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Elevated TGF-β1 concentration in bronchoalveolar lavage fluid from patients with primary lung cancer

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Abstract

Introduction: Transforming growth factor (TGF)-β is one of numerous inhibitory factors produced by cancer cells that regulate antitumor immunity. The aim of this study was to evaluate TGF-β1 levels and lymphocyte subsets in the broncholaveolar lavage fluid (BALF) of patients with primary lung cancer and to analyze the interdependence of these parameters.
Materials and Methods: BALF samples were collected from 38 patients with primary lung cancer prior to treatment and from 23 healthy volunteers. Concentrations of TGF-β1 were measured in two independent lots of samples using a commercially available sandwich ELISA kit after concentration of the supernatants. Differential cell counts in the BALF were performed on slides stained with the May Grünwald Giemsa method. Flow cytometry with monoclonal antibodies was applied for lymphocyte phenotyping.
Results: A higher level of TGF-β1 in the BALF of patients compared with the healthy subjects was observed in both lots of samples (3.23±2.96 pg/ml vs. 1.05±0.95 pg/ml, $p<0.05$, and 16.1±19.3 pg/ml vs. 10.1±11.1 pg/m,, respectively, difference not significant). There was significant positive correlation of the TGF-β1 level with the proportion of lymphocytes and negative correlation with both the proportion of macrophages and the percentage of cytotoxic and activated T lymphocytes.
Conclusions: Our findings confirmed that TGF-β takes part in the local response in the course of primary lung cancer.

Key words: lung cancer, bronchoalveolar lavage, TGF-β, lymphocytes.

Abbreviations: AM – alveolar macrophage, BALF – bronchoalveolar lavage fluid, ELISA – enzyme-linked immunoabsorbent assay, FITC – fluorescein isothiocyanate, IL-2 – interleukin 2, PBS – phosphate-buffered solution, PE – phycoerythrin, TGF-β – transforming growth factor β.

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INTRODUCTION

Lung cancer is now the most common fatal malignancy in the world. Despite considerable efforts, only 5–10% of patients survive 5 years. A better understanding of the immunology of lung cancer may contribute to the creation of new therapeutic methods to increase survival and improve the quality of life of cancer patients.

Bronchoalveolar lavage (BAL) is a useful procedure to sample the cellular and humoral constituents of the lung microenvironment. Changes in the cell profile of the bronchoalveolar lavage fluid (BALF) reflect immunologic reactions of the lung in pulmonary malignancies [8, 30]. Among the several different cytokines, transforming growth factor (TGF)-β is known to be present in the tumor environment and to have a suppressive effect on host defense [2, 10, 16, 28]. TGF-β belongs to a family of dimeric (disulfide linked) 25-kDa polypeptides. Three isoforms exist in mammals: TGF-β1, TGF-β2, and TGF-β3, with nearly identical properties and biological functions. TGF-β1 is active in the inhibition or activation of lymphocytes and macrophages and takes part in fibrotic processes [27, 28]. One of the biological effects of TGF-β is inhibition of the proliferation of epithelial cells, including some malignant cells [4]. Elevated concentrations of TGF-β and specific mRNA have been found in samples from resected tumors and in the cancer cell lines [2, 4, 19]. The aim of this study was to evaluate the concentrations of TGF-β1 in the BALF of patients with lung cancer in comparison with healthy subjects and to determinate the correlation of TGF-β1 concentration with macrophages, lymphocytes, and lymphocyte subpopulations.

MATERIALS AND METHODS

Subjects

The group of patients with histologically confirmed primary lung cancer consisted of 38 subjects. The investigation was performed prior to any treatment. The patients did not suffer from chronic obstructive lung disaease, and the mean FEV1%VC was >70% predicted. Twenty-three healthy volunteers were evaluated as a control group. Subjects with any infection or chronic pulmonary disease were excluded from the study. The investigation was approved by the Ethics Committee of the Warsaw Medical University. All healthy volunteers and patients signed an informed consent form prior to the examination. The characteristic of the investigated groups of subjects are summarized in Table 1.

Table 1. Characteristic of the study population

	Patients (n=38)	Healthy (n=23)
Sex F/M	14/24	9/14
Age (years)	58	39
Mean (range)	(28–72)	(19–62)
Smokers/ex-smokers/non-smokers	27/3/8	23/0/0
Pack/years (mean ±SD)	38±18.3	18.7±16.2
Histology of cancer		
squamous cell carcinoma	12	
adenocarcinoma	8	
small-cell lung cancer	2	
non-small-cell lung cancer*	16	
Stage of disease		
I	8	
II	7	
III	16	
IV	7	

* Cases of undifferentiated small-cell type in histology or diagnosed as non-small-cell type in the small cytological samples (brushings, fine needle aspirates, or bronchial washings).

BAL procedure

BAL was performed in the patients and controls during bronchofiberoscopy after premedication with atropine and lidocaine upper respiratory tract anesthesia. In the group of patients the BAL was taken during the diagnostic procedure from the lobe involved by the tumor in cases with peripheral mass or from the lobe adjacent to the involved lobe in cases with a central mass. In the control group the right middle lobe was lavaged. Four aliquots of 50 ml of 0.9% NaCl solution pre-heated to 37°C were sequentially instilled and immediately removed.

Analysis of the BALF cells

The BALF was analyzed after rejection of the first aliquot. After filtration through two layers of gauze and measurement of the volume, the fluid was centrifuged at 4°C at 250×g for 10 min. The supernatant was stored at –70°C until analysis. The cell pellet was washed and resuspended in 1 ml of cold phosphate-buffered saline (PBS). Cell viability was determined by the trypan blue exclusion test. Mean viability of the cells was 90%. The BAL cells were counted using a Bürker chamber. Differential cell counts were examined on smears stained by the May Grünwald Giemsa method. From each sample, 300 cells were counted.

The BALF cells were analyzed by the two-color flow cytometry method with a combination of monoclonal antibodies directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). The following mixtures of antibodies were used: anti-CD45-FITC and anti-CD14-PE, negative isotype controls (Ig2a-FITC and Ig2b-PE), anti-CD3-FITC and anti-CD19-PE, anti--CD3-FITC and anti-CD4-PE, anti-CD3-FITC and anti-CD8-PE, anti-CD3-FITC and anti-HLA-DR-PE, anti-CD3-FITC and anti-CD16+CD56-PE, anti-CD3--FITC and anti-CD25-PE, all from Becton Dickinson Immunocytometry System, San Jose, CA, USA. Each mixture of two antibodies was added to 50 µl of cells suspended in RPMI-1640 culture medium (Gibco-BRL, Gaithesburg, MD, USA) and incubated for 30 min in the dark at 4°C. Then the cells were washed twice with PBS, suspended in 0.4 ml of PBS with 1% paraformaldehyde, stored at 4°C, and analyzed within 24 h. The samples were analyzed using a FACS Calibur flow cytometer (Becton- Dickinson, San Jose, CA, USA). The cells were collected by CELLQuest software and a minimum of 8000 events were acquired. Two-color fluorescence intensity was displayed and analyzed on 1024 channels. Lymphocytes were identified using a combination of CD45/CD14 and light-scatter (FSC/SSC) characteristics as small, non-alveolar cells with CD45 expression.

For analysis of the TGF-β1 level in the BALF, the samples with similar recovery volume (about 50% of the instilled fluid) were selected. BAL supernatant was concentrated prior to TGF-β1 analysis. The first lot of samples was concentrated using Ultrafree-15 protein concentrators (Millipore, USA) with a molecular weight cut-off of 5 kDa. The mean concentration factor was in the range 10–30 times. The second lot of samples was concentrated using Amicon Ultra-15 (Millipore, USA) with molecular weight cut-off value of 10 kDa. The mean concentration factor ranged 5–10 times. The levels of TGF-β1 were measured using a commercially available sandwich ELISA kit (Bender MedSystem) according to the manufacturer's instructions. The application of two kinds of concentrators with different ultrafiltration membranes meant that the TGF-β1 values measured by Elisa had to be analyzed separately in each lot of samples.

Statistical analysis

For data comparisons the non-parametric Mann Whitney U test was used and median values were com-

pared, a p value <0.05 being regarded as significant. To analyze the TGF-β1 measurements, statistical analysis was applied separately in each lot of samples. The relationships between TGF-β1 concentration and the proportions of BALF cells were tested by the Spearman correlation test.

RESULTS

The results of the total cell count, differential cell count, and the median percentages of lymphocyte subsets analyzed by flow cytometry in the BALF of patients and controls are presented in Table 2. There were some differences between the group of patients and the group of controls in the proportions of cells, this being the subject of a previous study [8]. We found a higher concentration of TGF-β1 in the BALF from patients with lung cancer than from the control group in both lots of samples; however, the difference was significant only in the first lot (Fig. 1). In the first lot of samples the mean concentration of TGF-β1 in the patients group was 3.23±2.96 pg/ml vs. 1.05±0.95 pg/ml in the group of healthy ($p<0.05$). In the second lot of samples the values were 16.1±19.3 pg/ml and 10.1±11.1 pg/ml, respectively. There were no significant differences between active smokers and non-smokers. The highest levels of TGF-β1 were observed in two patients: one with small-cell lung cancer (77.7 pg/ml) and the second with adenocarcinoma in stage IV of disease (55.4 pg/ml). However, we did not observe any significant differences in cell counts, cell types, or TGF-β1 concentration between histological types, but a tendency towards positive correlation of the concentration of TGF-β1 with the stage of the disease can be seen (Fig. 2). We did not find any significant difference in TGF-β1 concentration between patients with "central mass" and those with peripheral mass.

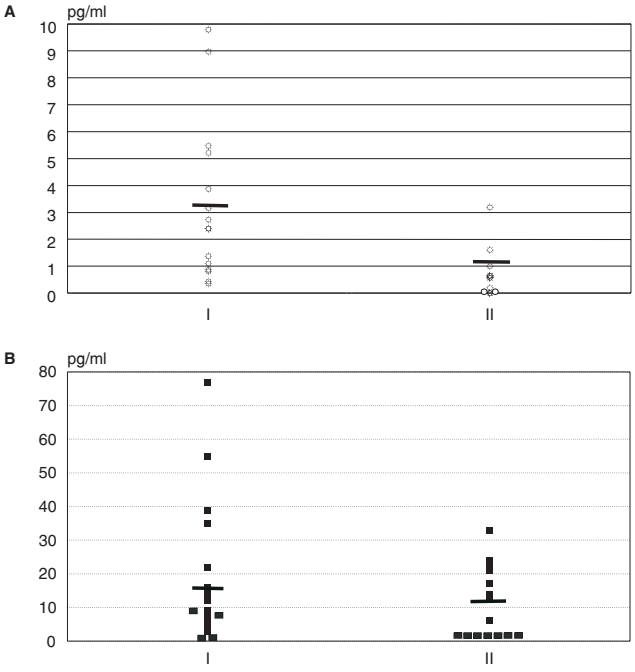

Fig. 1. TGF-β1 concentration in the BALF of patients with lung cancer (**I**) and in the BALF of healthy persons (**II**). Mean values shown as a line (**–**). **A** – the first lot of samples, concentrated with a 5-kDa ultrafiltration membrane. Number of patients: 14, number of healthy controls: 13 ($p<0.05$). **B** – the second lot of samples, concentrated with a 10-kDa ultrafiltration membrane. Number of patients: 24, number of healthy controls: 10. Difference not significant.

Table 2. Total and differential counts of BALF cells from patients and healthy subjects

	Patients (n=38)	Healthy (n=23)
Instilled fluid (ml)	200	200
Recovery fluid (ml)	96.4±14.7	118.6±26.7
Total cell count ($\times 10^6$)	11.6±12.4	11.4±4.8
Alveolar macrophages (%)	81.1±17.1	94.1±2.9
Lymphocytes (%)	15.4±15.6	4.6±2.7
Neutrophils (%)	3.6±3.3	1.2±1.4
T cells $CD3^+$	89.9±6.1	88.9±7.0
B cells $CD19^+$	1.9±1.0*	0.7±0.6*
NK cells $CD3^-$ $CD16^+56^+$	8.1±6.7	10.8±7.2
T helper cells $CD3^+$ $CD4^+$	49.3±12.6	41.8±14.2
T cytotoxic/suppressor cells $CD3^+$ $CD8^+$	40.1±16.3	52.2±18.3
T activated cells $CD3^+$ $HLA\text{-}DR^+$	25.8±19.8	28.3±19.5
T cytotoxic cells $CD3^+$ $CD16^+56^+$	5.2±4.2*	14.3±6.7*
T cells with IL-2R $CD3^+$ $CD25^+$	11.9±6.6	12.6±3.1

Lymphocyte subpopulations expressed as percentage of lymphocytes.
Data expressed as mean ±SD.
* $p<0.05$ in Mann Whitney U test, patients vs. healthy.

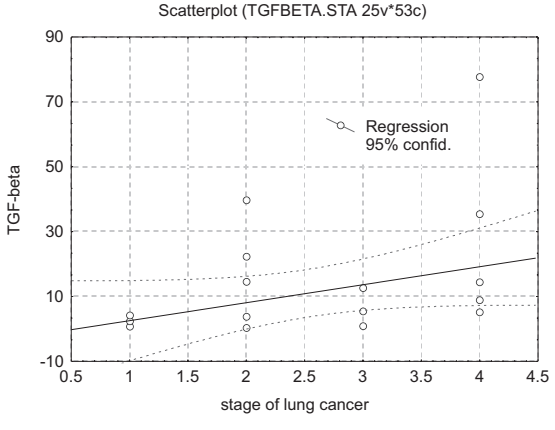

Fig. 2. Correlation of TGF-β1 concentration in the BALF of patients with stage of the disease ($R=0.1$, $p>0.05$).

Some correlations of TGF-β1 concentration with the proportions of BALF cells were found. There was significant positive correlation of TGF-β1 concentration with the proportion of lymphocytes ($R=0.5$, $p<0.05$) and significant negative correlation of TGF-β1 level with the proportion of macrophages ($R=-0.6$, $p<0.05$) in the group of patients. The negative correlation of TGF-β1 with cytotoxic lymphocytes ($R=-0.9$, $p<0.05$) and with the number T lymphocytes with a receptor for IL-2 ($R=-0.9$, $p<0.05$) was significant in the group of healthy subjects.

DISCUSSION

In this study we report for the first time a significantly elevated concentration of TGF-β1 in the BALF of lung cancer patients. In several studies, TGF-β was shown to be an immunosuppressive cytokine in lung cancer [10, 16]. TGF-β is also involved in different processes which are observed in malignancies, such as angiogenesis, fibroblast activation with remodeling of the extracellular matrix, and inflammation [1, 3, 6, 15, 22]. Elevated concentrations of this cytokine have been found in tumor surroundings [2, 4, 19, 21]. In our study, BALF from the lung affected by cancer was used to evaluate the concentration of TGF-β1. Analysis of BALF cells and extracellular components allows a desripction of the immune processes in the airway microenvironment [8, 14]. We found an elevated concentration of TGF-β1 in the BALF of patients with confirmed lung cancer which was significantly higher than in the control group. In our study a tendency towards positive correlation with the stage of the disease was observed (Fig. 2). We observed that the highest levels of TGF-β1 were measured in the BALF of patients with stage IV of disease and with types of lung cancer, small-cell and adenocarcinoma, known to be very aggressive. The role of TGF-β1 as a prognostic factor in lung cancer is still unclear. A high level of TGF-β in some studies corresponded with better prognosis [4, 6], while in others with worse [25]. Increased production of TGF-β1 by cells of small-cell lung cancer was observed in the study of Fischer et al. [11, 12], but Lagadec et al. [20] showed that TGF-β1 did not modify the proliferation of small-cell cancer cells.

TGF-β is produced by epithelial cells, tumor cells, and immune cells. One of the major sources are alveolar macrophages (AMs) [23]. Suppression of AMs in the course of lung cancer was observed by others [30]. In our study, negative correlation of TGF-β1 level with the proportion of AMs was found. TGF-β as a product of tumor cells has a potent immunosuppressive effect on AMs [18, 30]. As was shown in a study by Bingisser et al. [5], TGF-β may down-regulate apoptosis of alveolar macrophages, and the possible negative feedback may be induced by AMs themselves.

TGF-β is also an active cytokine in the activation and inhibition of lymphocytes [7, 13, 19, 23, 26, 29]. The role of lymphocytes in the immunology of lung cancer was documented previously [9, 12]. Positive correlation of TGF-β1 concentration and the percentage of lymphocytes could be seen in our study. Salez et al. [24] observed a significant correlation between the number of lymphocytes and TGF-β1 concentration in the BALF of patients with sarcoidosis. It is necessary to point out that the effect of TGF-β1 on cellular immune pathways is more complicated; for example, immature cells are stimulated by TGF-β, whereas activated representatives of the same cell populations may be inhibited by TGF-β [7, 13, 19, 28,2 9]. In our observation, an elevated level of TGF-β1 was accompanied by a higher proportion of T-activated lymphocytes, but there was negative correlation with natural killer (NK) cells and cytotoxic lymphocytes. This correlation was significant in the group of controls. It is possible that this reflects physiological circumstances and homeostasis, in which the coexistence of stimulatory and inhibitory processes persists [28], and the lack of significant correlation in cancer patients reflects pathological changes in the tumor environment. Gray et al. [13] confirmed in their study that TGF-β has a suppressive effect on cytotoxic cells. Lee et al. [21] recently confirmed that TGF-β1 is responsible for the impaired function of NK cells in patients with lung cancer, which is in agreement with our results (non-significant negative correlation of TGF-β1 concentration with the proportion of NK cells, data not shown). Likewise, negative correlation with T lymphocytes with a receptor for interleukin (IL)-2 was confirmed by Kehrl et al. [19]. In their study the addition of exogenous TGF-β to a culture of T lymphocytes inhibited IL-2-dependent T cell proliferation. Several recent reports indicate the role of $CD4^+/CD25^+$ T cells (regulatory T cells, Treg cells) and TGF-β in the induction of peripheral tolerance [17, 31]. Little is known about the presence of Treg cells in the BALF. The role of these cells in local immune tolerance in lung cancer requires further investigations.

In conclusion, our analysis showed an elevated concentration of TGF-β1 in the BALF of patients with lung cancer and may indicate a possible participation of TGF-β1 in local immunity of neoplastic disease.

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