Agnieszka Urbaniak[1, 2], Marek Zięba[1], Anna Zwolińska[3], Urszula Szkudlarek[3], Maria Łuczyńska[3], Krzysztof Noweta[4], Sylwia Kwiatkowska[2]

[1]Provincial Complex of Healthcare Facilities "Centre for the Treatment of Lung Diseases and Medical Rehabilitation" in Lodz, Poland
Director: Janusz Kazimierczak, BSc
[2]Clinical Department of Pneumonology, Medical University of Lodz, Poland
Head: S. Kwiatkowska, MD, PhD, Professor of the Medical University of Lodz
[3]Department of Experimental and Clinical Physiology, Medical University of Lodz, Poland
Head: Prof. D. Nowak, MD, PhD
[4]Department of Pneumonology and Allergy, Medical University of Lodz, Poland
Head: Prof. P. Górski, MD, PhD

Comparison of selected markers of local and systemic inflammation in patients with community-acquired pneumonia and pneumonia co-existing with lung cancer

A study funded by the Medical University of Lodz, Poland, from internal grant 502-18-523

Abstract

Background: The aim of the study was to compare markers of local and systemic inflammation in patients with community-acquired pneumonia (CAP) and pneumonia co-existing with lung cancer.

Material and methods: We enrolled 17 patients with CAP (Group 1), 14 patients with pneumonia co-existing with lung cancer (Group 2), 24 patients with lung cancer (Group 3) and 16 healthy individuals (Group 4, the control group). We evaluated the concentrations of hydrogen peroxide (H_2O_2), vascular endothelial growth factor (VEGF) and tumour necrosis factor-alpha (TNF-α) in exhaled breath condensate (EBC) and serum concentrations of VEGF and TNF-α.

Results: We found significantly higher EBC concentrations of VEGF and TNF-α in patients with pneumonia co-existing with lung cancer than in CAP patients (317.83 $\pm$ 77.78 *v.* 30.20 $\pm$6.56 and 1.98 $\pm$ 0.13 *v.* 0.31 $\pm$ 0.05, respectively). Although the EBC concentration of H_2O_2 in patients with pneumonia co-existing with lung cancer was higher than that in CAP patients (0.96 $\pm$ 0.16 *v.* 0.66 $\pm$ 0.09), the difference was not significant ($p > 0.05$). In CAP patients, however, we observed significantly higher serum concentrations of both cytokines compared to patients with pneumonia co-existing with lung cancer (VEGF: 1112.62 $\pm$ 244.38 *v.* 392.9 $\pm$ 78.2; TNF-α: 2.6 $\pm$ 0.48 *v.* 1.6 $\pm$ 0.2).

Conclusions: Patients with pneumonia co-existing with lung cancer show a distinct tendency towards local oxidative stress and a significantly increased local response compared to CAP patients. On the other hand, systemic inflammation in patients with pneumonia co-existing with lung cancer is markedly reduced compared to patients with CAP.

Key words: pneumonia, lung cancer, hydrogen peroxide, vascular endothelial growth factor, tumour necrosis factor alpha

Pneumonol. Alergol. Pol. 2011; 79, 2: 90–98

Corresponding author: Agnieszka Urbaniak, MD, Provincial Complex of Healthcare Facilities "Centre for the Treatment of Lung Diseases and Medical Rehabilitation, Okólna St. 181, 91–520 Łódź, Poland; tel.: +48 42 617 7374; fax: +48 42 659 0416; e-mail: agnes.urbaniak@gazeta.pl

Received on 16 June 2010

ISSN 0867–7077

Introduction

Community-acquired pneumonia (CAP) is one of the most common infectious diseases. The incidence of CAP increases with age of a given population. Among individuals over the age of 70 the incidence rate reaches about 30 per 1000 inhibitants per year [1]. Although the course of CAP is often mild, about 20% of the patients still require hospitalisation, which renders this condition the sixth most common cause of death in developed countries despite the advances in antibiotic therapy [2, 3]. The development and course of inflammation is undoubtedly affected by the immune status, which in turn is determined, at least partially, by the patient's co-morbidities.

Multiple studies have demonstrated elevated levels of proinflammatory cytokines in patients with CAP—both locally, in the respiratory system, and systemically, in the blood. Bronchoalveolar lavage fluid (BALF) from patients with community-acquired bacterial pneumonia has been shown to contain, in addition to interleukin 1-beta (IL-1β) and IL-6, elevated levels of tumour necrosis factor-alpha (TNF-α) [4], while patients with severe pneumonia and acute respiratory distress syndrome (ARDS) were characterised by a systemic increase in the levels of IL-6, IL-8 and TNF-α [5].

Microorganisms that grow at the site of local inflammation are potent stimulators of the respiratory burst of phagocytes which generates enormous amounts of reactive oxygen species (ROS). Hydrogen peroxide (H_2O_2) formed in free-radical-related processes which has not been neutralised by the antioxidant system and has not undergone further transformations, collected in exhaled breath condensate (EBC), is a good measure of local oxidative stress. It has been shown to be markedly elevated in exhaled air both in patients with community-acquired bacterial pneumonia [6] and in patients with lung cancer. Many studies demonstrate an increased local oxidative stress, which is directly manifested by elevated exhaled H_2O_2 in patients with non-small-cell lung cancer (NSCLC) and indirectly manifested by increased lipid peroxidation in tumour-invaded tissues [7, 8].

While in patients with CAP mainly the damaging effects of ROS on pathogenic microorganisms are observed, their role in lung cancer patients is not so clear. ROS have been shown to play a role in carcinogenesis, both in the initiation and in the promotion phase [9]. Furthermore, thanks to its involvement in angiogenesis, a significant role in the spread of cancer is played by vascular endothelial growth factor (VEGF) [10]. Overexpression of VEGF in tumour tissue or elevated serum levels of this factor have been demonstrated in patients with breast, gastric, colorectal and lung cancers [11–14]. For this reason antiangiogenic agents that inhibit the activity of VEGF and block its receptors have been introduced for the treatment of cancer in the recent years [15]. The role of TNF-α in lung cancer patients continues to be investigated, as this cytokine has been shown to suppress tumours in some studies and to promote tumours in others: TNF-α may suppress clonal proliferation of tumour cells but it may potentially facilitate the pathway of carcinogenesis by increasing the formation of ROS, which are known to be mutagenic [16, 17].

Based on the changes occurring in the pro- and antioxidant systems, which are paralleled by changes in the levels of selected markers, we decided to evaluate local and systemic inflammation in patients with CAP compared to patients with pneumonia co-existing with lung cancer. We therefore evaluated:

— local oxidative stress by measuring the concentration of H_2O_2 in EBC,
— local inflammation by measuring the concentrations of VEGF and TNF-α in EBC,
— systemic inflammation by measuring the concentrations of VEGF and TNF-α in the serum,
— association between local and systemic inflammation and selected clinical parameters.

Material and methods

Patients' characteristics

We enrolled 55 patients hospitalised at the Clinical Department of Pneumonology, Medical University of Lodz, Poland, in 2008 and 16 healthy volunteers, all subjects were divided into four groups:

— The group of 17 patients with CAP (9 men and 8 women), mean age 60 ± 6 years, mean tobacco exposure 17 ± 3 pack-years, mean number of pulmonary fields affected by inflammation 2.12 ± 0.23.
— The group of 14 patients with pneumonia co-existing with lung cancer (8 men and 6 women), mean age 76 ± 8 years, mean tobacco exposure 74 ± 3 pack-years. This group included 10 patients with NSCLC (3 patients with stage IIIB and 7 with stage IV disease) and 4 patients with small-cell lung cancer (SCLC) (2 patients with limited disease [LD] and 2 with extended disease [ED]; 2 patients with stage IIIB and 2 with stage IV disease).

— The reference group consisting of 24 patients with lung cancer without co-existing pneumonia (16 men and 8 women), mean age 61 ± 2 years, mean tobacco exposure 42 ± 5 pack-years. The group included 17 patients with NSCLC (7 patients with stage IIIB and 10 with stage IV disease) and 7 patients with SCLC (1 patient with LD and 6 with ED; 1 patient with stage IIIB and 6 with stage IV disease).
— The control group consisting of 16 healthy volunteers (11 men and 5 women), mean age 49 ± 3 years, mean tobacco exposure 19 ± 3 pack-years.

All study subjects were current smokers. Manifestations of chronic bronchitis were present in all the study groups but they did not meet the diagnostic criteria for chronic obstructive pulmonary disease (COPD).

In patients with pneumonia the diagnosis was based on the signs and symptoms of lower respiratory tract infection, clinical signs (fever [body temperature >38°C], tachycardia [heart rate >100 bpm], tachypnoea [breathing rate >24/min]), focal abnormalities on chest auscultation and specific findings on a chest X-ray.

Lung cancer was diagnosed based on cytology or histopathology. Cases of NSCLC were staged according to the new TNM classification published in 2009 and cases of SCLC were divided into LD and ED and also staged according to the TNM classification.

EBC concentrations of H_2O_2, VEGF and TNF-α were measured in all the study subjects within the first three days of hospitalisation. Serum concentrations VEGF and TNF-α were also determined.

The subjects had provided written informed consent and the study had been approved by the Ethics Committee of the Medical University of Lodz, Poland.

Exhaled breath condensate

EBC was collected using a method described previously [18, 19]. The device used for collecting on of the condensate consisted of a mouthpiece connected to a glass Liebig tube whose length was 55 cm, internal diameter was 10 mm and shield diameter was 36 mm (Labmed, Lodz, Poland; catalogue number 6010). The Liebig tube was cooled with ethanol at a temperature of –9°C which was pumped into the closed external circulation with MultiTemp III (Pharmacia Biotech AB, Sweden). EBC was collected into 10-ml plastic tubes (Sarstedt), which were then placed in a container with ice.

The examination, which lasted 20 minutes, was performed between 8.00 and 10.00 in the morning. The subject exhaled through a mouthpiece and inhaled beyond the mouthpiece. An average of 5 ml of EBC was obtained, which was stored at –80°C for no more than 2 weeks until analysis.

Measurement of the concentration of H_2O_2 in exhaled breath condensate

The concentration of H_2O_2 was measured according to the method described by Ruch et al. [20]: 10 μl of EBC was mixed with 90 μl of phosphate-buffered saline (PBS) at pH of 7.4 and with 100 μl of horseradish peroxidase (HRP) solution (1 U/ml), which contained 400-μM homovanillic acid (HVA). The mixture was incubated at 37°C for 60 min, which was followed by the addition of 300 μl of 0.1-M glycine/NaOH buffer at pH of 12.0 and 25-mM ethylenediaminetetraacetic acid (EDTA). A luminescence spectrophotometer (Perkin Elmer Luminescence Spectrometer, Beaconsfield, UK) was used, excitation was set at 312 nm and emission was measured at 420 nm. The lower limit of detection for VEGF was 9 pg/ml and for TNF-α — 0.06 pg/ml.:

$$Y = 0.012 \times (X - X^{\circ}) - 0.007$$

where: Y — concentration of H_2O_2 in μM, X — emission at 420 nm in U. The lower limit of detection for H_2O_2 was 0.083 μM.

Measurement of the concentrations of VEGF and TNF-α in EBC and in the serum

The concentrations of VEGF and TNF-α in EBC and in the serum were measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Quantikine, R&D Systems, USA) according to the manufacturer's instructions. Peripheral blood collected into an anticoagulant-free test tube was left for 30 min for clotting and centrifuged at 1000 rpm for 10 min. The resulting serum was retained at –20°C until analysis. The lower limit of detection was 9 pg/ml for VEGF and 0.06 pg/ml for TNF-α.

Statistical analysis

The results were expressed as means ± standard errors of the mean (SEM). The distributions of variables in individual groups were evaluated with the Shapiro-Wilk test. Correlations were evaluated with the Pearson test (r) and the Spearman test (R). The statistical significance of the differences was evaluated with the t-Student test and the Kolmogorov-Smirnoff test. A significance level of $p<0.05$ was adopted in all the cases.

Results

Exhaled breath condensate

The concentration of H_2O_2 in exhaled breath condensate

The mean concentrations of H_2O_2 in EBC were significantly higher in all the three patient groups than in the group of healthy individuals ($p<0.05$) (Table 1). Although the highest concentration was found in patients suffering from pneumonia co-existing with lung cancer, which was significantly higher than in patients with lung cancer ($p < 0.05$), it did not differ significantly from that observed in patients with CAP ($p > 0.05$). No significant difference was found in the concentrations of H_2O_2 between patients with CAP and patients with lung cancer ($p>0.05$) (Figure 1).

The concentrations of VEGF in exhaled breath condensate

The mean concentrations of VEGF in EBC in all the three patient groups showed a distinct trend towards higher values than those observed in healthy volunteers. However, they were significantly higher only in patients suffering from pneumonia co-existing with lung cancer and in patients with lung cancer ($p<0.05$). The highest concentration of VEGF was observed in patients suffering from pneumonia co-existing with lung cancer and it was significantly higher than that in patients with CAP ($p<0.05$) and patients with lung cancer ($p<0.05$) (Table 1). The mean concentration of VEGF in EBC in patients with CAP did not differ significantly from that in patients with lung cancer alone ($p>0.05$).

The concentrations of TNF-a in exhaled breath condensate

The presence of TNF-α in EBC was shown in all the three patient groups (Table 1). The highest concentration of this cytokine was found in the group of patients suffering from pneumonia coexisting with lung cancer and was significantly higher than, the concentrations in the remaining two patients groups' (Groups 1 and 3) ($p<0.05$). The concentration of TNF-α in healthy volunteers was below the lower level of detection (Figure 2).

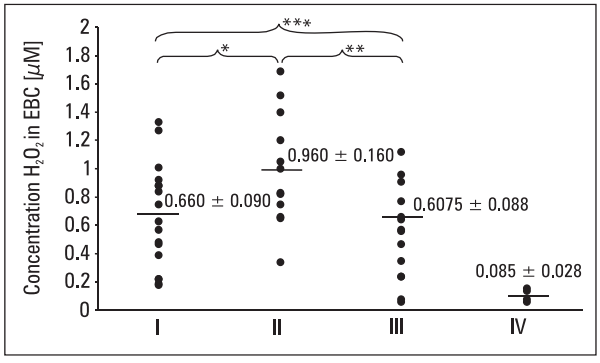
The concentrations of proinflammatory cytokines in the serum

The concentrations of VEGF in the serum

Compared to the group of healthy volunteers, significantly higher concentrations of VEGF were

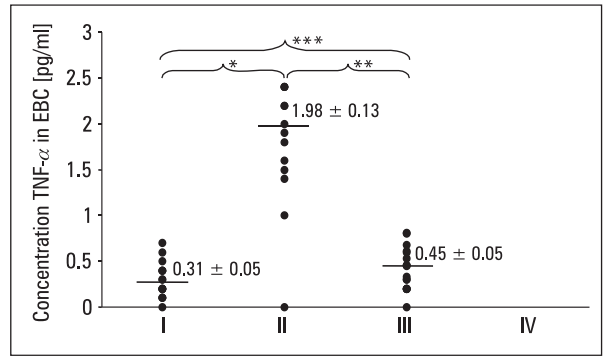
Table 1. Concentration of H_2O_2, VEGF and TNF-α in exhaled breath condensate

Study groups	VEGF [pg/ml]	TNF-α [pg/ml]	H_2O_2 [μM]
I Patients with community-acquired pneumonia	30.20 ± 6.56	0.31 ± 0.05	0.66 ± 0.09
II Patients with pneumonia and lung cancer	317.83 ± 77.78	1.98 ± 0.13	0.96 ± 0.16
III Patients with lung cancer (reference group)	53.96 ± 5.13	0.45 ± 0.05	0.6075 ± 0.088
IV Healthy volunteers (control group)	16.47 ± 0.96	0	0.085 ± 0.028



I — patients with community-acquired pneumonia
II — patients with pneumonia and lung cancer
III — patients with lung cancer
IV — healthy volunteers
* $p > 0.05$ I *v.* II
** $p < 0.05$ II *v.* III
*** $p > 0.05$ III *v.* I

Figure 1. Concentration of H_2O_2 in exhaled breath condensate



I — patients with community-acquired pneumonia
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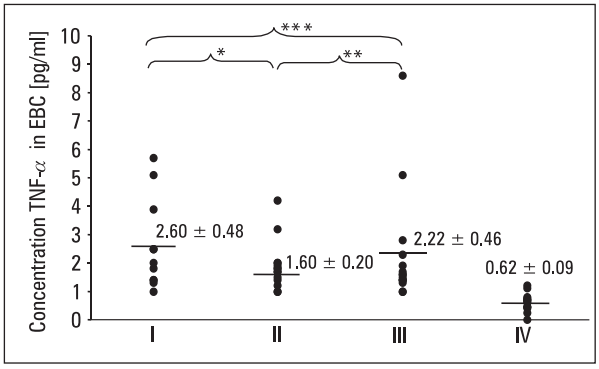
III — patients with lung cancer
IV — healthy volunteers
* $p < 0.05$ I *v.* II
** $p < 0.05$ II *v.* III
*** $p > 0.05$ III *v.* I

Figure 2. Concentration of TNF-α in exhaled breath condensate

Table 2. Serum concentration of VEGF and TNF-α

Study groups	VEGF [pg/ml]	TNF-α [pg/ml]
I. Patients with community-acquired pneumonia	1112.62 ± 244.38	2.6 ± 0.48
II. Patients with pneumonia and lung cancer	392.9 ± 78.2	1.6 ± 0.2
III. Patients with lung cancer (reference group)	704.70 ± 157.77	2.22 ± 0.46
IV. Healthy volunteers (control group)	183.27 ± 18.57	0.62 ± 0.09



I — patients with community-acquired pneumonia
II — patients with pneumonia and lung cancer
III — patients with lung cancer
IV — healthy volunteers
*p < 0.05 I *v.* II
**p > 0.05 II *v.* III
***p > 0.05 III *v.* I

Figure 3. Serum concentration of TNF-α

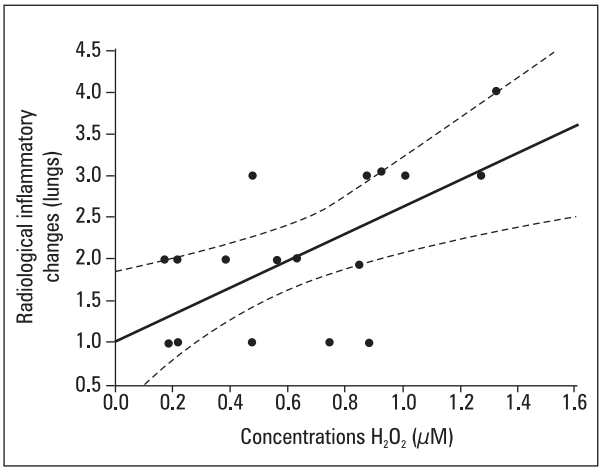


Figure 4. Positive correlation between the concentration of H_2O_2 in exhaled breath condensate in patients with community-acquired pneumonia and the extent of radiological inflammatory changes

observed in patients with CAP ($p<0.05$) and patients with lung cancer ($p<0.05$) and were the highest in patients with CAP. The lowest concentrations of VEGF in serum were observed in patients with pneumonia co-existing with lung cancer and they were significantly lower than those in patients with CAP (Table 2). No significant differences were observed in the concentrations of VEGF between patients suffering from pneumonia co-existing with lung cancer and patients with lung cancer ($p > 0.05$) or between patients with CAP and patients with lung cancer ($p > 0.05$).

The concentrations of TNF-α in the serum

The concentration of TNF-α in the serum was significantly higher in patients with CAP and in patients with lung cancer than in healthy individuals ($p<0.05$). The lowest concentration of TNF-α, which was observed in patients suffering from pneumonia with co-existent lung cancer, was significantly lower than that in patients with CAP ($p<0.05$) and comparable to that in patients with lung cancer ($p>0.05$). No significant difference in the concentration of TNF-α was observed between patients with pneumonia and patients with lung cancer ($p>0.05$) (Table 2, Figure 3).

Correlations between the study variables

In patients with pneumonia, a positive correlation was found between the concentration of H_2O_2 in EBC and the extent of inflammatory changes on a chest X-ray as measured by the number of affected pulmonary fields ($r=0.62$, $p<0.05$) (Figure 4). No correlation was found between the parameters characterising local changes and markers of systemic response. No statistically significant differences were found in age, except for Group 2, where patients were significantly older than individuals in the control group. There were, however, significant differences between the patient groups in the number of pack-years ($p<0.05$). However, the analysis of correlation between age, number of pack-years and the investigated parameters (VEGF, TNF-α, H_2O_2) in EBC and in the serum evaluated with the Pearson test and the Spearman test showed no statistical significance ($p>0.05$).

Discussion

Our study showed that both patients with pneumonia and patients with pneumonia co-existing with lung cancer were characterised by severe local inflammation compared to the control group. Oxidative stress, measured by the concen-

tration of H_2O_2 in EBC, was significantly higher in patients than in healthy smokers. The elevated concentration of H_2O_2 was paralleled by a local increase in the concentrations of VEGF and TNF-α (Table 1). We also found that the highest concentration of the investigated markers was present in patients with pneumonia co-existing with lung cancer, while the systemic response in this group was similar to that observed in healthy volunteers. On the other hand, patients with isolated pneumonia had significantly higher concentrations of VEGF and TNF-α than patients with pneumonia coexisting with lung cancer and had the most pronounced systemic response.

Other studies also demonstrate an increased oxidative stress in the airways of patients with pneumonia. Majewska et al. showed increased concentrations of H_2O_2 and thiobarbituric acid reactive substances (TBARs) in EBC in patients with bacterial CAP compared to the group of asymptomatic smokers [6]. In their study, the concentration of H_2O_2 correlated with the white blood cell count and the serum concentration of C-reactive protein (CRP). Similarly, Kietzmann et al. observed high concentrations of exhaled H_2O_2 in patients with ARDS caused among others by pneumonia [21]. Our results are consistent with these reports and confirm the predominance of local oxidative processes over antioxidant processes in patients with pneumonia and suggest a positive correlation between the concentration of H_2O_2 in EBC and the extent of radiological abnormalities. The more pronounced trend towards an increased concentration of H_2O_2 in EBC in patients with pneumonia co-existing with lung cancer is particularly interesting and noteworthy. The chronic inflammation resulting from smoking, which affects the airways and is associated with local accumulation of macrophages and neutrophils that are the main source of proinflammatory cytokines and ROS, may lead to the development of cancer [22, 23]. Free radicals have been shown to cause point mutations and chromosomal aberrations in the respiratory epithelium, leading to neoplastic transformation. This is confirmed by the increased concentration of H_2O_2 in EBC found in both healthy smokers [24] and patients with lung cancer [7]. The increased concentration of H_2O_2 in EBC in patients with pneumonia co-existing with lung cancer should therefore be attributed to the overlap of acute inflammation, smoking-induced chronic inflammation and the presence of cancer. In this case, the sources of ROS are not only leukocytes which accumulate at the site of acute inflammatory response but also immune cells present in the vicinity of the tumour.

Similarly to the intensity of oxidative stress, the highest concentrations of VEGF and TNF-α in EBC were observed in patients with pneumonia coexisting with lung cancer. Although the role of the pleiotropic cytokine TNF-α and that of VEGF have already been studied extensively, the concentration of both of these markers in the course of inflammatory processes have not been investigated to our knowledge. By stimulating lymphocytes to produce antibodies and by activating neutrophils and macrophages, TNF-α is involved in the immune response to multiple microorganisms which often cause pneumonia, such as *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The protective role of this factor in inflammation has also been shown in a mouse model [25], as administration of anti-TNF-α to mice was associated with increased inflammation leading to death. Similarly, we observed presence of TNF-α in EBC from patients with CAP, while the concentrations of this cytokine were indeterminable in healthy smokers. We found the highest concentrations of this cytokine in patients suffering from pneumonia with co-existing lung cancer, may result from a local mobilisation of immune cells leading to an increased formation of proinflammatory cytokines cause of chronic inflammation associated with the presence of the very tumour. Other studies have shown that in patients with lung cancer, tumour-associated macrophages (TAMs) are the source of multiple proinflammatory markers, such as TNF-α, IL-1 and IL-6 [26, 27]. Carpagano et al. not only showed a local increase in the concentrations of TNF-α as well as of IL-1 and leptin in patients with NSCLC compared to healthy ex-smokers but also a correlation between the investigated markers and the severity of cancer [28]. On the other hand, given the role played by VEGF in the process of tumour growth and spread, it is not surprising that we observed the highest concentrations of this factor in patients with pneumonia co-existing with lung cancer. It was over 10-fold higher than the value characteristic of patients with isolated pneumonia. In our study population, only the patients with cancer (Groups 2 and 3) had significantly higher concentrations of VEGF in EBC compared to healthy individuals. Gessner et al. [29] have recently analysed markers of angiogenesis, including VEGF, in EBC in various groups of patients and have even concluded that they may be useful for early diagnosis of lung cancer. The significantly increased concentration of TNF-α and VEGF in EBC in patients with pneumonia with coexisting lung cancer versus patients with CAP in our study suggests extremely advanced local in-

flammatory processes that are both associated with the acute response to the pathogens and with the presence of the tumour, although an association with local progression of the cancer cannot be ruled out.

The systemic inflammatory response has a different course than the local response. The highest concentrations of the investigated markers were observed in patients with CAP, while patients with pneumonia co-existing with lung cancer had only slightly ($p>0.05$) elevated concentrations of TNF-α and VEGF compared to the control group of healthy smokers. Among patients with isolated pneumonia, on the other hand, in addition to the elevated concentration of TNF-α, an exceptionally high concentration of VEGF was observed. Choi et al. also demonstrated in a population of paediatric patients with CAP a significantly elevated serum concentration of VEGF compared to the control group [30]. This value significantly rose if the pneumonia was complicated by pleural effusion and showed a positive correlation with erythrocyte sedimentation. On the other hand, the serum concentrations of TNF-α and VEGF in patients suffering from pneumonia co-existing with lung cancer which were similar to those seen in the control group suggest a decreased systemic inflammatory response in these patients. Interestingly, the concentrations of the investigated cytokines in patients from Group 2 also showed a downward trend ($p>0.05$) compared to the values seen in patients with lung cancer, which suggests that the overlapping inflammatory process no longer stimulated further increase of the investigated markers in the serum. This is associated with decreased systemic counts and activities of immune cells which have already been involved in the intensive local inflammation. Already in the 1970s an impaired immune response in patients with lung cancer was observed, which was manifested by a decreased circulating T cell count and a weak delayed-type cell-mediated hypersensitivity response [31]. It was shown that patients with this sort of immune system impairment were characterised by a more advanced form of cancer and a poorer response to chemotherapy. For this reason the development of pneumonia in a patient with lung cancer further impairs the pre-existing abnormalities of systemic immune response and is manifested by a marked systemic decrease in the formation of both studied markers we. This is consistent with the clinical observations of patients with lung cancer of demonstrate respiratory tract infections are one of the main causes of death in these patients [32, 33].

Conclusions

1. Patients with pneumonia co-existing with lung cancer show a distinct tendency towards local oxidative stress and a significantly increased local response compared to CAP patients.
2. Systemic inflammation in patients with pneumonia co-existing with lung cancer is markedly reduced compared to patients with CAP.
3. The concentration of H_2O_2 in EBC in patients with CAP shows a positive correlation with the extent of the inflammatory changes.

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