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Serum from patients with chronic obstructive pulmonary disease promotes proangiogenic behavior of the vascular endothelium

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Abstract. – OBJECTIVE: It has been documented that COPD is a risk factor for lung cancer. In COPD patients, changes in lung angiogenesis – a critical process in the development of lung cancer – have been poorly investigated. We aimed to determine whether serum from COPD patients could promote the proangiogenic capabilities of endothelial cells *in vitro*.

PATIENTS AND METHODS: The research was carried out using sera from COPD patients and healthy volunteers, endothelial cells EA.hy926, and bronchial epithelial cells. The concentration of angiogenic molecules was quantified using ELISA tests. The proliferation and migration of EA.hy926 were tested using fluorescence-based methods. Tube formation was analyzed with a commercially available assay.

RESULTS: Sera from COPD patients and conditioned media generated by epithelial cells exposed to these sera stimulate proliferation, but not migration, of EA.hy926. This coincided with increased tube formation in both experimental regimens. The sera from COPD patients contained increased levels of CCL2, CCL21, and HGF, whereas the conditioned media generated by epithelial cells treated with these sera exhibited increased levels of CCL2, CCL21, CXCL8, FGF, and sICAM-1. The concentration of angiogenic markers in the sera and conditioned media, and their effect on the behavior of the endothelium were independent of smoking status (COPD and controls), stage of obstruction, and disease group (COPD).

CONCLUSIONS: The increased incidence of lung malignancy in COPD patients may be associated, at least to some extent, with the direct and indirect proangiogenic activity of their sera (via alterations in the secretome of epithelial cells).

Key Words
Angiogenesis, Endothelial cells, COPD, Lung cancer.

Introduction

Chronic obstructive pulmonary disease (COPD) is a very prevalent condition with many comorbidities, including lung cancer[1,2]. The estimated risk of developing lung neoplasm in COPD patients ranges from 1.23 to as high as 6.81 in the study population[2-4]. The coexistence of both diseases seems to be associated with more aggressive tumor outcomes[5]. One of the most significant mechanisms linking COPD with lung cancer is chronic inflammation with the activation and recruitment of many types of cells, and the release of a wide array of cytokines, many of which may show angiogenic activity[6].

Angiogenesis, the process of formation of new blood vessels from pre-existing vessels, is a fundamental step in primary tumor progression and dissemination. At least four important steps, including basement membrane injury, migration, proliferation of endothelial cells, and the vascularization (tube formation) process are critical parts of this phenomenon[7]. The key players in the angiogenesis are the cellular elements (endothelial cells, epithelial cells, fibroblasts, immune cells, cancer cells) and acellular elements (extracellular matrix) of a tumor microenvironment[8,9]. Numerous chemokines that alter tumor pathophysiology are major regulators of angiogenesis[10]. Some of these, including CCL2, CCL21, vascular endothelial growth factor (VEGF), and CXCL8 may be important in certain aspects of tumor progression in non-small-cell lung cancer[11]. Although the role of angiogenesis in lung cancer is clear, data on this process in COPD are sparse. What is currently known

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about angiogenesis in COPD is that this process is involved in vessel remodeling and thereby in the alteration of bronchial and pulmonary circulation[12]. Accumulating evidence suggests that inflammation is a turning point in initiating vessel remodeling. The exertion of very large amounts of angiogenic chemokines (enhanced additionally through hypoxia), such as CCL2, CCL21, CXCL8, fibroblast growth factor (FGF), VEGF, soluble intercellular adhesion molecule-1 (sICAM-1), may play a significant role in angiogenic stimuli in COPD[2,13-15].

Taking into account that many of these markers are common to both COPD and lung cancer, and also participate in angiogenesis, it seems to be highly probable that this process is a connecting link between these two diseases.

In this report, we determine the proangiogenic capabilities of COPD serum and its capacity to stimulate angiogenesis in a model resembling the tissue milieu of the respiratory system, plausibly leading to increased progression of lung cancer. We suggest that the serum may exert its activity directly (by intensifying endothelial cell proliferation) or indirectly (by altering the secretory phenotype of bronchial epithelial cells), leading to the increased motility of endothelial cells.

Patients and Methods

Patients

The study was performed using serum samples obtained from COPD patients (n = 32; female n = 10, male n = 22; mean age 63.69 ± 7.12 years), diagnosed according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria with all stages of airflow obstruction (1: mild, n = 4; 2: moderate, n = 5; 3: severe, n = 12; 4: very severe, n = 11), and with all disease groups based on combined disease assessment (A, n = 4; B, n = 4; C, n = 3; D, n = 21), both current (n = 13) and former smokers (n = 16) with a history of more than 20 pack-years, as well as nonsmokers (n = 3) and healthy volunteers (n = 20; female n = 4, male n = 16; mean age 64.40 ± 8.24 years): current smokers (n = 6), former smokers (n = 7) and nonsmokers (n = 7). Patients were recruited with stable disease status (without exacerbations in the six weeks prior to the study), during planned hospitalizations at the Department of Pulmonology, Allergology, and Respiratory Oncology, or during planned visits to the Outpatient Clinic. The exclusion criteria were an inability to perform the spirometry tests, a history of asthma, tuberculosis, lung interstitial diseases, pulmonary thromboembolism, and other chronic pulmonary diseases. A control group was recruited among patients' relatives and friends. The exclusion criteria for the controls included any pulmonary disease.

The serum samples were taken in a fasting state and centrifuged immediately after collection. The serum was then stored in aliquots at -80°C until required. Clinical evaluation was performed once written informed consent was obtained, with the approval of the Bioethics Committee at Poznań University of Medical Sciences for research on human subjects (consent number 979/12). The methods were carried out in accordance with the relevant guidelines and regulations.

Chemicals

Unless otherwise stated, all chemicals and plastics were from Sigma-Aldrich (St. Louis, MO, USA). Cell culture plastics were from Nunc (Roskilde, Denmark). The kits for analyzing the concentrations of CCL2, CCL21, CXCL8, FGF, hepatocyte growth factor (HGF), interleukin-6 (IL-6), sICAM-1, and VEGF were from R&D Systems (Abingdon, UK). The Angiogenesis Assay Kit was from Bio-Tech. (Minneapolis, MN, USA).

Cell Culture and Experimental Conditions

Human bronchial epithelial cells (HABECs) (PromoCell, Heidelberg, Germany) were cultured in a serum-free Airway Epithelial Cell Growth Medium, as recommended by the vendor. The medium was supplemented with bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone, epinephrine, triiodo-L-thyronine, transferrin, and retinoic acid. The cells were seeded at low density onto the culture dishes and allowed to attach for 24 h. The cells were then exposed to 10% serum from COPD patients and healthy volunteers for 48 h. After incubation, the cells were subjected to further analysis. In some experiments, the cells exposed to the serum were carefully washed and subjected to a fresh, serum-free medium for 24 h, in order to generate a conditioned medium (CM) for the immunoenzymatic assays.

EA.hy926 endothelial cells were obtained from ATCC (Rockville, MD, USA) and cultured

in DMEM medium supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), glucose (4500 mg/L), sodium pyruvate (110 mg/L), and antibiotics.

Measurement of Endothelial Cell Proliferation

Endothelial cell proliferation was examined using a Cell Proliferation Kit I (PromoKine; Heidelberg, Germany). In brief, EA.hy926 cells were seeded into culture dishes at low density (5×10^3 cells per well), allowed to attach for 2 h, and then growth-synchronized by serum deprivation for the next 4 h. Afterwards, the cells were exposed either to 10% serum from COPD patients and from healthy volunteers, or to the conditioned media generated by bronchial epithelial cells subjected to these sera (10%, for 48 h). After incubation, the endothelial cells were probed with (5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester, and CFDA-SE (5 μM, for 15 min at 37°C). The cells were then washed with culture medium and incubated for 5 min at 37°C to hydrolyze the free dye. Finally, the fluorescence of carboxyfluorescein, a product of CFDA-SE transformation, was recorded using a Synergy H1 spectrofluorimeter at excitation and emission wavelengths of 495 nm and 519 nm, respectively.

Measurement of Endothelial Cell Migration

The migration of endothelial cells through a polycarbonate membrane (8 μM pores) towards the chemotactic gradient generated by the 10% serum from COPD patients and from healthy volunteers, or the conditioned media generated by bronchial epithelial cells subjected to these sera (10%, for 48 h), was examined using ChemoTx chambers (Neuro Probe, Gaithersburg, MD, USA). In brief, endothelial cells were probed with calcein-AM (5 μM, 45 minutes, 37°C), and then 4×10^4 cells were suspended in serum-free growth medium and applied to the top side of the filter to form a hemispherical drop. Afterward, the cells were allowed to migrate toward the chemoattractant for 60 minutes at 37°C. After incubation, the membranes with cells that did not migrate were removed, and the intensity of fluorescence emitted by the cells that did migrate through the membranes was recorded using a Synergy H1 spectrofluorimeter at excitation and emission wavelengths of 485 nm and 535 nm, respectively.

Tube Formation Assay

Tube formation analysis was performed with a Cultrex *in vitro* Angiogenesis Assay Tube Formation Kit (Trevigen, Gaithersburg, MD, USA). In brief, EA.hy926 cells were grown in a 25 cm^2 tissue culture flask in Roswell Park Memorial Institute-1640 (RPMI-1640) medium with 10% fetal bovine serum (FBS) until they reached 90% confluency. Afterwards, the cells were serum starved for 24 h. On the day of the experiment, the plates were coated with Reduced Growth Factor Basement Membrane Extract (BME), and subsequently incubated at 37°C for 1 h. In the meantime, the endothelial cells were harvested and added (4×10^4) into RPMI-1640 medium enriched with the 10% serum from COPD patients or from healthy volunteers, or to the conditioned media generated by bronchial epithelial cells exposed to these sera (10% for 48 h). The cells were then incubated on the BME matrix at 37°C for 16 h. After incubation, the efficiency of tube formation was examined using an AxioVert. A1 light microscope (Carl Zeiss, Jena, Germany).

Statistical Analysis

The results were analyzed using GraphPad Prism v.5.00 software (GraphPad Software, La Jolla, CA, USA). The groups were compared using the Mann-Whitney test and Kruskal –Wallis analysis followed by Dunn's Multiple Comparison post-test, when appropriate. The results were expressed as means ± SEMs. Differences with a p-value < 0.05 were considered to be statistically significant.

Results

The Effect of Sera from COPD Patients and Healthy Volunteers and Conditioned Media From HABECs Treated with These Sera on the Proliferation and Migration of EA.hy926 Cells

The endothelial cells proliferated more efficiently after exposure to COPD serum than after incubation with serum from the control donors. There were no differences in the migration effect when the endothelium was stimulated through COPD or control serum. Endothelial cell proliferation was fueled more efficiently after exposure to the CM from HABECs treated with COPD serum than to the serum from controls. There were no differences in the migration effect when the endothelium was stimulated with

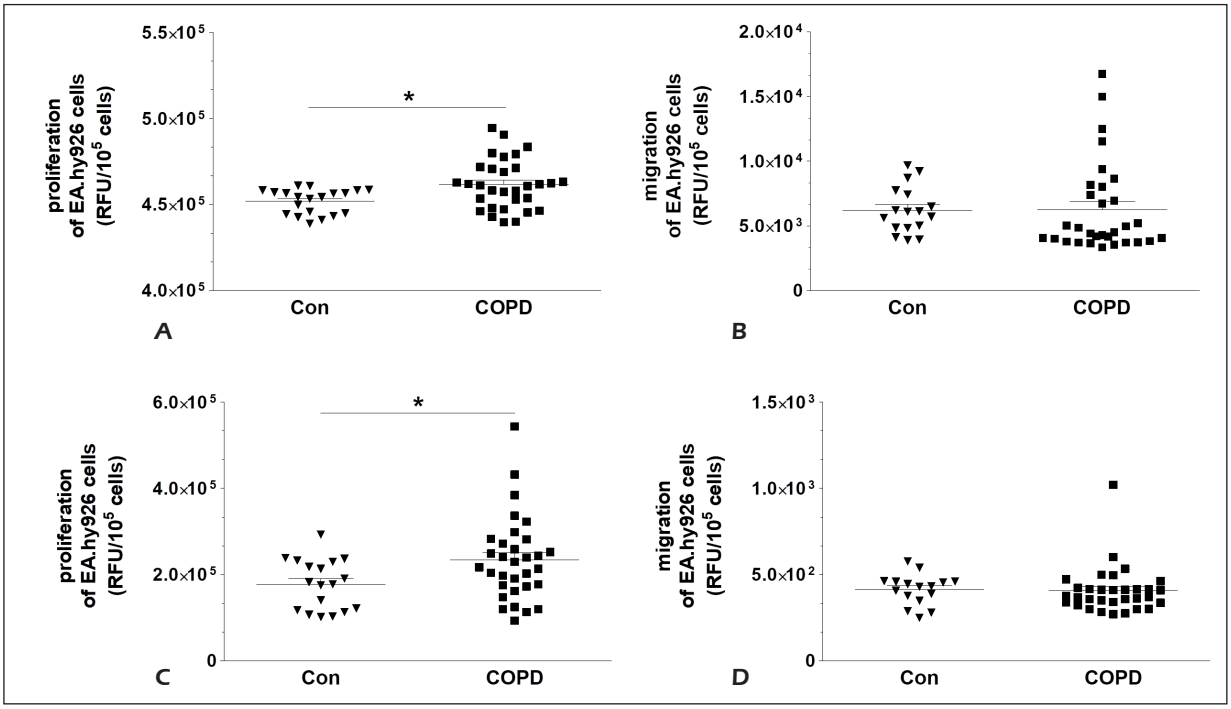

Figure 1. Effect of sera from COPD patients (COPD) and healthy volunteers (Con), and conditioned media from HABECs treated with these sera on the proliferation (**A**, **C**) and migration (**B**, **D**) of EA.hy926 endothelial cells. The results are expressed as means ± SEMs. HABECs: human bronchial epithelial cells; RFU: relative fluorescence units.

the secretome of HBECs treated with COPD or control serum (Figure 1). The effect of clinical factors, including smoking status, stage of obstruction, and disease group on the functional capabilities of endothelium were considered. The proliferation of EA.hy926 did not differ significantly after exposure to serum (Figure 2 ABC) or to CM from HABECs treated with serum (Figure 3 ABC) between smokers, former smokers, and nonsmokers (COPD and controls), as well as between stages of obstruction and disease groups (COPD patients). The proliferation of EA.hy926 cells was significantly more intense after exposure to serum from COPD current smokers in comparison to serum from the nonsmoking controls (Figure 2A). EA.hy926 cells proliferated more intensely after exposure to the serum of severely obstructed patients in comparison to the controls (Figure 2B). The intensity of the proliferation of EA.hy926 cells did not change when the endothelium was exposed to CM from HABECs treated with sera from patients with various smoking statuses, different stages of obstruction, and different disease groups (Figure 3).

Effects of Sera from COPD Patients and from Healthy Volunteers, and of Conditioned Media Harvested from HABECs Treated with These Sera, on the Tube Formation Process in EA.hy926 Cells

Researches using endothelial cells grown in a 3D environment formed *in vitro* by the Basement Membrane Extract (BME) revealed that the efficiency of the formation of tubular structures was visibly higher when the cells were maintained in sera from the COPD patients, or in the conditioned media generated by epithelial cells exposed to these sera, than in the presence of serum from healthy donors or the conditioned media from the epithelial cells subjected to the sera from these individuals (Figure 4).

Concentration of Soluble Proangiogenic Agents in Sera from Patients with COPD and from Healthy Volunteers

The concentrations of eight arbitrarily selected angiogenic agents, CCL2, CCL21, CXCL8, FGF, HGF, IL-6, sICAM-1, VEGF, were measured in the sera of COPD patients and healthy

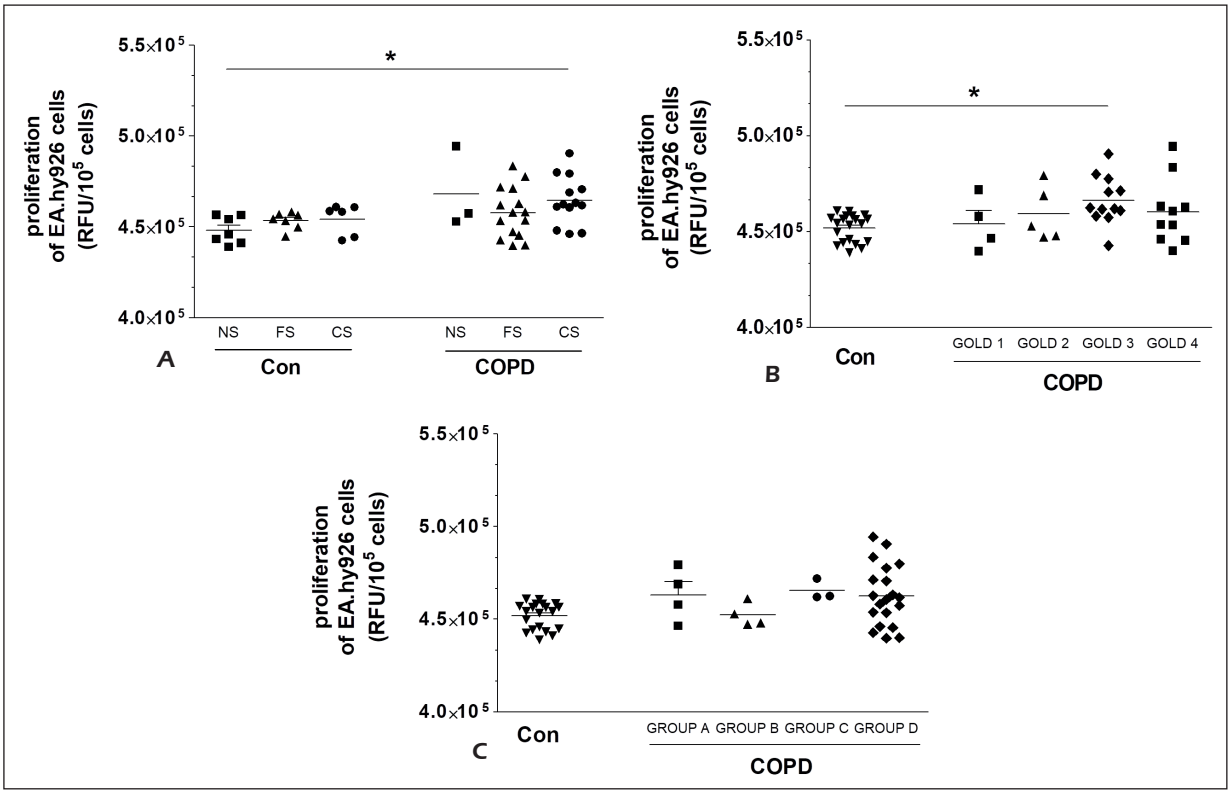

Figure 2. Effects of serum from COPD patients (COPD) and healthy volunteers (Con) on the proliferation of EA.hy926 endothelial cells in relation to smoking status (**A**), stage of obstruction (**B**), and COPD group (**C**). Results expressed as means ± SEMs. NS: nonsmokers, FS: former smokers, CS: current smokers; RFU: relative fluorescence units.

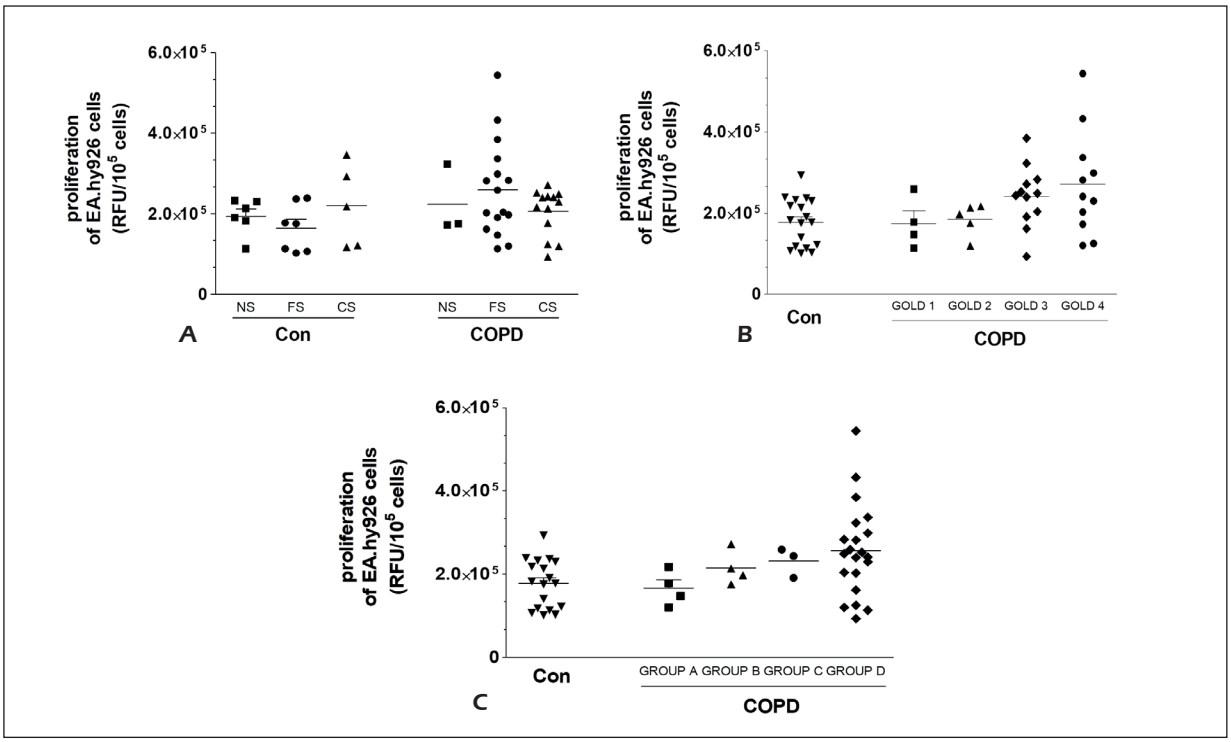

Figure 3. Effects of conditioned media from HABECs treated with sera of COPD patients (COPD) and healthy volunteers (Con) on the proliferation of EA.hy926 endothelial cells in relation to smoking status (**A**), stage of obstruction (**B**), and COPD group (**C**). Results expressed as means ± SEMs. NS: nonsmokers, FS: former smokers, CS: current smokers; RFU: relative fluorescence units.

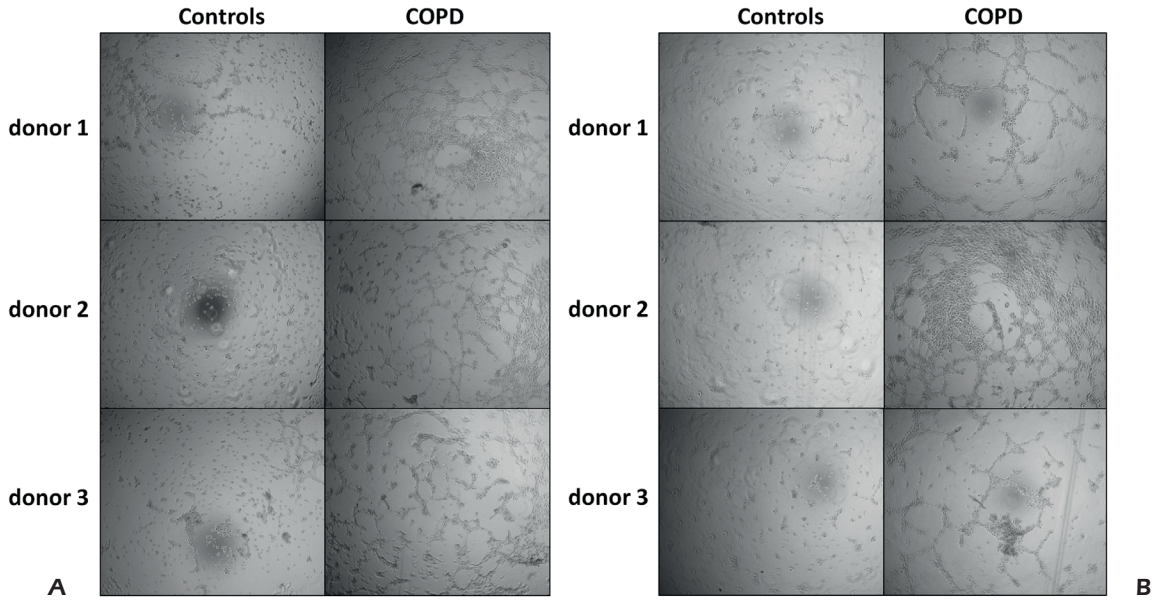

Figure 4. Analysis of tube formation in response to sera and conditioned media. Representative images of tube formation using endothelial cells exposed to sera from COPD patients and healthy controls (**A**), or to conditioned media harvested from HABECs treated with these sera (**B**). The experiment was performed with n = 20 from each group.

volunteers. Quantitative analysis using ELISA tests revealed that sera from COPD patients contained increased concentrations of CCL2, CCL21, and HGF, as compared to those from healthy individuals (Table I). The effect of clinical factors, including smoking status (COPD and controls), stage of obstruction, and disease group (COPD) on the concentrations of upregulated proangiogenic agents in the serum was determined (Table II). There were no significant differences in the concentrations of CCL2, CCL21, and HGF between current smokers, former smokers, and nonsmokers (COPD and controls), or between stages of obstruction and disease groups (COPD patients). The HGF concentration was higher in patients with very severe obstruction and from group D, as compared to the controls.

Table I. Concentration of proangiogenic agents in sera from patients with COPD and from healthy volunteers.

Soluble factor (pg/ml)	Control	COPD
CCL2	33.65 ± 8.85	99.38 ± 20.62*
CCL21	34.70 ± 2.68	37.44 ± 1.55*
CXCL8	ND	ND
FGF	29.05 ± 5.19	25.38 ± 6.44
HGF	215.90 ± 55.40	476.90 ± 53.22*
IL-6	9.40 ± 12.76	8.34 ± 2.25
sICAM-1	75.70 ± 20.51	142.30 ± 23.74
VEGF	47.50 ± 4.29	47.34 ± 6.10

The results are derived from analysis of the serum of 32 COPD patients and 20 controls, and are expressed as means ± SEMs. *$p<0.05$ *vs*. control; ND: undetectable.

Concentrations of Proangiogenic Agents in Conditioned Media Harvested from HBECs Exposed to Sera from COPD Patients and from Healthy Volunteers

The same group of angiogenic agents as in the case of the sera was quantified in the CM harvested from HABECs exposed to sera from the COPD patients and healthy volunteers. The results showed that the CM from epithelial cells that had undergone exposure to sera from the COPD patients were characterized by significantly increased levels of CCL2, CCL21, CXCL8, FGF, and sICAM-1, as compared to the CM generated by cells exposed to sera from the control donors (Table III). The effect of clinical factors, including smoking status (COPD and controls), stage of obstruction, and disease group (COPD) on the concentrations of upregulated proangiogenic agents in CM exposed to COPD sera and to the sera of healthy volunteers was examined (Table IV). There were no significant differences in the concentrations of CCL2, CCL21, CXCL8, FGF, and sICAM-1 when current smokers, former smokers, and nonsmokers were compared (COPD and controls), or between stages of obstruction and disease groups (COPD patients). CCL2 was signifi-

Table II. Concentration of proangiogenic agents in serum from patients with COPD, divided by smoking status, stage of obstruction, and disease group, and from healthy volunteers.

Soluble factor (pg/ml)	CCL2		CCL21		HGF	
	Control	COPD	Control	COPD	Control	COPD
Smoking						
Nonsmokers	15.00 ± 4.58	95.33 ± 76.29	36.71 ± 6.37	37.67 ± 3.48	174.0 ± 97.00	430.7 ± 75.86
Former smokers	54.00 ± 19.32	76.06 ± 21.90	37.14 ± 4.34	38.44 ± 2.50	335.4 ± 111.9	518.9 ± 83.69
Current smokers	31.67 ± 16.18	129.0 ± 40.16	29.50 ± 0.43	36.15 ± 2.21	125.3 ± 52.92	436.0 ± 81.55
GOLD	33.65 ± 8.85		34.70 ± 2.68		215.9 ± 55.40	
1		42.00 ± 14.70		42.50 ± 6.12		390.0 ± 90.27
2		172.2 ± 77.84		35.40 ± 2.42		438.8 ± 192.4
3		79.00 ± 32.57		37.83 ± 2.27		442.7 ± 57.52
4		109.4 ± 31.94		36.09 ± 2.99		563.3 ± 112.9*
COPD group	33.65 ± 8,85		34.70 ± 2.68		215.9 ± 55.40	
A		146.0 ± 95.81		34.75 ± 1.31		244.0 ± 79.68
B		132.8 ± 50.73		37.50 ± 3.52		452.5 ± 250.2
C		25.67 ± 10.11		49.33 ± 8.74		416.7 ± 107.5
D		94.67 ± 24.41		36.24 ± 1.73		534.6 ± 61.96*

The results are derived from analysis of the serum of 32 COPD patients and 20 controls, and are expressed as means ± SEMs. *p<0.05 vs. control; #p<0.05 difference between groups with different smoking status, stage of obstruction, or disease group.

cantly higher in CM exposed to COPD sera than in CM that had undergone exposure to serum from the controls and former smokers. Some of the examined markers (CCL2, FGF, sICAM-1) were upregulated in the CM after exposure to sera of COPD patients with severe obstruction, others (CCL2, CCL21) were upregulated after exposure to the sera of COPD patients with very severe obstruction, in comparison to the CM that had undergone exposure to sera from the controls. The same effect was noted for group D of the disease, where all angiogenic molecules, excluding CXCL8, were higher in the CM exposed to those sera than in the CM exposed to sera from the controls.

Table III. Concentration of proangiogenic agents in the conditioned media harvested from HBECs exposed to serum from COPD patients and from healthy volunteers.

Soluble factor (pg/ml)	Control	COPD
CCL2	43.45 ± 8.11	419.1 ± 102.7*
CCL21	699.9 ± 191.4	3082 ± 574.3*
CXCL8	15.10 ± 1.07	35.28 ± 6.79*
FGF	6.10 ± 0.46	8.72 ± 0.47*
HGF	ND	ND
IL-6	24.80 ± 1.54	23.78 ± 2.19
sICAM-1	22.55 ± 4.42	42.81 ± 3.64*
VEGF	52.05 ± 7.77	46.66 ± 3.92

The results are derived from analysis of CM from 32 COPD patients and 20 controls and are expressed as mean ± SEM. *p<0.05 *vs*. control; ND-undetectable.

Discussion

Numerous epidemiological studies have shown that COPD patients are at a higher risk of developing lung cancer[2-4,16]. Angiogenesis, the growth of new capillary blood vessels, is one of the most crucial steps in the effective progression of neoplasms[7], and also contributes to vessel remodeling in COPD patients[6]. Although the role of angiogenesis in both diseases has been examined, it has never been studied from the point of view of sharing involvement in the progression of lung cancer in COPD patients. In this report, we have focused on sera from COPD patients, which we have treated as an environment highly specific to this disease[17], and which has the capacity to stimulate angiogenesis in the respiratory system. The same experimental system has been used twice by our group, when we discovered that sera from COPD patients stimulate the migration of lung cancer in the CCL21-dependent manner[18], and that they induce a senescence-related phenotype in the bronchial epithelial cells, thereby promoting processes related to tumor invasiveness[19]. We here developed a model in which endothelial cells (EA.hy926, commonly used in experimental studies[20,21]) were subjected to the serum from COPD patients and healthy volunteers, and to a conditioned medium from the bronchial epithelial cells exposed to both kinds of serum. Analysis of the proliferation and migration of endothelial cells (the most important stages in tumor angio-

Table IV. Concentration of proangiogenic agents in the serum from patients with COPD divided by smoking status, stage of obstruction, and disease group, and from healthy volunteers.

Soluble factor (pg/ml)	CCL2		CCL21		CXCL8		FGF		sICAM-1	
	Control	COPD	Control	COPD	Control	COPD	Control	COPD	Control	COPD
Smoking										
Non-smokers	60.43 ± 21.16	571.0 ± 449.3	1060 ± 507.4	3794 ± 1974	16.00 ± 1.34	44.67 ± 32.17	6.14 ± 0.67	7.00 ± 1.15	17.71 ± 5.53#	33.67 ± 8.37
Former smokers	32.14 ± 7.14	553.9 ± 171.5*#	583.1 ± 189.9	4108 ± 984.0*	14.43 ± 2.49	22.38 ± 5.78	5.57 ± 0.81#	8.25 ± 0.70	24.14 ± 9.12	37.31 ± 4.10
Current smokers	36.83 ± 5.49#	218.2 ± 91.32	416.2 ± 58.50	1655 ± 412.7	14.83 ± 1.70	49.00 ± 12.94	6.67 ± 0.99	9.69 ± 0.65#	26.33 ± 8.91	51.69 ± 6.66#
GOLD	43.45 ± 8.11		699.9 ± 191.4		15.10 ± 1.07		6.10 ± 0.46		22.55 ± 4.42	
1		175.8 ± 25.71		2157 ± 623.6		16.00 ± 3.56		9.25 ± 0.85		40.00 ± 8.69
2		274.2 ± 189.6		2581 ± 851,1		55.80 ± 25.15		7.80 ± 0.58		43.80 ± 6.30
3		392.2 ± 140.0*		2419 ± 862.5		40.08 ± 12.40		9.16 ± 0.89*		48.67 ± 7.96*
4		602.8 ± 241.6*		4369 ± 1285*		27.73 ± 8.60		8.45 ± 0.90		37.00 ± 4.52
COPD group	43.45 ± 8.11		699.9 ± 191.4		15.10 ± 1.07		6.10 ± 0.46		22.55 ± 4.42	
A		110.0 ± 43.77		2488 ± 857.9		50.75 ± 32.42		9.25 ± 0.63		44.50 ± 9.95
B		519.3 ± 227.0		2677 ± 763.3		29.25 ± 14.63		8.00 ± 0.91		38.50 ± 4.79
C		94.33 ± 39.00		1164 ± 76.0		48.00 ± 31.05		10.00 ± 2.31		43.33 ± 13.25
D		505.3 ± 145.7*		3546 ± 831.3*		31.67 ± 7.38		8.57 ± 0.62*		43.24 ± 5.01*

The results are derived from analysis of CM from 32 COPD patients and 20 controls and are expressed as mean ± SEM. *p<0.05 vs. control; #p<0.05 difference between groups with various smoking status, stage of obstruction or COPD group.

genesis[22]) grown in these environments showed that both the sera from COPD patients and the conditioned media from the epithelial cells exposed to these sera proliferated more efficiently. At the same time, there was no difference in the efficiency of migration between the COPD and control groups. Clinical factors (smoking status in COPD patients and controls, obstruction stage and disease group in COPD patients) did not significantly change the intensity of proliferation; the conclusion is, therefore, that this is associated only with the disease process. The next piece of evidence of proangiogenic activity of the serum was the increased efficiency of tube formation; this test was the equivalent of angiogenesis in 3D conditions[23]. The formation of tube-like structures was much more pronounced in cultures where the endothelial cells had been incubated with sera from COPD or with conditioned media from epithelial cells subjected to these sera. The experiments with tube formation confirmed that there were changes in the functional capabilities of the endothelial cells and a switch into invasive behavior characteristic of progressive tumors[21,22]. Because our 2D experiments showed that the sera from COPD patients stimulated only the proliferation of endothelial cells, increased tubulogenesis was probably also a similar effect. At the moment, we can only speculate as to why the sera from the COPD patients and the CM generated in response to these sera promoted the proliferation while leaving migration unchanged. It can be hypothesized that this activity is associated with the specific pattern of changes that occurred within the soluble angiogenic agents present in the sera and the CM. In the next stage of the study, we attempted to verify this scenario. To this end, we quantified eight arbitrarily selected angiogenic agents in samples of sera and CM. These include CCL2, CCL21, CXCL8, FGF, HGF, IL-6, sICAM-1, and VEGF. It should be emphasized that CCL2, CCL21, HGF, sICAM-1, and CXCL8 are known to be upregulated in COPD patients[2,14,15,18], and that CCL21, VEGF, CXCL8 have already been linked to the development of lung cancer[18,24,25]. Our findings showed that the sera from COPD patients contained increased levels (compared to the control group) of CCL2, CCL21, and HGF. In the case of the conditioned media from the bronchial epithelial cells subjected to these sera, our analysis revealed increased concentrations of CCL2, CCL21, CXCL8, FGF, and sICAM-1. It is worth emphasizing that some of the angiogenic agents found in serum can upregulate those in CM, which makes them potential promoters of epithelial cell secretome. The expression of ICAM-1 in human lymphatic endothelial cells was mediated by CCL2 in a time-dependent manner up to 18 h[26]. In fact, the soluble form of ICAM-1 (sICAM-1) is known to be a product of cell-bound shedding[27], which means that there may be a direct link between increased serum CCL2 and increased sICAM-1 in the CM.

The results that we obtained are in line with those of other groups. For example, CCL2, which is an important biomarker related to COPD[14], showed a significant increase in patients with the emphysema phenotype, as compared to chronic bronchitis[28]. At the same time, there is strong evidence that the prevalence of lung cancer is higher in emphysematous COPD patients[1]. Taking into account the above data, the increased level of CCL2 in COPD serum in our study may be an important risk factor for the progression of lung cancer. CXCL8 is, in turn, an important cytokine with documented roles in the initiation, progression, angiogenesis, and metastasis of many tumors, including lung cancer[25]. When circulating in the blood, CXCL8 may increase the risk of lung cancer by as much as 45%-86%, and it is thereby an important prognostic factor[29,30]. The key property of all the examined molecules and a potential element linking COPD with lung cancer is COPD's contribution to the angiogenesis process[21,29,31-34]. The upregulation of angiogenic markers in COPD patients may be the consequence of hypoxemia and thus tissue hypoxia[35]. Most of the COPD patients we examined were in advanced stages of the disease, which means that they were possibly exposed to hypoxemia during exacerbations and periodic worsening of breathing. It is intriguing that the level of VEGF – the most important angiogenic marker[36,37] – did not increase, and was lower in the COPD sera and CM than in the controls. This could be associated with a commonly used treatment that can modify VEGF production. Glucocorticosteroids can reduce VEGF secretion by structural cells, and β2-agonists limit the vascularity of airways in asthma *in vivo*[38,39]. On the other hand, the low level of VEGF may be significant for the occurrence of emphysema, which is a risk factor for lung cancer[40,41]. In the study of Kasahara et al[42], VEGF concentration was lower in patients with emphysema than in those without. Moreover, blocking the VEGF receptor in rats resulted in the development of emphysema-like changes in the

lung parenchyma[43]. It is worth noting that VEGF is the regulator of the first phase of endothelial cell migration during angiogenesis (chemotaxis), along with another two mechanisms, haptotaxis, and mechanotaxis. The ineffective migration process in our experiment may be associated with the lowered level of VEGF[44]. Analysis of the angiogenic agents upregulated in the sera and CM did not show any differences between smokers, former smokers, and past smokers (COPD and controls), or between patients with mild, moderate, severe, or very severe obstruction, or between groups A, B, C, or D (COPD). On the basis of this data, which confirms the lack of effect of clinical factors on the proangiogenic activity of COPD serum, it is highly speculative that this is only the consequence of the specific inflammation assigned to obstructive disease. These results are in line with those we obtained in our proliferation experiment, which excluded the effects of the examined clinical components. The changes between the levels of some molecules (HGF in serum; CCL2, CCL21, FGF, and sICAM-1 in CM) and controls in the advanced stages of obstruction or disease category is probably the result of these groups having the largest numbers of patients; their selection was random and compatible with the distribution of patients in the population[45].

Conclusions

We suggest that serum in COPD patients may be a potent inducer of angiogenic reactions in vascular endothelial cells, which under certain (probably patient-specific) conditions may predispose to the development of lung cancer. Our results are worthy of further research, in which altered angiogenesis would be confronted with a precise follow-up based on the analysis of cancer incidence in the study group. Another important question that should also be answered is whether serum from COPD patients may be used for diagnostic purposes in COPD patients, in the manner described here and in our previous reports[18,19].

Contribution Statements:

BKK contributed to the design and implementation of the research, interpreted the data, wrote the manuscript, and is the corresponding author; JMP supervised the development of the work; EM performed cell culturing and measurements of endothelial cell proliferation and migration; NM performed ELISA tests; MM dealt with tube formation; AT supervised the project; HBG supervised the project; KK conceived the original idea, supervised the development of work, and helped to evaluate and edit the manuscript. All authors edited and approved the final version of the manuscript.

Conflict of Interests:

The authors declare that they have no conflict of interests.

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