V M
VIA MEDICA

Monika Kosacka[1], Irena Porębska[1], Anna Korzeniewska[1], Renata Rubinsztajn[2], Marcin Grabicki[3], Renata Jankowska[1], Halina Batura-Gabryel[3], Ryszarda Chazan[2]

[1]Chair and Department of Pulmonology and Lung Cancer, Wroclaw Medical University, Poland
[2]Department of Internal Diseases, Pneumonology and Allergology, Medical University of Warsaw, Poland
[3]Department of Pulmonology, Allergology and Respiratory Oncology, University of Medical Sciences, Poznan, Poland

Serum levels of apoptosis-related markers (sFasL, TNF-α, p53 and bcl-2) in COPD patients

The authors declare no financial disclosure.

Abstract

Introduction: Taking into account important role of apoptosis in COPD pathogenesis, we wanted to asses the serum levels of markers involved in apoptosis regulation, including apoptosis inducers such as TNF-α, sFasL or p53 protein and apoptosis inhibitor bcl-2 and, in addition, to compare these markers with selected COPD parameters.

Material and methods: In 181 patients (60 women) with COPD (age was 62.2 ± 9.37 years; FEV_1% 55.2 ± 19.98 %) and in 29 controls (11 women), serum levels of TNF-α, sFasL, p53 and bcl-2 were evaluated by the enzyme-linked immunosorbent assay (ELISA) method.

Results: In COPD patients the mean sFasL level was 0.092 ± 0.077 ng/ml and mean TNF-α level was 2.911 ± 3.239 pg/ml. There were no differences in serum sFasL and TNF-α in COPD patients and control group. TNF-α and sFasL did not correlate with COPD parameters such as FEV_1%, BMI, RV% (percentage of predicted value of residual volume) or BODE. Although we tried to evaluate bcl-2 and p53 protein serum levels with two different tests, measurable levels of bcl-2 were only detected in 15 patients and p53 in only 3 patients. Bcl-2 values were from 0.418 to 11.423 ng/ml and p53 from 90.772 to 994.749 pg/ml.

Conclusions: We didn't observe any differences in serum levels of pro- and antiapoptotic markers in COPD patients and the control group or correlations between the markers studied and COPD parameters.

Key words: apoptosis, bcl-2, COPD, p53, sFasL, TNF-α

Introduction

The role of apoptosis in the pathogenesis of COPD is complicated and still remains underexplained, which is why many aspects of this process form the main topic of most recent studies.

The majority of studies concentrate on apoptosis of structural cells. Segura-Valdez et al. presented that first of all endothelial cells from capillaries and arterioles exhibited patterns of apoptosis. Less frequently these authors also revealed apoptotic alveolar epithelial cells, interstitial and inflammatory cells in lung tissue of COPD patients [1]. Other authors proved markers of apoptosis, including caspase-3, Bax and Bad in emphysematous lungs [2]. Hodge et al., after assessing cells obtained from bronchial brushing and bronchoalveolar lavage revealed increased apoptosis in former and current smokers with COPD compared to the non-smoking control groups [3]. Some authors indicated that deregulation of granulocyte apoptosis could lead to sustained inflammation [4]. The most recent studies concen-

trate on the importance of apoptosis outside the lungs. Increased apoptosis of the skeletal muscle is responsible for the skeletal muscle atrophy [5]. Moreover apoptosis interacts with other pathomechanisms involved in the development of COPD such as proteinase-antiproteinase imbalance, inflammation and oxidative stress [6].

One of the major pathways involved in the regulation of programmed cell death is death receptor ligation. Apoptosis can be activated in response to extracellular signals and is mediated by binding members of the tumor necrosis factor family (e.g. Fas Ligand and TNF-α) to death receptors on the cell surface [6].

The P53 tumor suppressor gene and its product p53 protein possess many biological functions, one of them is the ability to induce apoptosis in response to cellular stress such as DNA damage, hypoxia or oncogene activation. P53 stimulates a wide network of signals that act through two major apoptotic pathways. The activation of extrinsic apoptotic pathway by p53 can be performed through the induction of genes encoding transmembrane proteins. One of these proteins is the cell-surface receptor Fas. P53 could also induce Fas mRNA expression [7, 8].

The apoptosis inhibitor bcl-2 prevents mitochondrial permeability transition pore opening and the release of apoptogenic proteins from mitochondria. On the one hand, bcl-2 may also block the p53-mediated apoptosis [9, 10] and, on the other hand, protein p53 plays a critical role in regulating of Bcl-2 family proteins [11].

The importance of the Fas/FasL system in the regulation of apoptosis is well known. The Fas transmembrane receptor is one of the death receptors and belongs to the TNF superfamily. The Fas receptor is activated by binding to its Fas ligand [11].

Taking into account the important role of apoptosis in COPD pathogenesis, we wanted to test the hypothesis that the development of COPD could be associated with alterations in serum levels of markers involved in apoptosis regulation, including apoptosis inducers such as TNF-α, sFasL or p53 and apoptosis inhibitor bcl-2.

Material and methods

Patients

A total of 181 patients with COPD were enrolled in the study. The group examined comprised 121males and 60 females. All patients were in stable disease (no during exacerbation). The mean age was 62.2 ± 9.37 years. COPD was diagnosed on the basis of the Global Initiative for Chronic Obstructive Lung Disease criteria. Mean $FEV_1\%$ was 55.20% ± 19.98. The majority of subjects were overweight and obese with mean BMI 29.16 ± 7.37. 17 patients were in stage IV, 57 in stage III, 88 in stage II and 19 in stage I of COPD. Almost all patients were current or former smokers.

COPD patients had many concomitant diseases: 140 had hypertension, 40 diabetes, 30 stable ischemic heart disease and 2 had a previous stroke history. All patients with co-morbidities received standard treatment according to international recommendations In majority of cases hypertension was treated with ACE-inhibitors, usually in combination with drugs from other groups as diuretics or calcium blockers. Diabetes treatment constituted in almost all patients oral hypoglycemic agents. Only one patient received insulin treatment.

A control group constituted 29 healthy subjects, including 11 females. Mean age was 49.48 ± 13.68 and mean BMI 29.56 ± 3.81.

Measurement of serum levels: TNF-α, sFasL, p53 and bcl-2

Blood samples were collected from fasting subjects in the morning. After centrifugation for 10 minutes at 1467 RCF, the serum was removed and stored at $-80^{\circ}C$. The serum levels of the markers assessed were measured using the enzyme-linked immunosorbent assay (ELISA) method. The following ELISA kits were used:

— for TNF-α: Human TNF-α Quarantikine High Sensitivity Kit- R&D Systems. Minimum detectable dose (MDD) ranged from $0.038-0.191$ pg/ml, mean MDD 0.106 pg/ml.
— for p53 protein two different kits were used: Human p53 Platinum ELISA eBioscience (the limit of detection 0.33 U/ml) and p53 pan ELISA ROCHE (the lower limit of detection is 9 pg/ml).
— for bcl-2 protein two different kits were used: Human bcl-2 Platinum ELISA eBioscience and Human bcl-2 ELISA Biovendor (limit of detection is 0.5 ng/ml).
— for sFasL protein two different kits were used: sFasL Platinum ELISA eBioscience (the limit of detection 0.07 ng/ml) and Human sFasL R&D Systems (MDD ranged from $1.01-8.05$ pg/ml, mean MDD was 2.66 pg/ml).

The tests were performed according to the manufacturer's specifications. We used the ELISA microplate reader from MRXe Dynex Technologies.

Table 1. TNF alpha and sFasL serum levels in the groups examined

	COPD I n = 19	COPD II n = 88	COPD III n = 57	COPD IV n = 17	All COPD patients	Control	p
TNF alpha pg/ml	2.474 ± 2.87	2.529 ± 1.55	3.23 ±3.42	4.44 ± 7.14	2.911 ± 3.23	2.550 ± 1.48	NS
sFasL ng/ml	0.090 ± 0.08	0.098 ± 0.08	0.080 ± 0.06	0.084 ± 0.06	0.092 ± 0.07	0.071 ± 0.02	NS

Statistical analysis

Statistical analysis was performed using the CSS Statistica software for Windows (version 5.0). Spearman's test was used to assess the relationship between two variables and the Mann-Whitney U test to compare values between two groups.

Differences between samples were considered significant at $p < 0.05$.

The work has been approved by the appropriate ethical committee related to the institutions (The Bioethical Committee in Warsaw).

The study was a part of the grant from The National Centre for Research and Development: "Chronic obstructive pulmonary disease (COPD) -systemic disease, the biggest threat of XXI century (No. 13003406/2009)

Results

In COPD patients, the mean sFasL level was 0.092 ng/ml ± 0.077. We didn't observe differences in sFasL levels between COPD patients and the control group (p = 0.956). The mean values of sFasL in the different stages of COPD are shown in Table 1. We didn't demonstrate any correlations between sFasL serum levels and selected parameters as BMI, BODE, FEV_1 or RV% (Table 2).

In COPD patients, the mean TNF-α level was 2.911 ± 3.239 pg/ml. We didn't find differences between TNF-α levels in COPD patients and the control group (p = 0.391). We observed that the average TNF concentration increased with the severity of the COPD, however the differences did not reach statistical significant levels (p = 0.288) (Table 1). We also didn't observe correlations between TNF-α serum levels and selected parameters (Table 2).

Moreover we didn't find differences in sFasL (p = 0.620) and in TNFalpha levels (p = 0.547) between both genders.

Although we tried to evaluate bcl-2 serum levels with two different tests, measurable levels were only detected in 15 patients. The values ranged from 0.418 to 11.423 ng/ml. It was a very heterogonous group of patients. Percentage of FEV_1 predicted value (FEV_1%) ranged in these

Table 2. Correlation coefficients between sFasL and TNF alpha serum levels and selected parameters

	sFasL		TNF alpha	
	r_s	p	r_s	p
BMI	0.106	NS	0.121	NS
BODE	−0.034	NS	0.108	NS
FEV₁%	0.036	NS	−0.056	NS
RV%	0.067	NS	0.305	NS

patients from 25% to 76% and BMI from 18.6 to 44.8.

Similar to bcl-2, two tests were used for the p53 measurement. We only detected p53 protein in 3 serum samples, all of which were from females. The range of values was 90.772−994.749 pg/ml. Two of the three 3 patients had low BMIs: 16.7 and 18.6, low FEV_1% 35 and 37% and 3−4 exacerbations every year in their medical history. The third patient had higher FEV_1% —74%, BMI 20,2 and 1 exacerbation per year.

Discussion

As mentioned in the introduction, the majority of studies concentrate on examining the role of apoptosis on cells or tissue. In the lung tissue of COPD smokers and non-COPD smokers, Siganaki et al. examined apoptosis markers in lung tissue using the western blot and immunochemistry. These authors proved increased p53 expression in type II pneumocytes in COPD patients, but there were no differences in p53 expression in alveolar macrophages and in lymphocyte-like cells. However, expression of bcl2 didn't differ between the groups studied. This imbalance between pro-apoptotic p53 and anti-apoptotic bcl2 in type II pneumocytes may indicate enhanced apoptosis in the alveolar epithelium of COPD patients [12].

We only detected p53 protein in serum samples from 3 females. Among them 2 of the 3 patients were underweight , had low FEV_1% and many exacerbations per year. This could sugge-

sts that the serum p53 level may also be helpful in better understanding of the role of apoptosis in pathogenesis of COPD progression, however this group was too small for any conclusions to be drawn. We realize that p53 is an intracellular protein and it's in sera evaluation of non neoplasmatic subjects could be disputable. However, a few studies indicate its importance in different non-neoplasmatic disorders, including COPD. Targowski et al. compared p53 concentrations in 3 groups: patients with non small cell lung cancer, COPD and control group [13]. In this study p53 concentration was measured using ELISA method and the same ROCHE test as in our study. These authors demonstrated increased serum concentration of p53 protein in 34% of patients with moderate and severe COPD (without any data about comorbidites). Moreover, they observed a higher prevalence of p53 protein in the serum of COPD patients compared with healthy subjects [13]. Dincer et al. demonstrated that p53 serum levels were higher in acute myocardial infarction patients compared to the control group. These authors also observed a time-dependent decrease in p53 levels. Such a relationship could suggest, that p53 may be a marker of apoptosis after myocardial infarction [14]. In addition, it was demonstrated that expression of proapoptotic genes including p53 was higher in unstable than in stable atherosclerotic plaques [15]. These findings in ischemic heart disease could suggest that apoptosis process may increase in COPD exacerbation comparing with stable disease. Unfortunately we were not able to test this hypothesis in our study, because all patients examined were in stable disease, but two of 3 patients with detected p53 had many exacerbations in medical history.

Rumora et al. analyzed the expression of Bcl-2 and Bax in the leucocytes using the western blotting. These authors demonstrated that expression of Bcl-2 was significantly decreased, especially in COPD smokers, but also in COPD ex-smokers and healthy smokers as compared with healthy non-smokers [16]. According to our knowledge, bcl-2 serum levels were not evaluated in COPD patients. Some studies showed differences in bcl-2 serum levels in lung cancer as well as in non-neoplasmatic diseases, which suggest its important role in the pathogenesis of many disorders. It has been shown that in the majority of patients with advanced lung cancer (even in 96%) bcl-2 serum levels were increased and bcl-2 levels were higher in lung cancer subjects than in the control group [17]. In addition, lupus nephritis was associated with glomerular expression and increased serum levels of bcl-2, which suggests its role in glomerular injury [18]. In addition, patients with multiple sclerosis in the active phase had higher bcl-2 levels than the control group or patients in the inactive phase [19].

The interpretation of TNF-alpha serum levels in the examined group is much more complicated. TNF-alpha, originally described as a factor produced by the endotoxin stimulated macrophages, is now one of the most extensively studied cytokines. TNF-alpha is not only one of the apoptosis mediators, but it is also a powerful proinflammatory cytokine [20]. TNF-alpha was often examined in COPD. Some studies suggest that this cytokine is involved in the development of cachexia in COPD patients [21]. Some authors also indicate a difference between local and systemic TNF-alpha production. It has been shown, that the sputum cells of patients with COPD produced less TNF-alpha than the control group and this could lead to impaired local defense. However, these authors didn't observe differences in TNF-alpha production by blood cells between the COPD group and the control group [22]. Some studies suggest that serum TNF-alpha could correlate with steroid use and $FEV_1\%$ [23]. This could be in accordance with our results indicating for higher TNF levels to be achieved by patients with more advanced COPD.

We didn't find any changes in sFasL levels between COPD patients and the control group or in the different COPD stages: similar observations were described by other authors. Takabatake et al. didn't find any differences in serum levels of sFasL or plasma levels of sFas. These authors didn't observed any correlations between sFasL or sFas levels and clinical variables in COPD patients either [24]. Yasuda et al. demonstrated that plasma sFas levels increased in severe COPD, however these authors didn't observe changes in sFasL levels between COPD patients and the control group [25].

We are aware, that our study has several limitations. First of all, group examined and control group were not good matched especially in age, what could have influence on TNF-alpha analysis. It is well known that TNF-alpha levels increase with age [26]. In addition many COPD patients were with concomitant diseases. This could also have influence on analyzed parameters [14].

Conclusions

We didn't observe differences in serum levels of pro- and antiapoptotic markers in COPD patients and the control group or correlations between the markers studied and COPD parameters.

Conflict of interest

References:

1. Segura-Valdez L, Pardo A, Gaxiola M, Uhal BD, Becerril C, Selman M. Upregulation of gelatinasas A and B, collagenases 1 and 2, and increased parenchymal cell death in COPD. Chest 2000; 117: 684−694.
2. Imai K, Mercer BA, Schulman LL, Sonett JR, D'Armiento JM. Correlation of lung surface area to apoptosis and proliferation in human emphysema. Eur Respir J 2005; 25: 250−258.
3. Hodge S, Hodge G, Holmes M, Reynolds PN. Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. Eur Respir J 2005; 25: 447−454.
4. Haslett C. Granulocyte apoptosis and its role in the resolution and control of lung inflammation. Am J Respir Crit Care Med 1999; 160: S5−11.
5. Plataki M, Tzortzaki E, Rytila P et al. Apoptotic mechanisms in the pathogenesis of COPD. Int J Chron Obstruct Pulmon Dis 2006; 1: 161−171.
6. Demendts IK, Demoor T, Bracke KR et al. Role of apoptosis in the pathogenesis of COPD and pulmonary emphysema. Respir Res 2006; 30: 53.
7. Haupt S, Berger M, Goldberg Z, Haupt Y Apoptosis-the p53 network. J Cell Sci 2003; 116: 4077−4085.
8. Shen Y, White E. p53-dependent apoptosis pathways. Adv Cancer Res 2001; 82: 55−84.
9. Reed JC, Jurgensmeier JM, Matsuyama S. Bcl-2 family proteins and mitochondria. Biochim Biophys Acta 1998; 1366: 127−137.
10. Gustafsson AB, Gottlieb RA. Bcl-2 family members and apoptosis, taken to heart. Am J Physiol Cell Physiol 2007; 292: C45−51.
11. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol 2007; 35: 495−516.
12. Siganaki M, Koutsopoulos AV, Neofytou E et al. Deregulation of apoptosis mediators p53 and bcl-2 in lung tissue of COPD patients. Respir Res 2010; 11: 46. doi: 10.1186/1465-9921-11-46.
13. Targowski T, Janda P, Owczarek W et al. Evaluation of occurrence frequency of circulating p53 protein in serum of patients with chronic obstructive pulmonary diseases and non-small cell lung cancer. Pol Merkur Lekarski 2010; 28: 265−267.
14. Dincer Y, Himmetoglu S, Bozcali E, Vural VA, Akcay T. Circulating p53 and cytochrome c levels in acute myocardial infarction patients. J Thromb Thrombolysis 2010; 29: 41−45. doi: 10.1007/s11239-009-0328-0.
15. Rossi ML, Marziliano N, Merlini PA et al. Different quantitative apoptotic traits in coronary atherosclerotic plaques from patients with stable angina pectoris and acute coronary syndromes. Circulation 2004; 110: 1767−1773.
16. Rumora L, Milevoj L, Popovic-Grle S, Barisić K, Cepelak I, Grubisić TZ. Levels changes of blood leukocytes and intracellular signaling pathways in COPD patients with respect to smoking attitude. Clin Biochem 2008; 41: 387−394. doi: 10.1016/j.clinbiochem.2007.
17. Tas F, Duranyildiz D, Oguz H et al. The value of serum Bcl-2 levels in advanced lung cancer patients. Med Oncol 2005; 22: 139−143.
18. Fathi NA, Hussein MR, Hassan HI, Mosad E, Galal H, Afifi NA. Glomerular expression and elevated serum Bcl-2 and Fas proteins in lupus nephritis: preliminary findings. Clin Exp Immunol 2006; 146: 339−343.
19. Sakai T, Inoue A, Kohn CS Osame M. Serum levels of apoptosis-related molecules in patients with multiple sclerosis and human T-lymphotropic virus Type I-associated myelopathy. Interferon Cytokine Res 1999; 19: 999−1004.
20. Petrescu F, Voican SC, Silosi I. Tumor necrosis factor-alpha serum levels in healthy smokers and nonsmokers. Int J Chron Obstruct Pulmon Dis 2010; 9: 217−222.
21. Di Francia, Barbier D, Mege JL, Orehek J. Tumor necrosis factor-alpha levels and weight loss in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1994; 150: 1453−1455.
22. Dentener MA, Louis R, Cloots RH, Henket M, Wouters EF. Differences in local versus systemic TNFalpha production in COPD: inhibitory effect of hyaluronan on LPS induced blood cell TNFalpha release. Thorax 2006; 61: 478−484.
23. Liu SF, Chin CH, Wang CC, Lin MC. Correlation between serum biomarkers and BODE index in patients with stable COPD. Respirology 2009; 14: 999−1004. doi: 10.1111/j.1440-1843.2009.01608.x.
24. Takabatake N, Nakamura H, Inoue S et al. Circulating levels of soluble Fas ligand and soluble Fas in patients with chronic obstructive pulmonary disease. Respir Med 2000; 94: 1215−1220.
25. Yasuda N, Gotoh K, Minatoguchi S et al. An increase of soluble Fas , an inhibitor of apoptosis, associated with progression of COPD. Respir Med 1998; 92: 993−999.
26. Bruunsgaard H, Skinhoi P, Pedersen AN, Schroll M, Pedersen BK. Ageing, tumour necrosis factor-alpha (TNF-alpha) and atherosclerosis. Clin Exp Immunol 2000; 121: 255−260.