk factor for COPD – tobacco smoke – is well known since many years, the cellular and molecular mechanisms that are involved in the pathogenesis of COPD have not yet been fully elucidated [1]. Recently, it has been reported that soluble Granzyme B levels and the proportion of T cells expressing intracellular Granzyme B or perforin were increased in the BAL of both current and ex-smokers with COPD [4]. Granzyme B is proposed to play a role in pathogenesis of chronic obstructive pulmonary disease [8].

Subjects and methods

Eighty subjects were included in this study which comprised of forty COPD patients and forty age matched apparently healthy subjects as controls. This study was carried out at the Chest department of the Benha University Hospital from April 2011 to May 2012.

Inclusion criteria

1. All COPD patients and healthy smoker controls must be current heavy cigarette smokers.
2. By spirometry: post bronchodilator improvement in FEV1 is less than 12% in all patients.
3. Patients should be in a stable state and not in exacerbations.

Exclusion criteria

1. History of cancer in the past five years.
2. Active liver disease.
3. Heart disease.
4. Kidney disease.
5. Diabetes Mellitus.
6. Collagen vascular diseases.
7. Non-COPD pulmonary conditions.20 of COPD patients were in GOLD (2011) stage II (moderate) while the other 20 patients were in stage III (severe). 20 of the healthy subjects were heavy cigarette smokers while the other 20 were never smokers.

All cases were subjected to the following:

1. Full history taking including:
   History of smoking and its severity by smoking index (number of packs/day × number of years smoking).
   History of other co-morbidities that may affect the level of Granzyme B.
2. Clinical examination:
   General and local examination.
3. Laboratory investigations:
   Fasting and 2 h post prandial blood glucose.
   Kidney function tests.
   Liver function tests.
4. Radiological examination:
   Plain postero-anterior and lateral chest X-rays were done to exclude any chest lesion if present.
5. Ventilatory function test (spirometry):
   Done before and after bronchodilatation.
6. Collection and processing of blood sample [9]:
   a. Venous blood samples were taken from arm veins in the right way and labeled with patient name and number.
   b. *Serum preparation:* Serum is obtained by allowing the blood to clot in the original closed container at room temperature (20–30 min). Centrifugation for serum is 10 min at an RCF of 1000 in the stoppered container.
7. Granzyme B assay (Granzyme B ELISA kit by Abcam, Cambridge, England, ab46142):
   a. *Principle of assay method:*Granzyme B kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific for Granzyme B has been coated onto the wells of the microtiter strips provided. Samples, including standards of unknown Granzyme B concentrations and unknowns are pipetted into these wells. During the first incubation, the Granzyme B antigen and a biotinylated monoclonal antibody specific for Granzyme B are simultaneously incubated. After washing, the enzyme (streptavidin-peroxidase) is added. After incubation and washing to remove all unbound enzyme, a substrate solution which is acting on the bound enzyme is added to induce a colored reaction product. The intensity of this colored product is directly proportional to the concentration of Granzyme B present in the samples.
   b. *Data analysis:*Generate a linear standard curve by plotting the average absorbance on the vertical axis versus the corresponding Granzyme B standard concentration on the horizontal axis. The amount of Granzyme B in each sample is determined by extrapolating O.D. values to Granzyme B concentrations using the standard curve.

Results

Tables 1–10

Discussion

The results in the present study as regards age and sex were in agreement with Peder et al. In their survey, they studied the prevalence of COPD in Copenhagen and included 6236 participants. All non-COPD participants aged 35 years or older with adequate lung function data were included for the final prevalence analyses. COPD staging was done according to the GOLD criteria. They found that COPD prevalence increases with age and was higher among males.

Granzyme B values were higher in patients than in controls. Values were also higher in severe disease (GOLD III) than in moderate disease (GOLD II). Also, Granzyme B values were higher in smoker controls than in nonsmoker controls.

In the present study, Granzyme B values were significantly higher in patients than in controls ($p < 0.001$) (Table 4). This result is supported by the work of Hodge et al. [4] who studied intracellular Granzyme B in blood derived cytotoxic T

Table 1 Age and sex distribution in COPD patients.

	Moderate group I	Severe group II	All patients
– Age			
• Mean	52.8	67.7	60.25
• SD	5.96	8.26	10.37
• Range			
30–49	6	0	6
50–79	14	19	33
⩾80	1	1	1
– Sex			
• Male	19 (95%)	20 (100%)	39 (97.5%)
• Female	1 (5%)	0 (0%)	1 (2.5%)

The table shows that the majority of patients were in 6th, 7th and 8th decades of life.
All patients were males except a female.

Table 2 Age and sex distribution among nonsmoker and smoker controls.

	Smokers group III	Nonsmokers group IV	All controls
– Age			
• Mean	45.4	44.3	44.85
• SD	6.52	7.28	6.76
• Range			
30–49	13	13	26
50–79	7	7	14
⩾80	0	0	0
– Sex			
• Male	20 (100%)	19 (95%) ⩾ 80	39 (97.5%)
• Female	0 (0%)	1 (5%)	1 (2.5%)

The table shows that controls studied were between 4th and 8th decades of life. All controls were males except a female.

Table 3 Descriptive statistics of Granzyme B (pg/ml).

	Mean ± SD	Range	*N*
Patients	287.08 ± 118.43	70.1–568.1	40
Controls	88.65 ± 59.68	0–226	40
Severe COPD	346.21 ± 109.76	167.54–568.1	20
Moderate COPD	227.95 ± 96.85	70.1–384.06	20
Smoker controls	127.99 ± 54	57.11–226	20
Nonsmoker controls	49.3 ± 33.7	0–111.25	20

The table shows statistical values of Granzyme B (pg/ml) in different groups studied.

Table 4 Comparison of Granzyme B (pg/ml) between patients and controls.

Groups	Mean ± SD	Range	Mean diff.	*t*	*p*
Patients	287.08 ± 118.43	70.1–568.1	198.43	10.91	0.00
Controls	88.65 ± 59.68	0–226			

The table shows that Granzyme B levels were significantly higher in patients than controls.

Table 5 Comparison of Granzyme B between severe and moderate COPD.

Groups	Mean ± SD	Range	Mean diff.	*t*	*p*
Severe COPD	346.21 ± 109.76	167.45–568.1	118.264	5.38	0.00
Moderate COPD	227.946 ± 96.85	70.1–384.06			

The table shows that Granzyme B levels were significantly higher in severe COPD patients than those with moderate disease.

lymphocytes and Natural Killer cells in COPD subjects and asymptomatic controls. In blood there was a significant increased expression of Granzyme B in cytotoxic T lymphocytes in COPD than in healthy controls. Most circulating NK cells expressed Granzyme B, with the median fluorescence intensity of staining increased in both COPD groups and asymptomatic smokers. Hodge et al. [4] also studied the percentage of T cells expressing Granzyme B in bronchoalveolar lavage. There was a significant correlation between Granzyme B expression in BAL and apoptosis of bronchial epithelial cells. As regards the role of Granzyme B in COPD severity, the results in the present study show that Granzyme B values were significantly higher in severe COPD (GOLD III) than in moderate (GOLD II) patients ($p < 0.001$) (Table 5). These results agree with Vickerman et al. [13]. The main objective of the study done by Vickerman et al. [13] was to assess the relation between plasma Granzyme B level and the severity of COPD. Plasma Granzyme B was measured in 100 COPD patients (mean age 67 ± 8; mean FEV1% 47 ± 23%) by ELISA; 50 with GOLD II disease and 50 with GOLD III and IV disease. They detected increasing plasma Granzyme B levels in more advanced cases of COPD (GOLD III and IV) compared to less severe cases. Urbanowicz et al. [12] studied the cytotoxic activity of NK cells in smoker COPD patients, healthy smokers and healthy nonsmokers. In this study, proportions of NK cells expressing Granzyme B were significantly higher in COPD patients than in healthy controls. Also the proportions of same cells were significantly higher in healthy smokers than in nonsmokers. They also studied the correlation between the proportions of NK cells expressing Granzyme B and FEV1% where an inverse relation was detected between them. This inverse relation supports our result in which Granzyme B was significantly higher in patients with severe disease (GOLD III) than those with moderate disease (GOLD II) ($p < 0.001$) (Table 5). Results of Urbanowicz et al. [12] also support our finding of a significant inverse relation between FEV1% and Granzyme B was detected in patients ($p < 0.001$) and smoker controls ($p < 0.05$) (Table 7). A significant inverse relation was also detected between FEF25–75% and Granzyme B in patients ($p < 0.001$) and smoker controls ($p < 0.05$) (Table 8) indicating that Granzyme B is not only related to disease severity but also related to small airway disease. Contrary to our results, Morissette et al. [7] studied Granzyme B in T lymphocytes derived from blood and found no relation between Granzyme B and COPD severity. Twenty-nine subjects matched for age and sex were included in their study: nine were active smokers with evidence of emphysema and airflow limitation defined according to the GOLD criteria (FEV1 < 80% predicted value, FEV1 /FVC < 70% predicted value, and reversibility ⩽ 12% and

Table 6 Comparison of Granzyme B between moderate COPD and smoker controls.

Groups	Mean ± SD	Range	Mean diff.	*t*	*p*
Moderate COPD	227.946 ± 96.85	70.1–384.06	99.95	3.934	0.001
Smoker controls	127.99 ± 54	57.11–226			

The table shows that Granzyme B (pg/ml) level was significantly higher in patients with moderate COPD than smoker controls.

Table 7 Correlation between FEV1% and Granzyme B (pg/ml).

	R	*t*	*p*
Patients (All)	−0.698	6.013	0.000
Controls (All)	−0.519	3.739	0.001
Severe COPD	−0.499	2.446	0.025
Moderate COPD	−0.698	4.134	0.001
Smoker controls	−0.546	2.764	0.013
Nonsmoker controls	0.137	0.587	0.565

The table shows a significant inverse relation between FEV1% and Granzyme B (pg/ml) in all groups except non smoker controls where no significant relation was observed.

Table 8 Correlation between FEF25–75% and Granzyme B (pg/ml).

	R	*t*	*p*
Patients (All)	−0.711	6.235	0.000
Controls (All)	−0.700	6.044	0.000
Severe COPD	−0.668	3.804	0.001
Moderate COPD	−0.565	2.903	0.009
Smoker controls	−0.474	2.286	0.035
Non smoker controls	−0.048	0.203	0.841

The table shows a significant inverse relation between FEF25–75% and Granzyme B in all groups except for nonsmoker control group where no significant relation was observed.

Table 9 Correlation between age and Granzyme B.

	R	*t*	*p*
Patients (All)	0.674	5.626	0.000
Controls (All)	0.637	5.094	0.000
Severe COPD	0.472	2.271	0.036
Moderate COPD	0.595	3.142	0.006
Smoker controls	0.548	2.782	0.012
Non smoker controls	−0.118	0.505	0.619

The table shows a significant direct relation between age and Granzyme B (pg/ml) in patients and smoker control groups, but no significant relation was detected in nonsmoker control group.

200 ml after salbutamol inhalation), 10 were smokers with normal lung function, and 10 were nonsmokers with normal lung function. In subjects with airflow limitation, the presence of emphysema was further confirmed by a radiologist following CT scanning analyses and a diffusion capacity (DLCO) $<80\%$ predicted value for men and $<70\%$ predicted value for women. Subjects were excluded from the study if they have a history of cancer in the past five years, active liver, heart or kidney disease, diabetes or any other non-emphysematous pulmonary condition. There was no significant difference in Granzyme B levels between all groups.

Table 10 Correlation between smoking index and Granzyme B (pg/ml).

	R	*t*	*p*
Patients (All)	0.740	6.790	0.000
Severe COPD	0.694	4.085	0.001
Moderate COPD	0.662	3.749	0.001
Smoker controls	0.498	2.438	0.025

The table shows a significant direct relation between smoking index and Granzyme B (pg/ml) in patients, and smoker control group.

The present study detected a significant direct relation between age and Granzyme B both in patients ($p < 0.001$) and smoker control ($p < 0.05$) groups (Table 9). This result is supported by Janssens et al. [6] who mentioned that alveolar dilatation and impairment of gas exchange are linked with lung aging, and the expression of Granzyme B in type II pneumocytes and also stated that alveolar macrophages may represent one contributing mean by which alveolar cell apoptosis occurs. Also, in support to our result, Boivin et al. [2] mentioned that Granzyme B is associated with pathogenesis of other aging related diseases such as atherosclerosis and skin wrinkling. It is noted by Rahman and Adcock [10] that reactive oxygen species, such as those derived from cigarette smoke, drive inflammation via activation of redox-sensitive transcription factors, including nuclear factor-kB (NFkB) and activator protein-1, which in turn promote the upregulation of several pro-inflammatory molecules. The consequent enhanced inflammation would purportedly perpetuate the accelerated aging process through the activation of apoptotic pathways by mediators such as Granzyme B.

The classical epidemiologic studies of Fletcher and Peto [3] demonstrated that death and disability from COPD were related to an accelerated decline in lung function with time, with a loss of 50 to 100 ml in FEV1 per year, but even in healthy volunteers there is a loss of 20 ml per year with aging. Janssens et al. [6] demonstrated that physiologic aging of the lung is associated with dilatation of alveoli with an enlargement of airspaces and a decrease in gas exchange surface area, together with a loss of supporting tissue for peripheral airways (“senile emphysema”), resulting in decreased static elastic recoil of the lung and increased residual volume and functional residual capacity. This age-dependent loss of elastin fibers is similar to the loss of skin elasticity and wrinkling of the skin that occurs with age. Thus, aging lungs exhibit both structural and functional alterations [5].

In the present study, a significant direct relation was detected between Granzyme B values and smoking index in patients ($p < 0.001$) and smoker controls ($p < 0.05$) (Table 10).

Hodge et al. [4] studied Granzyme B and perforins in current and ex-smoker COPD. They measured granzyme levels intracellularly in T-cells isolated from blood and BAL as well as soluble levels in BAL. They found soluble levels to be increased in BAL both in smoker COPD as well as asymptomatic smokers which supports our result. They also found persistence of apoptotic activity induced by granzymes after smoking cessation.

On the other hand, Morissette et al. [7] found no difference between emphysematous smokers, normal smokers and normal nonsmoker subjects in expression of Granzyme B by T-lymphocytes.

Conflict of interest

The authors declare that there is no conflict of interest.

References

[1] P. Barnes, S. Shapiro, R. Pauwels, Chronic obstructive pulmonary disease: molecular and cellular mechanisms, Eur Respir J 22 (2003) 672–688.
[2] W. Boivin, R. Cruz, H. Zhao, P. Rauniyar, C. Walker, M. McManus, et al, Abstract 5488: Granzyme B contributes to extracellular matrix degradation and advanced atherosclerotic plaque formation, Circulation 118 (2008), S-559-b.
[3] C. Fletcher, R. Peto, The natural history of chronic airflow obstruction, Br Med J 1 (1977) 1645–1648.
[4] S. Hodge, G. Hodge, J. Nairn, M. Holmes, P.N. Reynolds, Increased airway granzyme b and perforin in current and ex-smoking COPD subjects, COPD 3 (2006) 179–187.
[5] K. Ito, Does lung aging have an impact on chronic obstructive pulmonary disease?, J Organ Dysfunction 3 (2007) 204–220
[6] J. Janssens, J. Pache, L. Nicod, Physiological changes in respiratory function associated with ageing, Eur Respir J 13 (1999) 197–205.
[7] M. Morissette, J. Parent, J. Milot, Perforin, granzyme B, and FasL expression by peripheral blood T lymphocytes in emphysema, Respir Res 8 (2007) 62.
[8] D. Ngan, S. Vickerman, D. Granville, The possible role of granzyme B in the pathogenesis of chronic obstructive pulmonary disease, Ther Adv Respir Dis 3 (2009) 113–129.
[9] R. Pincus, A. McPherson, Henry's Clinical Diagnosis and Management by Laboratory Methods, vol. 22, Elsevier Saunders, 2011, pp. 30–34.
[10] I. Rahman, I. Adcock, Oxidative stress and redox regulation of lung inflammation in COPD, Eur Respir J 28 (2006) 219–242.
[11] E. Rogot, Z. Hrubec, Trends in mortality from chronic obstructive pulmonary disease among U.S. veterans: 1954 to 1979, Am Rev Respir Dis 140 (3 Pt. 2) (1954) S69–S75.
[12] A. Urbanowicz, R. Lamb, I. Todd, M. Corne, C. Fairclough, Enhanced effector function of cytotoxic cells in the induced sputum of COPD patients, Respir Res 11 (2010) 76.
[13] S. Vickerman, H. Zhao, Y. Li, D. Ngan, P. Hiebert, D. Granville, et al, Abstract A2941: characterization of granzyme b protein expression in blood from COPD patients, Am Thorac Soc Int Conf Abstr 10 (2009) 1164.