Myeloid and lymphoid dendritic cells and cytotoxic T lymphocytes in peripheral blood of non-small cell lung cancer patients – a pilot study

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Abstract

Purpose: Dendritic cells (DCs) and cytotoxic T lymphocytes (CTLs) are the first protecting barrier against different pathogens (viruses, bacteria and neoplasms cells). Immature myeloid- and lymphoid dendritic cells possess ability to phagocytose and present antigens to lymphocytes. They have also ability to produce IL-12, which is also known as natural killer cell stimulatory factor or cytototoxic lymphocyte maturation factor. The aim of the study was to demonstrate the relationship between percentage of immature dendritic cells and percentage of CTLs subtypes in peripheral blood of non-small cell lung cancer (NSCLC).

Material and methods: The study population consisted of 10 patients suffered from NSCLC (the mean age: 61.8±10.55). The monoclonal antibodies and three-color flow cytometry technique was applied to determine the cells phenotype in peripheral blood.

Results: Significant negative correlation ($R=-0.693$, $p<0.05$) between percentage of lymphoid DCs and percentage of CTLs was shown. The myeloid to lymphoid DCs ratio significantly positively ($R=+0.638$, $p<0.05$) correlated with the percentage of CTLs. The significant negative correlation between the percentage of myeloid DCs and the percentage of CTLs-IL-12R-positive cells, as well as expression of this receptor were also ascertained ($R=-0.68$, $p<0.05$ and $R=-0.757$, $p<0.01$, respectively).

Conclusions: In presented pilot study we demonstrated clearly relationship between the percentage of immature DCs and the percentage and the phenotype of CTLs in peripheral blood of lung cancer patients.

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Abbreviation list: DCs – dendritic cells; CTLs – cytotoxic T lymphocytes; NSCLC – non-small cell lung cancer; IL-12R – interleukin 12 receptor; PBDCs – peripheral blood dendritic cells; BDCA – blood dendritic cells antigen.

Introduction

Almost one million of new cases of different types of lung cancer are recorded every year in the world [1]. In Poland, the number of the lung cancer cases systematically increases and the incidence of this disease is about 70 per 100.000 subjects. An increase in the number of cancer patients results not only from the influence of the environmental factors, with a leading strongly negative effect of tobacco smoking, but also of the genetic and immunologic disturbances [1].

More and more investigations refer to the problems in the antitumour immunological response. In response to cancer antigens, dendritic cells (DCs) are the most effective antigen presenting cells [2]. DCs are rare, heterogeneous population of cells that are principally involved in the antigen presentation and stimulation of lymphocytes [2]. Antigenic fragments, after antigens processing in DCs, are exposed at the cell surface through major histocompatibility complex (MHC) responsible for an efficacious antigen presentation [2]. DCs bear costimulatory molecules and possess ability to produce critical cytokines (chemokines, IL-12), thus ensuring the initiation and the fate of acquired immunity [3].

IL-12 stimulates the cell-mediated immune response [4]. This cytokine, in co-operation with IL-2, causes proliferation and activation of Th_1 cells and the cytotoxic activity induction against microbial pathogens and tumour antigens [2,4]. The interaction of NK cells and cytotoxic T lymphocytes (CTLs) with tumour cells involves, at least partly, the binding of the CD95 (Fas/APO-1) antigen present on tumour cells by the CD95 Lig-

Table 1. **Phenotype of lymphocyte in peripheral blood of examined lung cancer patients and in the healthy subjects**

	% granulocyte	% lymphocyte	% CD3+ cells	% CD19+ cells	% CD4+ cells	% CD8+ cells	CD4:CD8 ratio	% NK cells	% CTLs
Lung cancer patients	73.6±9.7%	15.8±7.6%	64.6±10.7%	12.1±8.2%	35.4±11.9%	34.6±10.7%	1.24±0.79	15.4±8.1%	13.1±7.3%
Healthy donors	55.5±7.6	32.2±7.3%	67.6±8.1%	10.9±4.5%	40.2±10.0%	32.1±7.8%	1.31±0.57	14.4±6.3%	10.9±5.5%

and (FasL) present on NK cells and CTLs [5]. The activation of the CD95 antigen results in tumour cell apoptosis [4-7].

Tumour cells are capable of evading the immune system by numerous methods [8]. Tumour-associated macrophages and tumour cells as well as specific type of DCs may secrete immunomodulatory cytokines, such as IL-10, which antagonise the activities of IL-12 [8,9]. IL-10 is a main factor promoting the commitment of naive lymphocytes to Th_2-type profile of cytokine production, which is associated with humoral immune response and immune tolerance [9-11].

The aim of the study was to demonstrate the relationship between percentage of immature dendritic cells and percentage of CTLs subtypes in peripheral blood of non-small cell lung cancer.

Material and methods

The study population comprised of 10 non-small cell lung cancer subjects (the mean age: 61.8±10.55). Five men suffered from squamous lung cancer. The giant cell carcinoma was diagnosed in one man and in one woman. The adenocarcinoma appeared in three men. IIIb stage of lung cancer was diagnosed in five patients, stage IIIa in three patients and stage II in two subjects. The phenotype lymphocytes and CTLs status was also performed in peripheral blood of 22 healthy volunteers.

Peripheral blood samples were collected in heparinised tubes. Mononuclear cells were isolated by density gradient centrifugation on Gradisol L (Aqua Medica, Poland). Interphase cells were removed, washed twice in PBS without Ca^{2+} and Mg^{2+} (Biomed, Poland) containing 0.5% bovine serum albumin (BSA) and 2 mM EDTA (Sigma, Germany) and than resuspended in the mentioned buffer for future immunostaining of DCs.

Three-colour flow cytometry technique was used to establish peripheral blood leukocytes subtypes. The following directly conjugated monoclonal antibodies were used: Simultest (Becton Dickinson), mouse anti-human CD57 FITC, CD8 PerCP, CD19 CyChrome, CD123 PE, CD25 PE, CD69 PE, IL-10 Receptor PE and IL-12 Receptor PE (Becton Dickinson, Pharmingen, USA), CD95Ligand PE (Caltag, USA), BDCA-1 FITC, BDCA-2 FITC (Miltenyi Biotec, Germany). Cells were collected in a FACS Calibur flow cytometer (Becton Dickinson, USA) and their phenotype was analysed using Cell Quest Software. An acquisition gate was established based on FCS and SSC that included all mononuclear cells. Density of antigens on cell surface was determined as mean fluorescence intensity (MFI).

The CTLs were identified as double CD8 and CD57 positive cells. The expression of activation markers and FasL, as well as presence of IL-12 and IL-10 receptor on CTLs was determined. Immunofluorescent staining was performed according to manufacturers' protocol using Lysing Solution (Becton Dickinson, USA). Each measurement contained 30 000 total events.

Myeloid DCs were defined as BDCA-1-positive and CD19-negative cells and lymphoid DCs as BDCA-2 and CD123 double positive cells. Non-specific staining was inhibited by adding 20 μl of FcR Blocking Reagent to 10^7 cells resuspended in the buffer prior the labeling. The cells were incubated for 10 min in the dark at 4°C and washed in the buffer afterwards. Each flow cytometry measurement contained 300 000 total events [12].

Statistical analysis was performed using non-parametric Spearman correlation test, Wilcoxon matched pair test as well as Statistica 5.0 software.

Results

In lung cancer patients group, the percentage of myeloid DCs was slightly higher than the percentage of lymphoid DCs (0.19±0.15% and 0.16%±0.20%, respectively). The myeloid to lymphoid DCs ratio was 3.05±4.40.

The peripheral blood lymphocyte subtypes were represented by the following percentage of cells: T lymphocytes – 64.6±10.7%, B lymphocytes – 12.1±8.2%, T helper cells – 35.4±11.9%, suppressor-cytotoxic T cells – 34.6±10.7%, NK cells – 15.4±8.1%. CTLs CD8 and CD57 double positive cells was 13.10%. 45.7±13.0% of CD8-positive cells had CD57 antigen (*Tab. 1*).

FasLigand was expressed on 13.31±14.42% of CTLs. Activation markers (CD69, CD25) appeared on 18.05±12.32% and 5.02±6.05% of CTLs. Slightly lower percentage of CTLs expressed receptor for IL-12 (65.10±21,87%) than receptor for IL-10 (72.96±17.75%). Expression of FasLigand and receptors for IL-12 as well as IL-10 on CTLs surface was following: 46.84±61.51 MFI, 29.73±10.42 MFI, 31.01±15.89 MFI (*Tab. 2*).

The percentage of granulocyte was significantly ($p<0.005$) higher and the percentage of lymphocyte was significantly ($p<0.005$) lower in PB of lung cancer patients than of healthy donors. The CD4:CD8 ratio was slightly ($p=0.12$) lower in lung cancer group compared to the control group. The percentage of early activated, double positive CD8, CD69 lymphocytes was significantly ($p<0.05$) higher in lung cancer patients than in the control group. There were no significant differences in the percentage of CTLs with IL-2R, CD69 and FasL expression between the lung cancer and the control groups (*Tab. 1, 2*). The expression of FasL on CTLs as well as the percentage of

Table 2. **CTLs status characteristic in the patients with lung cancer and in the healthy donors**

	% CTLs among CD8+ cells	% FasL+ CTLs	Exp. FasL on CTLs (MFI)	% CD69+ CTLs	% CD25+ CTLs	% IL12R+ CTLs	Exp. IL-12R on CTLs (MFI)	% IL10R+ CTLs	Exp. IL-12R on CTLs (MFI)
Lung cancer patients	45.7±13.0%	13.31±14.42%	46.84±61.51	18.05±12.32%	5.02±6.05%	65.10±21.87%	29.73±10.42	72.4±18.2%	31.01±15.89
Healthy donors	40.6±14.3%	15.8±12.0%	29.9±26.8	20.2±15.9%	3.9±3.9%	70.8± 20.7%	28.51±8.22	79.7±17.5%	36.44±17.37

Figure 1. **Correlation between percentage of lymphoid DCs and percentage of CTLs in peripheral blood of lung cancer patients**

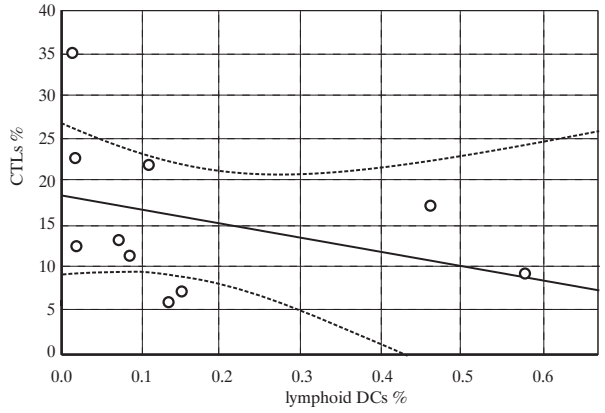
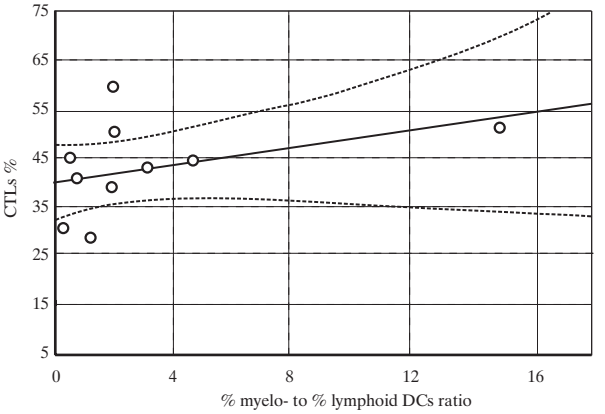


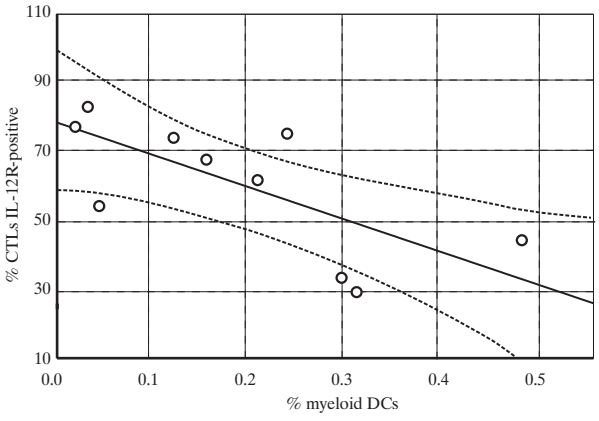
Figure 2. **Correlation between myeloid- to lymphoid DCs ratio and percentage of CTLs in peripheral blood of lung cancer patients**



CTLs with IL12R and IL10R were slightly higher in lung cancer patients than in healthy subjects.

Significant negative correlation ($R=-0.693$, $p<0.05$) between the percentage of lymphoid DCs and the percentage of CTLs was shown (*Fig. 1*). The myeloid to lymphoid DCs ratio significantly positively ($R=+0.638$, $p<0.05$) correlated with the percentage of CTLs (*Fig. 2*). The significant negative correlation between the percentage of myeloid DCs and the percentage of CTLs with IL-12R (*Fig. 3*) as well as expression of this receptor were also ascertained ($R=-0.68$, $p<0.05$ and $R=-0.757$, $p<0.01$, respectively).

Figure 3. **Correlation between percentage of myeloid DCs and percentage of CTLs with expression of IL-12R in peripheral blood of lung cancer patients**



Discussion

Dendritic cells are highly effective antigen-presenting cells [14]. They are raised from hematopoietic stem cells in the bone marrow and are widely distributed as immature DCs into both lymphoid and non-lymphoid tissues [15,16]. The potential mechanisms controlling the production of DCs or their progenitors within bone marrow are not known [2,16,17]. Nor it is known whether these putative controls are autonomous, exclusive of bone marrow, or dependent on exogenous-paracrine influences, or affected by external environmental stimuli like microbial products [2].

The DCs population represents only a minute subpopulation (less than 1%) of the peripheral blood mononuclear cells [12,18]. The recent studies have demonstrated that in human peripheral blood exist at least two subpopulations of circulating immature dendritic cells: myeloid and lymphoid subsets [13,18,19]. These two subpopulations of PBDCs (peripheral blood dendritic cells) are characterized by expression of specific markers for circulating DCs – blood dendritic cells antigens: BDCA-1 and BDCA-2 [12,13]. BDCA-1, which in cluster differentiation correspond to CD1c antigen, is expressed on human myeloid subpopulation of PBDCs [12]. BDCA-1-possitive cells express myeloid markers and secrete IL-12 after stimulation of foreign antigens [12]. BDCA-2 is specifically expressed on human plasmacytoid (lymphoid) dendritic cells and it is postulated that BDCA-2-positive cells produce a large amount of IL-4 after antigen stimulation [13]. These two subsets of peripheral blood dendritic cells were claimed to stimulate Th1 and Th2 types of immune responses,

respectively, and hence, were termed by some investigators as DC1 and DC2 [12,16]. However, other reports suggest that differentiation of naïve T cells is also determined by cytokine environmental (IL-12 versus IL-4) and the activation state of DCs [16,19].

Our recent investigation confirmed that the percentage of both myeloid and lymphoid DCs was lower in patients with NSCLC than in the healthy subjects. Furthermore, percentage of immature DCs in peripheral blood of cancer patients may correspond to the type of neoplasm [20]. In another study, we showed expression of IL-12R and IL-10R on peripheral blood CTLs in lung cancer patients. Percentage of IL-12R-positive cells was lower in lung cancer patients than in the healthy persons. The phenotype of CTLs was depended on immunological system status (presence of inflammation process) and clinical manifestation of the disease [10,21,22].

Our observation presented in this paper is a pilot study on the quantitative analysis of dendritic cells and allows us to conclude that the connection between percentage of immature DCs and CTLs appeared in peripheral blood of lung cancer patients. We affirmed negative correlation between percentage of myeloid DCs and percentage of CTLs with expression of IL-12 receptor. We hypothesised, that this relationship is in connection with compartmentalisation of immunological response in lung cancer. In NSCLC patients, myeloid DCs may migrate from peripheral blood to tumour tissue and metastatic lymph nodes. Mature DCs in lymph nodes could produce IL-12 and stimulate CTLs. However, it is postulated that the mature DCs may be malfunctioning in the cancer-bearing patients [8,22].

The correlation between percentage of DCs and percentage of CTLs may be also independent phenomenon. In this coincidence, numerous of factors connected with development of lung cancer may posses the independent ability to influence on percentage of bone marrow-derived DCs and CTLs [8]. Third hypothesis is based on the data, that not only mature DCs seeded in tissues and lymph organs, but also immature DCs in peripheral blood could process the tumour antigens and produce IL-12 [9]. Associated with mentioned facts, we concluded that direct relationships between percentage of immature DCs and phenotype and percentage of CTLs in peripheral blood of NSCLC patients may exist. Therefore, establishing these correlation is still an open question and further studies are clearly needed to examine these relationships.

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