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Cytokines in Patients with Lung Cancer

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Abstract

Lung cancer is one of the most common malignant diseases and is amongst the leading causes of death. Cell-mediated immune response and cytokines could play an important role in antitumour immunity. The aim of the study was to evaluate the cytokines', tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6, releasing capacity in patients with lung carcinoma and benign lung disease. A group of 41 patients were tested for the production of TNF-α, IL-1β and IL-6 in bronchoalveolar lavage (BAL) and blood. The levels of cytokines in the lung cancer patients were: (1) in BAL – IL-6, 173 ± 85 pg/ml; TNF-α, 170 ± 116 pg/ml; and IL-1β, 473 ± 440 pg/ml; (2) in the blood – IL-6, 197 ± 53 pg/ml; TNF-α, 311 ± 202 pg/ml; and IL-1β, 915 ± 239 pg/ml. Alveolar macrophages of the patients with a lung cancer secreted significantly more cytokines, IL-6 ($P = 0.0004$) and IL-1β ($P = 0.0047$), than alveolar macrophages of the patients with a nonmalignant lung cancer. However, significantly lower levels of cytokine production by the BAL cells were found in patients with small cell lung cancer. This production decreased further in phase IV of nonsmall cell lung cancer.

Introduction

Lung cancer is the most common malignant disease occurring in males and the second most common malignant disease occurring in females. Amongst 10 leading causes of death in Croatia, it is rated at the third position in males and at the ninth position in females, according to the report of the Croatian National Public Health Institute, 2000.

Despite surgical treatment, chemotherapy and radiotherapy, a 5-year period of surrival is minimal in nonsmall cell lung cancer, it does not exceed 15% and in small cell lung cancer 2% [1].

The occurrence of a large number of the tumours in immunocompromised patients indicates a role of the immune system in the control of tumour growth. The significance of the immune surveillance in the control of tumour growth has still not been sufficiently studied. Both immunocompetent cells and tumour cells secrete many cytokines, which have an important role in the stimulation of cellular and humoral activity. Cytokines mediate numerous physiological and immune reactions, which are manifested in various biological effects, including tumouricidal activity. Activated macrophages secrete many mediators, which regulate host defence by stimulating cellular immunity. As a response to the stimulation, the macrophages secrete pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6 and IL-12, and oxidants, such as nitric oxide [2, 3]. Conversely, the effect of the macrophages is controlled by the release of anti-inflammatory mediators such as IL-10, tumour growth factor-β and IL-11 [3–5]. The activated macrophages, producing cytokines such as IL-12, TNF-α and interferon-α (IFN-α) and IFN-β, are powerful activators of natural killer (NK) cells, which act cytotoxically on some tumours [6, 7]. T lymphocytes are also involved in the immune surveillance of tumour growth. T-helper 1 (Th1) lymphocytes secrete IL-2, IFN-γ and TNF-α, which activate the macrophages. IL-12, secreted by the activated macrophages and dendritic cells, stimulates the Th1 cells to secrete IFN-γ [8, 9]. Th2 lymphocytes secrete IL-4, IL-5, IL-10 and IL-13, which, apart from stimulating production, can also inhibit some macrophage activity [10].

Analysis of the cells obtained by bronchoalveolar lavage (BAL) gives a clear picture of immune events of the respiratory system [11, 12]. BAL enables the collection of live cells, proteins and enzymes for analysis, which contributes to the knowledge of the dynamics and kinetics of events occurring on the respiratory surface. In the diagnosis of lung tumours, the analysis of soluble and cellular elements of BAL is of particular importance [13].

The aim of the study was to evaluate the releasing capacity value of the cytokines TNF-α, IL-1β and IL-6, in patients with different histological types of lung carcinoma and in patients with benign lung disease.

Patients and methods

Infiltration changes on the chest radiograph of 41 patients were the inclusion criteria for the study. Patients were fibrobronchoscoped and divided into two groups:

1 patients in whom a malignant lung cancer was confirmed (cases);
2 patients in whom malignant disease was excluded, i.e. infiltration on the chest radiograph was the result of slow regression of pneumonia, or in whom bronchoscopy was performed because of haemoptysis, as a result of chronic bronchitis (controls).

Amongst the cases, there were 26 patients: 20 males and six females, aged from 40 to 77 years. Cases were further divided into three subgroups according to cytological findings:

1 patients in whom planocellular carcinoma (PL) was confirmed;
2 patients in whom adenocarcinoma (AD) was confirmed;
3 patients in whom microcellular carcinoma (MC) was confirmed.

Fifteen patients with nonmalignant lung diseases were considered as controls: 11 males and four females, aged from 47 to 82 years.

The samples for the study were collected by BAL and from peripheral venous blood. We determined the production of the cytokines TNF-α, IL-1β and IL-6.

BAL. We performed BAL during fibrobronchoscopy. The site of the lavage was chosen according to the changes seen on the chest radiograph. When no changes were found in the bronchi, the lavage was performed in the segment of the middle lobe on the right or the lingula on the left side. The lavage was performed by the instillation and collection of sterile physiological fluid, which was heated to room temperature. We instilled 20 ml of physiological fluid, up to a total of 100 ml, then aspirated them with a standard aspirator and stored them in an ice-cooled sterile container. Approximately, 60–80% of the instilled solution was aspirated.

Preparation of BAL cells for the determination of cytokines. The obtained BAL sample was rinsed by centrifugation twice for 10 min at 300 *g* in RPMI medium. The cells were resuspended in RPMI medium, enriched with 10% fetal calf serum, and stimulated with 10 μg/ml of lipopolysaccharide (LPS), up to one million cells, and then incubated them for 18 h at 37 °C and 5% CO_2, after which the sample was centrifuged and the obtained supernatant stored at −20 °C for further testing.

Preparation of cells of peripheral blood for the determination of cytokine secretion. Four millilitre of phosphate-buffered saline (PBS) was added to 2 ml of whole blood, with heparin, of which 2 ml was taken and 10 μg of LPS added per one million cells. They were then incubated in a test tube at 37 °C and 5% CO_2 for 18 h and centrifuged, and the supernatant (plasma) was stored at −20 °C for further testing.

The cytokine level in the samples was determined by enzyme-linked immunosorbent assay (ELISA) using the following: for IL-6, The Complete Immunotest (Boehringer Mannheim Biochemica, Mannheim, Germany); for TNF-α, Factor-Test hTNF-α ELISA Kit (Genzyme Corporation, Cambridge, MA, USA); for IL-1β Cistron Biotechnology, NY, USA.

Statistical analysis. We described cytokine levels by standard descriptive methods of the mean and its standard deviation. The levels were compared by Student's *t*-test for independent samples. We used STATISTICA for Windows by STATSOFT, at an alpha level of 0.05.

Results

Alveolar macrophages of the patients with a lung cancer secreted significantly more cytokines, IL-6 and IL-1β, than alveolar macrophages of the patients with a nonmalignant lung disease.

The levels of cytokines in the lung cancer patients were:

1 in BAL: IL-6, 173 ± 85 pg/ml; TNF-α, 170 ± 116 pg/ml; and IL-1β, 473 ± 440 pg/ml;
2 in the blood: IL-6, 197 ± 53 pg/ml; TNF-α, 311 ± 202 pg/ml; and IL-1β, 915 ± 239 pg/ml.

The levels of cytokines in the patients with benign lung disease were:

1 in BAL: IL-6, 59 ± 103 pg/ml; TNF-α, 109 ± 209 pg/ml; and IL-1β, 101 ± 252 pg/ml;
2 in the blood: IL-6, 157 ± 101 pg/ml; TNF-α, 317 ± 241 pg/ml; and IL-1β, 800 ± 414 pg/ml (Fig. 1).

Cytokine levels of BAL were significantly higher in cancer patients for IL-6 ($P = 0.0004$) and IL-1β ($P = 0.0047$), whilst differences between other samples were not significant (Table 1).

PL was diagnosed in 15 patients, AD in eight and MC in three patients. Comparing the levels of cytokines in the BAL cell cultures according to the type of tumour, we found differences between small cell lung cancer and nonsmall cell lung cancer.

Differences between MC and AD were significant in BAL for IL-6 ($P = 0.0007$), TNF-α ($P = 0.0231$) and IL-1β ($P = 0.0259$), as well as in blood, for IL-6 ($P = 0.0001$) and IL-1β ($P << 0.05$) (Table 2).

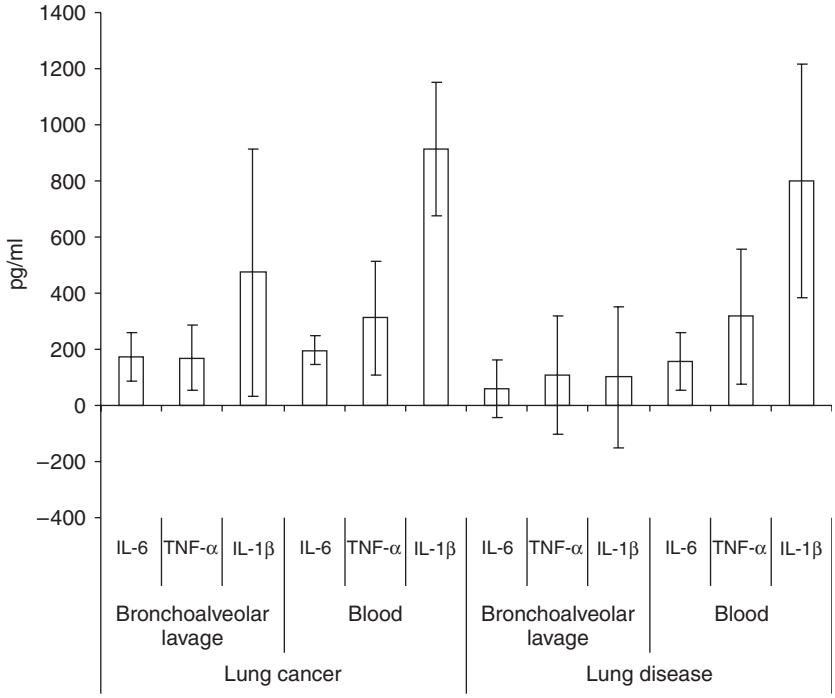

Figure 1 Cytokine levels in patients with lung cancer and benign lung disease.

Differences between MC and PL were not significant in BAL, but significant in blood for IL-6 ($P = 0.001$) and IL-1β ($P << 0.05$) (Table 3).

Differences between AD and PL were significant neither in BAL nor in blood (Table 4).

We were interested in finding out whether there was any difference in the secretion of TNF-α, IL-6 and IL-1β in BAL, with regard to the stage of the disease. Some decrease in the cytokine levels was observed during progression of the disease in the patients with nonsmall cell lung cancer (Fig. 2).

Discussion

Lung cancer is the leading cause of morbidity and mortality among malignant diseases. Incidence and mortality are

Table 1 Differences in cytokine levels between patients with lung disease and lung cancer

Sample	Cytokine	Disease (pg/ml)	Cancer (pg/ml)	*t*-value	*P*
Bronchoalveolar lavage	IL-6*	59 ± 103	173 ± 85	−3.84	0.0004
	TNF-α	109 ± 209	170 ± 116	−1.21	0.2335
	IL-1β*	101 ± 252	473 ± 440	−3.00	0.0047
Blood	IL-6	157 ± 101	197 ± 53	−1.66	0.1047
	TNF-α	317 ± 241	311 ± 202	0.09	0.9315
	IL-1β	800 ± 414	915 ± 239	−1.14	0.2633

*Significant at $P < 0.05$. IL-6, interleukin-6; TNF-α, tumour necrosis factor-α.

Table 2 Differences in cytokine levels between patients with microcellular carcinoma (MC) and adenocarcinoma (AD)

Sample	Cytokine	MC (pg/ml)	AD (pg/ml)	*t*-value	*P*
Bronchoalveolar lavage	IL-6*	73 ± 25	205 ± 42	−5.03	0.0007
	TNF-α*	83 ± 15	231 ± 90	−2.73	0.0231
	IL-1β*	87 ± 12	723 ± 400	−2.66	0.0260
Blood	IL-6*	97 ± 15	215 ± 32	−6.07	0.0002
	TNF-α	107 ± 12	354 ± 184	−2.25	0.0506
	IL-1β*	267 ± 58	1000 ± 0	−39.80	0.0000

*Significant at $P < 0.05$. IL-6, interleukin-6; TNF-α, tumour necrosis factor-α.

Sample	Cytokine	MC (pg/ml)	PL (pg/ml)	t-value	P
Bronchoalveolar lavage	IL-6	73 ± 25	176 ± 96	−1.80	0.0915
	TNF-α	83 ± 15	155 ± 126	−0.96	0.3505
	IL-1β	87 ± 12	418 ± 442	−1.27	0.2233
Blood	IL-6*	97 ± 15	207 ± 46	−4.02	0.0010
	TNF-α	107 ± 12	328 ± 214	−1.75	0.0994
	IL-1β*	267 ± 58	1000 ± 0	−56.80	0.0000

Table 3 Differences in cytokine levels between patients with microcellular (MC) and planocellular carcinoma (PL)

*Significant at $P < 0.05$. IL-6, interleukin-6; TNF-α, tumour necrosis factor-α.

Sample	Cytokine	AD (pg/ml)	PL (pg/ml)	t-value	P
Bronchoalveolar lavage	IL-6	205 ± 42	176 ± 96	0.82	0.4199
	TNF-α	231 ± 90	155 ± 126	1.51	0.1455
	IL-1β	723 ± 400	418 ± 442	1.62	0.1194
Blood	IL-6	215 ± 32	207 ± 46	0.46	0.6528
	TNF-α	354 ± 184	328 ± 214	0.29	0.7710
	IL-1β	1000 ± 0	1000 ± 0	—	—

Table 4 Differences in cytokine levels between patients with adenocarcinoma (AD) and planocellular carcinoma (PL)*

*IL-6, interleukin-6; TNF-α, tumour necrosis factor-α.

increasing rapidly, especially in southern and eastern European countries [14]. Despite early diagnosis, improved surgical techniques and advanced radiological and chemotherapeutic treatment, little has been done to halt the progression of the disease [15, 16].

The immune response of an organism to a tumour has been the subject of numerous investigations [17]. Tumour-associated antigens stimulate the immune system to cellular and humoral immune response [18]. Whether or not a significant response will be achieved, mediated by T lymphocytes, depends on the level of expression of major histocompatibility complex molecules and the expression of adhesive molecules, which depends on the ability of the tumour cells reaching the lymphoid tissue.

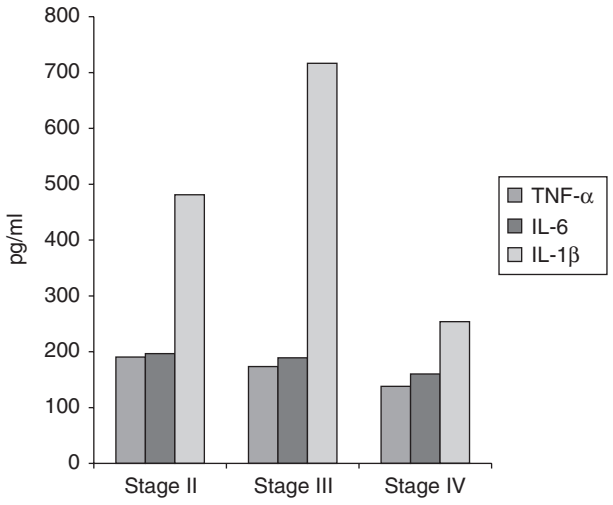

Figure 2 Changes of the cytokine levels in bronchoalveolar lavage during progression of nonsmall cell lung cancer.

In this regard, the role of cytokines is particularly important, owing to the fact that they can influence the response of potentially reactive lymphocytes and can have an influence on the activity of endothelial and stromal cells [19]. Cytokines, such as TNF, IL-1 and IL-6, are included in the immune reaction as important polypeptide mediators [20]. The lipopolysaccharide component of gram-negative bacteria is a powerful activator of macrophages during the production of cytokines [21, 22]. According to our results, following stimulation with LPS, the activated macrophages and monocytes release TNF, and this function is significantly more pronounced in alveolar macrophages than in monocytes. A possible explanation for this finding may be that different soluble mediators already activate the alveolar macrophages in patients with a tumour, which makes them more responsive to LPS. Another possibility is that a part of the cytokines secretes other cells contained in BAL. We found similar results in other studies [23].

Macrophages mediate the defence activity by releasing different soluble mediators, which are toxic to tumour cells [24]. Although alveolar macrophages release free radicals of oxygen, their role in the destruction of the tumour cells is probably less pronounced, because of the loss of myeloperoxidase, an enzyme that elevates the toxic effect of radical oxygen, which is lost during the maturation of macrophages [25]. The reactive products of oxygen, including NO, NO_2 and NO_3, can also be implicated in the cytolysis mediated by TNF [26]. They are cytotoxic to tumour cells, inactivating enzymes by releasing iron from aconitase and respiratory reductase enzymes [27, 28].

The question is whether in bronchogenic carcinoma the functional ineffectiveness of the macrophage precedes

the development of bronchogenic carcinoma, or whether the tumour products negatively regulate the activity of these cells. By determining the cytotoxic activity of alveolar macrophages *in vitro* in patients with bronchogenic carcinoma and comparing them with a control group, it was discovered that cytostatic activity decreases with the growth of the tumour. Our results also indicated lower levels of cytokines during stage IV of the disease, particularly for MC. After binding to their cell receptor, TNF-α begins lysis of tumour cells. Tumour cells can synthesize protein that protects them from lysis mediated by TNF-α, which is confirmed by the fact that inhibitors of the synthesis of these proteins, such as actinomycin D, elevate the sensitivity of target cells to TNF [29]. In the absence of metabolic inhibitors and the presence of TNF, characteristic apoptotic changes occur in the tumour cells [30]. Damaged activation of sphingomyelinase and the formation of ceramide may be the mechanisms by which tumours avoid apoptosis, which is mediated by TNF-α [30].

The growth of solid tumours is dependent upon their ability to stimulate the development of new blood vessels into the tumour mass. The angiogenic potential of the endothelial cell is carefully balanced between positive and negative regulations. Tumours have the potential of up- or downregulating controls, producing an environment in which new blood vessels are formed [31]. In lung cancer, vascular endothelial growth factor is associated with tumour vessel density, malignant pleural effusions and coagulation–fibrinolysis factors, and its serum levels are increased significantly, according to stage progression [32].

Inhibition of tumour vascularization represents one of the possibilities of preventing tumour growth [33]. The ability of TNF-α to cause haemorrhagic necrosis of a tumour *in vivo* is well known. Endothelial cells of tumour blood vessels are the target cells for the cytotoxic effect of TNF-α [7]. Apart from direct cytotoxicity, TNF-α can indirectly cause tissue damage through adhesive molecules such as intercellular adhesion molecule-1, which increases the interaction between the endothelium and leucocytes or platelets [34].

The investigation and development of different anti-angiogenesis and vascular targeting strategies are of interest, with respect to numerous tumour types, including lung carcinoma [31].

We found high levels of IL-1β in the cultures of BAL cells and blood in patients with a lung tumour. IL-1 acts synergistically with TNF-α [20, 35]. TNF-α can induce IL-1 synthesis [36]. The synergetic activity of these two cytokines has been determined in experimental investigations with regard to cytotoxicity to some tumour cells [37].

We also found elevated levels of IL-6 in patients with a lung tumour. Tumour cells produce IL-6 constitutively and others only when appropriately stimulated [38]. Elevated levels of IL-6 in a host with a tumour are also the result of increased production from T lymphocytes, predominantly of $CD4^+$ cells, which secrete Th2 cytokines [39]. IL-6 also regulates the function of lymphokine activated killer (LAK) cells by elevating the secretion of TNF-α and the expression of TNF receptor [40]. For successful defence of the tumour, the synergistic activity of cytokines is important. IL-1, IL-6 and TNF-α increase T-cell response [9, 41]. They also upregulate the secretion of IL-2 [42]. IL-2 activates NK cells because they have IL-2 receptors, through which it is possible to send intercellular signals, which activate the NK cells [30]. Significant levels of cytokine production by the BAL cells were found in patients with small cell lung cancer. This production decreased further in phase IV in nonsmall cell lung cancer. In spite of marked immune response to the tumour, progression of the disease frequently occurs.

The results of our investigation indicate a reduction in the levels of cytokines in the cultures of BAL cells and blood, with progression of the disease. In our patients, significant difference was observed in the levels of cytokines between small cell lung cancer and nonsmall cell lung cancer. The low levels can be explained by the fact that we diagnosed MC during the widespread stage of the disease. Similar results were found in a study in which TNF-α, IFN-γ and IL-6 were determined in the BAL of patients with small cell lung cancer and nonsmall cell lung cancer. In patients with small cell lung cancer, the levels of all the three cytokines were low [43].

One possible explanation for the undesirable course of malignant disease is that the tumour cells can spontaneously secrete immunosuppressive cytokines, which negatively regulate the immune system of the host [38]. Immunosuppressive cytokines can inhibit cytotoxic leucocyte mechanisms by negative regulation of perforins and B granzymes. Inhibition can also occur by the suppression of the production of cytokines, such as IFN-γ, TNF-α and IL-2, which are essential for strengthening cytotoxic activity [38].

Conclusion

In patients with a lung tumour, the immune system shows significant activity during attempts to destroy the tumour cells. The alveolar macrophages of patients with a lung tumour secrete significantly more pro-inflammatory cytokines than the alveolar macrophages of patients with nonmalignant lung disease after stimulation with LPS. Depending on the type of tumour, there is a significant difference between small cell lung cancer and nonsmall cell lung cancer. The BAL and blood cells of patients with small cell lung cancer secrete significantly less cytokines compared with the BAL and blood cells obtained from patients with nonsmall cell lung cancer. The secretion of cytokines in BAL cell cultures obtained from patients with nonsmall cell lung cancer decreases with progression of the disease.

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