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Endothelin, Endothelial Cells, and von Willebrand Factor in Peripheral Blood of COPD in the Context of Hypoxemia

Endotelina, komórki śródbłónka oraz czynnik von Willebranda a hipoksemia u pacjentów z przewlekłą obturacyjną chorobą płuc

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Abstract

Background. The pulmonary endothelium plays a significant role in the pathobiology of chronic obstructive pulmonary disease (COPD), a chronic inflammatory disease of the respiratory tract in which increased activity or even damage to the endothelium due to chronic inflammation, hypoxemic stress, and/or angiogenesis may occur. The authors hypothesized that patients with COPD have increased concentrations of endothelin (ET-1) and von Willebrand factor (vWF) and/or circulating endothelial cell (CEC) counts, markers of endothelial activity and/or damage.

Objectives. The aim of the study was to assess the concentrations of ET-1 and vWF and the number of CECs in the peripheral blood of COPD patients compared with healthy individuals and to investigate if hypoxemia or ventilation disturbances affect these parameters.

Material and Methods. The study included 20 patients with COPD in stage III (according to GOLD) as well as 15 healthy individuals as controls. ET-1 and vWF were assessed by an immunoenzymatic method. Immunofluorescence using the specific monoclonal endothelium antibody CLB-HEC 19 MoAb was applied to evaluate CECs.

Results. The concentration of ET-1 was higher in COPD patients than in controls, medians being 1.2 (range: 1.0–3.8) and 0.44 (0.2–0.7) pg/ml ($p < 0.001$), respectively. The number of CECs was higher in COPD patients than in controls (mean: 1.5, range: 0.6–3.1 vs. mean: 0.44, range: 0.32–1.11 cells/ml, respectively, $p < 0.001$). The concentration of vWF in COPD patients did not differ from that in the controls. There was positive correlation between CEC count and the concentrations of both the studied factors ($p < 0.05$). The number of CECs in the patients peripheral blood correlated significantly with the pO_2 value ($R_s: 0.36, p < 0.05$).

Conclusions. The increased number of CECs and concentration of ET-1 in peripheral blood may result from endothelial damage or activation occurring in COPD (Adv Clin Exp Med 2007, 16, 3, 395–401).

Key words: COPD, endothelial cells, endothelin, vWF.

Streszczenie

Wprowadzenie. W przebiegu p.o.ch.p. dochodzi do aktywacji śródbłónka naczyniowego i uwolnienia jego mediatorów, m.in. endoteliny 1, a równocześnie destrukcji z powodu towarzyszącego chorobie przewlekłego niedotlenienia. Markerami uszkodzenia śródbłónka są: czynnik von Willebranda i krążące komórki śródbłónka (CEC).

Cel pracy. Ocena tych czynników w p.o.ch.p. w kontekście zaburzeń wentylacyjnych oraz hipoksemii.

Materiał i metody. Zbadano 20 pacjentów w III stadium p.o.ch.p. (według GOLD) oraz 15 osób zdrowych. ET-1 i vWF oznaczano we krwi metodą immunoenzymatyczną, CEC oceniano za pomocą metody immunofluorescencyjnej z wykorzystaniem swoistego przeciwciała monoklonalnego CLB-HEC 19. Wszyscy chorzy mieli oznaczone wskaźniki wentylacyjne płuc oraz mierzone gazy krwi arterializowanej włośniczkowej.

Wyniki. Stężenie ET-1 we krwi obwodowej było wyższe w grupie pacjentów z p.o.ch.p. niż u osób zdrowych, mediana (Me) wynosiła odpowiednio: Me = 1,2 (1,0–3,8) i Me = 0,44 (0,2–0,7); $p < 0,001$. Liczba krążących komórek śródbłónka była również większa w grupie pacjentów z p.o.ch.p.: Me = 1,5, (0,6–3,1) i Me = 0,44, (0,32–1,11); $p < 0,001$. Nie wykazano różnicy stężeń czynnika vWF między grupami. Stwierdzono korelację między wartością

CECs i stężeniem obu pozostałych czynników ($p < 0,05$). Liczba CEC we krwi obwodowej korelowała z wartościami stężenia parcjalnego pO_2 w arterializowanej krwi włośniczkowej ($R_s = 0,36$, $p < 0,05$).

Wnioski. Ocena krążących komórek śródbłonna i ET-1 w p.o.ch.p. może być jednym ze wskaźników uszkodzenia naczyń w tej chorobie (*Adv Clin Exp Med* 2007, 16, 3, 395–401).

Słowa kluczowe: p.o.ch.p., komórki śródbłonna naczyniowego, czynnik von Willebranda.

Inflammation is an important issue in the pathogenesis of chronic obstructive pulmonary disease (COPD). Cellular inflammatory infiltrates in COPD are dominated by neutrophils, T lymphocytes ($CD8^+$), and macrophages [1, 2]. Recruitment of circulating cells to peripheral tissue (including the bronchi) occurs as an effect of adhesion between these cells and vascular endothelium. Thus the endothelium is not only a mechanical barrier between the blood stream and the vessel wall, but it also plays a role in immunological response, leukocyte mobilization to peripheral tissue during inflammation, and smooth muscle tonus in the respiratory tract. The markers of endothelial activity are endothelin (ET-1) and von Willebrand factor (vWF), secreted from the endothelium. ET-1 is a peptide controlling vascular tonus able to initiate bronchoconstriction as well as proliferation of smooth muscles [3, 4]. Higher concentrations of ET-1 have been demonstrated in bronchial biopsies and BAL (bronchoalveolar lavage) fluids from asthmatic patients and COPD patients than in healthy individuals [5, 6]. vWF is also produced by endothelium and is also a marker of endothelial dysfunction in several vascular and non-vascular diseases. In acute or chronic hypoxemia the concentration of vWF in the peripheral blood increases. In some diseases, such as pulmonary hypertension, the vWF concentration is a recognized indicator of vascular damage and appears together with an increased number of circulating endothelial cells (CECs) [8].

CECs have recently been evaluated and established as a novel marker of endothelial damage in a variety of disorders. Changes in the circulating population of endothelial cells can be found in hypertension, renal diseases, cytomegalovirus infection, septic shock, sickle cell anemia, and unstable angina and in renal transplant recipients [9–11]. Woywodt et al. recently established that elevated CEC number correlates with disease activity in antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis [12]. Though these conditions are diverse in their etiology and pathobiology, all share a common history of vascular injury.

The aim of this study was to investigate the markers of endothelial activity and damage, i.e. CEC counts and the concentrations of ET-1 and vWF in the peripheral blood of patients with

COPD. It is believed that such a complex experiment has not yet been published.

Material and Methods

The study involved 20 COPD patients in stage III (according to GOLD 2003) with a mean age of 69.5 ± 9.9 years. Disease duration and history of smoking are given in Table 1. Fifteen healthy individuals, 9 men and 6 women with a mean age of 31.5 ± 9 years, were also included in the study as controls. Concomitant hypertension, diabetes, or glomerulonephritis were considered exclusion criteria in both groups. The members of the two groups were comparable with regard to gender, but not age, the mean age in the control group being much lower. Every patient gave his/her informed consent before inclusion to this study and the protocol was approved by the Bioethics Committee of the Silesian Piasts University of Medicine in Wrocław.

Measurement of CECs in Whole Blood

The procedure of counting circulating endothelial cells was performed according to van Mourik et al. [13] and Sbarbati et al. [14] with the present authors' modification. The method for identifying CECs in blood samples involved Percoll density gradient centrifugation combined with immunofluorescent staining of subfractions.

Venous blood samples of 4.5 ml were drawn from the antecubital vein. The first 2 ml of the blood sample collected in the syringe was discarded as potentially polluted with endothelial cells from locally punctured tissues. The 4.5 ml of blood obtained was carefully mixed with 0.5 ml of 3.8% sodium citrate and gently stirred.

The blood samples were dripped over Percoll layers of 1.060 g/ml and centrifuged at $1000 \times g$ at room temperature for 10 minutes. The upper cell layer was collected and centrifuged at $400 \times g$ for 10 minutes. The supernatant was discarded and the cellular pellet was resuspended in phosphate buffered saline (PBS) and 2% bovine serum albumin (BSA), sedimented on glass coverslips by means of a cytocentrifuge (SHANDON, England), and processed for immunofluorescence. The cov-

Table 1. Demographic data, airway parameters, and blood gases of the COPD patients**Tabela 2.** Dane demograficzne, wyniki badań czynnościowych płuc i gazometrii u chorych na p.o.ch.p.

	COPD patients (Chorzy na p.o.ch.p.)
Demographic features (Dane demograficzne)	
sex M/F	12/8
age \pm SD (years)	69.5 \pm 9.9
duration of symptoms (years)	13.7 \pm 5.7
history of smoking (pack-years)	38.1 \pm 11.4
Airway parameters (Wyniki badań czynnościowych płuc)	
FEV ₁	0.94 \pm 0.18
FVC l	1.38 \pm 0.36
FEV ₁ /FVC%	60.32 \pm 12.70
Blood gases (Wyniki gazometrii)	
pO ₂ mm Hg	59.66 \pm 11.68

Values are expressed as the mean \pm SD.

Wartości wyrażono jako średnie \pm SD.

erslips had been previously coated with 1% gelatin fixed with 0.5% glutardialdehyde.

Immunofluorescence

Every cell sample from the Percoll gradient was divided and sedimented on three coverslips. Two were randomly chosen and stained with MoAb CLB-HEC19. The third coverslip was stained with rabbit serum as a control. Each staining was performed for 1 hour at room temperature. The slides were washed, fixed with methanol for 10 minutes, incubated with MoAbs, and washed once more. Then the slides were stained with fluorescein isothiocyanate (FITC)-conjugated polyvalent goat antimouse Ig (1 : 80) (DACO, Denmark) and embedded in 87% glycerol in PBS (9 : 1) and 1 mg/ml para-phenylene diamide (pH 8.6).

The slides with fluorescent CECs were screened using a fluorescence microscope. Each slide previously stained with CLB-HEC19 MoAbs was screened for the presence of immunofluorescent ECs. The sum of fluorescent cells on two slides (2/3 of the actual CEC number in one blood sample) was calculated and the result expressed as the number of circulating ECs in one ml of whole blood.

Monoclonal Antibody

The CLB-HEC19 MoAb is one of a series of monoclonal antibodies raised against cultured HUVECs (human umbilical vein ECs). CLB-

HEC19 recognizes an EC-specific plasma membrane protein with an apparent molecular weight of 100,000. This antibody does not react with blood cells, including erythrocytes, lymphocytes, monocytes, granulocytes, and blood platelets, or with cultured fibroblasts and smooth muscle cells. The immunohistochemical analysis of multiple tissue samples shows also that MoAb CLB-HEC19 exclusively stains ECs.

The Measurement of vWF Plasma Concentration

vWF was assayed in plasma using a commercially available immunoenzymatic kit (Asserachrom, Diagnostica Stago, France). In this assay, rabbit antibody fixed on titration microplates binds vWF. Later, the remaining antigen determinants of vWF bind to another rabbit antibody (peroxydase labeled). This complex produces a color reaction in the presence of orthophenylenediamine and H₂O₂. The result is interpreted as the percentage of the normal vWF concentration.

The Measurement of Endothelin Plasma Concentration

Endothelin was assayed in plasma using a commercially available immunoenzymatic method (Biomedica Gruppe, Austria) according to the manufacturer's protocol. In short, endothelin is bound in plasma by a polyclonal antibody previously attached in the polystyrene wells of the titration microplates. Bound endothelin is further detected by a mouse monoclonal antibody that in turn is bound by a peroxidase-labeled anti-mouse IgG. The complex polyclonal antibody – endothelin-labeled IgG is finally detected in an enzymatic reaction of peroxidase with the provided substrate. The color intensity after the reaction is directly proportional to the endothelin concentration.

Evaluation of gasometric parameters was carried out in capillary blood.

Statistical Analysis

The statistical significance of differences between groups regarding demographic data was determined using the Mann-Whitney nonparametric *U*-test. A *p* value < 0.05 was considered statistically significant. The concentrations of ET-1 and vWF and endothelial cell counts are expressed as medians and ranges. To compare values, the Spearman's rank (*R_s*) correlation test was used for assessing correlation. A *p* value < 0.05 were accepted as statistically significant.

Results

The demographic features, blood gases, and airway parameters of COPD patients are presented in Table 1. It was found that the concentration of ET-1 and the number of CECs in peripheral blood were higher in COPD patients than in healthy individuals, whereas the activity of vWF did not differ in the two groups (Table 2). The calculated correlation was significantly positive between CEC count and vWF plasma level and between CEC count and ET-1 level. Correlation between CEC count and partial oxygen pressure in capillary blood existed as well; CEC count in the patients' peripheral blood correlated significantly with the pO_2 value ($R_s: 0.36, p < 0.05$) (Fig. 1a). However, there was no correlation between ET-1 and the pO_2 value (Fig. 1b).

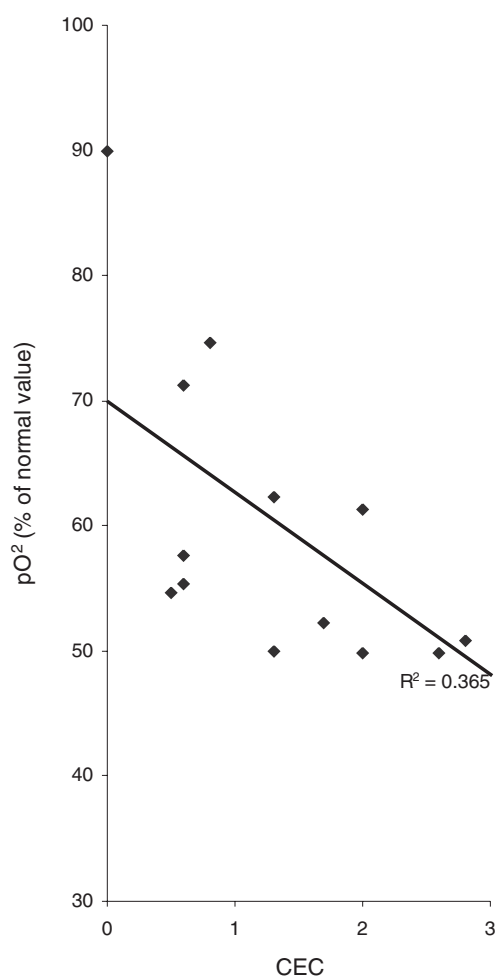


Fig. 1a. Correlation between partial oxygen pressure and CECs in COPD patients

Ryc. 1a. Korelacja między parcjalnym ciśnieniem tlenu a krążącymi komórkami śródbłonna u pacjentów chorych na p.o.ch.p.

Discussion

These experiments demonstrated an increased number of CECs in the peripheral blood of patients with COPD compared with healthy controls. The interpretation of this finding is difficult at the moment, especially since to the authors' knowledge there are no other published experiments addressing this issue. The demonstrated phenomenon does not have to be specific to patients with COPD and can be observed in patients with other respiratory diseases or in elderly persons. In arterial hypertension, chronic glomerulonephritis, and renal transplant rejection, increased CEC counts have been attributed to damage to the endothelium resulting from hypertensive stress and vascular stretching [8, 11, 15, 16]. It appears that the mechanism leading to an increased number of CECs in COPD is distinct from that in hypertension or renal transplant rejection and mainly depends on chronic hypoxemia and, probably, angiogenesis. The reason for the increase in angiogenesis needs to be further elucidated; however, it seems possible that chronic hypoxia in COPD can represent a causative or at least a conducive agent. The inverse correlation between oxygen partial pressure and CEC count in COPD patients supports this opinion.

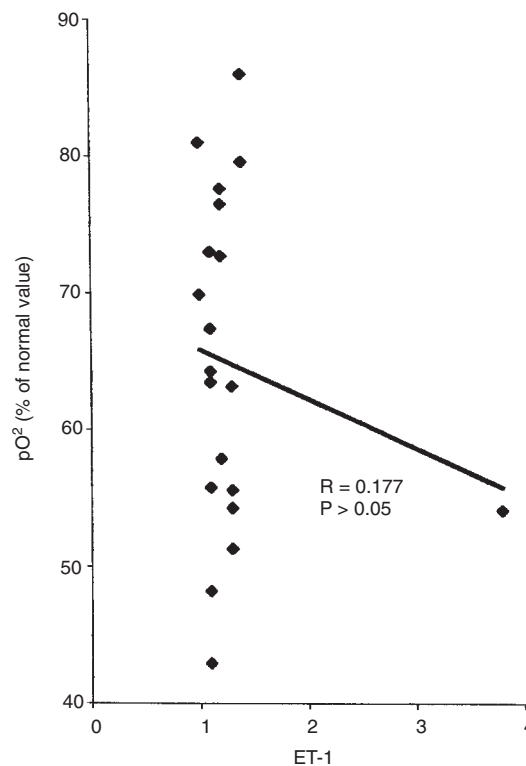


Fig. 1b. Correlation between partial oxygen pressure and ET-1 in patients with COPD

Ryc. 1b. Korelacja między parcjalnym ciśnieniem tlenu a endoteliną-1 u pacjentów chorych na p.o.ch.p.

Table 2. Endothelin (ET-1), endothelial cells (CECs), and von Willebrand factor (vWF) in the COPD patients and controls**Tabela 2.** Stężenie endoteliny (ET-1), czynnika von Willebranda i liczba krążących komórek śródbłonna u chorych na p.o.ch.p.

		ET-1 pg/ml	CECs N/ml	vWF %
COPD patients (Chorzy na p.o.ch.p.)	N	20	14	14
	Me	1.2	1.5	107.5
	Min	1.0	0.6	92
	Max	3.8	3.1	132
	$X \pm SD$	1.38 ± 0.61	1.59 ± 0.89	110.48 ± 13.92
Control patients (Grupa kontrolna)	N	15	15	15
	Me	0.44	0.44	97.85
	Min	0.2	0.32	60
	Max	0.7	1.10	110
	$X \pm SD$	0.45 ± 0.14	0.48 ± 0.3	102.8 ± 32.2
<i>p</i> value (Istotność statystyczna)	< 0.001	< 0.05	> 0.05	

N – number of patients.

Me – median.

 $X \pm SD$ – mean \pm standard deviation.

N/ml – number of CEC per ml.

N – liczba pacjentów.

Me – mediana.

 $X \pm SD$ – średnia \pm odchylenie standardowe.

N/ml – liczba krążących komórek śródbłonna na ml.

Hoshino and co-workers demonstrated that vascularization of the bronchi in asthmatics is much greater than in healthy controls [17]. Also in this case the exact mechanism remains obscure. Investigating the molecular basis for the phenomenon they found that the concentrations of VEGF (vascular endothelial growth factor) and the VEGF receptor were much higher in bronchial biopsies from patients with asthma than in normal controls. Additionally, the number of cells expressing mRNA for VEGF inversely correlated with bronchial caliber and hyperresponsiveness. In conclusion, VEGF seems to play an important role in angiogenesis and, consequently, in airway remodeling in asthma. This evidence indicates that the most probable answer to the question about the origin of the circulating endothelial cells is that they come partly from the process of angiogenesis. The first stage of this process is characterized by loosening of intercellular tight junctions, which allows the cells to migrate and create new blood vessels [18]. The cascade of intercellular reactions and CEC response occurring mainly during hypoxia has been described in detail by Michiels and co-workers [15].

In the present study it was not possible to identify the precise site of origin of the CECs in the COPD patients; the authors propose that one likely site is the pulmonary vasculature. This hypothesis is based on the observation that the CEC count correlates significantly with the severity of diseases (gasometric value) and in the patients of the present study the respiratory system, including the

pulmonary vascular bed, was the predominant site of the pathology. Boussat and co-workers investigated the expression of VEGF, a potent growth and permeability factor of CECs, on cultured human bronchial epithelial cells and showed a significant increase in VEGF expression after hypoxia. Increased levels of VEGF have been found to be associated with increased numbers of endothelial precursor cells in the peripheral circulation [19].

The increased number of CECs and its correlation with ET-1 concentration cast some light on the pathophysiology of bronchial narrowing in COPD. An elegant experiment by Celik and co-workers showed that in patients with pulmonary hypertension, concentrations of ET-1 in pulmonary arterial blood correlated with partial oxygen pressure [20]. At the same time, the ET-1 level in the peripheral blood was lower and correlation was not observed. This confirms the thesis about local ET-1 production and release.

vWF is a marker not only of pulmonary, but also of peripheral endothelial dysfunction. Increased concentrations of vWF were observed in acute bronchitis and in COPD. Chambers and colleagues assumed that the vWF level can be a marker of a sub-clinical lung injury and may be used to predict faster impairment in pulmonary ventilation [21]. The concentration of vWF in plasma rises in patients with pulmonary, especially primary, hypertension. Lopes and co-workers observed in a group of such patients that high levels of vWF correlated with shorter survival time [16]. This indicates that vWF may be not only

a disease marker, but also a prognostic factor. They confirmed this hypothesis in a follow-up study showing a relationship between vWF concentration and FEV₁ decrease over a longer period of time. Ferroni and coworkers also found an increase in vWF in COPD patients compared with healthy controls, but this difference did not reach statistical significance [22]. In the present experiments, no significant differences between healthy controls and patients were found. What was found was positive correlation between vWF concentration and CEC count and between CEC count and endothelin concentrations.

Also found in the present study were a significant increase in the number of CECs and the ET-1 concentration in COPD patients as well as a highly positive correlation between the number of endothelial cells and endothelin and vWF levels, which are recognized markers of endothelial activity and/or damage. This indicates that in chronic obstructive diseases the vascular endothelium can be affected. It will take further investigation to determine the exact origins of the CECs and the relationship of the increased number of these cells in angiogenesis.

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