Phenotypic and functional properties of generated dendritic cells in lung cancer patients

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ABSTRACT

The application of dendritic cells (DC) as a «natural adjuvant» is widely used in various fields of cancer immunotherapy. This study is aimed to investigate phenotypic and functional characteristics of dendritic cells (DCs) generated from monocytes of peripheral blood of healthy volunteers and cancer patients.

MATERIAL AND METHODS. Dendritic cells were generated from the monocytes of 40 patients with non-small cell lung cancer (NSCLC) at IIB-IIIA stages; the control group consisted of 10 healthy individuals. Monocytes were isolated from blood using Histopaque (1.077 g/mL) and cultured in RPMI-1640 medium supplemented with 1 % autologous plasma, 100 ng/ml of human GM-CSF and 20 ng/mL of IL-4 for 8 days. On the day 6 we used autologous lyophilized tumor cells as a source of tumor-associated antigens. One day later we added LPS (100 ng/mL) and 2αb-IFN (10,000 units/mL). Phenotypic and functional characteristics of DCs were identified by flow cytometry and real-time PCR.

RESULTS. We have found that in lung cancer patients generated DCs had moderate level of maturity and demonstrated more pronounced tolerogenic features in contrast to DCs of healthy volunteers: patients’ DCs had higher mRNA expression levels of TGF-β and IDO, and secreted lower amount of IL-12. Expression of CCR7 gene was particularly on the normal level in DCs of cancer patients, which indicates on saving of migratory properties of these cells. Expression of DCs’ maturity marker CD83 increased after each subsequent vaccine administration while TGF-β, IL-10 mRNAs levels decreased to the end of vaccine therapy course decreased to the level observed in DCs of healthy volunteers.

CONCLUSION. Thus, the study of biological characteristics of DCs will help to improve and develop the most effective protocols for rational use of DC vaccines. These data indicate the need for further optimization of technologies of DC generation in patients with lung cancer with emphasis on the stimulation of Th1-polarizing properties by increasing cytokine-secreting potential.

KEYWORDS: lung cancer; immunotherapy; dendritic cells; TGF-β; IDO; IL-12
The degree of maturity is considered an extremely important DCs’ characteristic in their use as a natural adjuvant in cancer vaccines [9-11]. This is because the use of DCs immature phenotype that have weak immunogenicity may lead to abortive proliferation and anergia of effector cells, induction of tolerance to tumor antigens as a result of activation of CD4+ and CD8+ regulatory T-cells (Treg), which secrete IL-10 and TGF-β.

However, any clear criteria for DCs standardization in creating cancer vaccines do not exist. They must meet a number of requirements that reflect their ability for antigen presentation and stimulation of T-cells: to be viable; to express high levels of markers of maturity of costimulatory and adhesion molecules; to secrete cytokines; to be able to activate CTLs and Th1-mediated immune response; as well as to express chemokine receptor CCR7, which is required for DCs migration to lymph nodes [12-14].

In cancer patients the functional activity of DCs derived from precursors is usually reduced, in particular, they are often resistant to many activating stimuli and remain immature. Anticancer chemotherapy and radiation therapy may also cause reduced effectiveness of formation and inhibition of DCs maturation, which can complicate and limit their application [15, 16]. At the same, immature or partially mature DCs are also an optimal population for their use in anticancer immunotherapy [17-19]. In particular, the first commercial vaccine Sipuleucel-T (Dendreon, USA) for the treatment of patients with hormone-refractory metastatic prostate cancer, approved by the FDA, contains a combination of immature and partially mature DCs loaded with peptides of prostate-specific antigen [20]. On the other hand, some authors note a significant drawback in mature DCs – a lack of a homing receptor CD62L for migration to the lymph nodes through the endothelium [21, 22].

At present, the optimal combination of DCs maturation inducers for their use in cancer immunotherapy is not described, and protocols to obtain functionally mature DCs are not standardized. Therefore, finding the most effective combinations of factors to produce functionally mature DCs, suitable for the use in cancer vaccines, remains an urgent problem. Much attention is paid to the use of toll-like receptors ligands (TLR), including lipopolysaccharide (LPS) or poly(I:C) [23, 25]. The use of TLR ligands in combination with cytokines provides DCs maturation, accompanied by phenotypic and functional changes, such as the acquisition of Th1 polarizing potential [24, 26].

Our previous studies have shown that the combination of two activating signals at the obtaining of monocyte-derived DCs – IFN-α and LPS, has a significant modulating effect on their cytokine- and chemokine secretory activity, causing prevalence of proinflammatory potential and improving viability [27]. The obtained results justified the use of this obtaining DCs technology for immunotherapy of non-small cell lung cancer (NSCLC) patients.

Assessment of efficacy of anticancer vaccines based on DCs in patients with NSCLC was conducted under phase III clinical trials «Randomized, double-blind, parallel-group study of the effectiveness of dendritic cell autovaccine in combination with standard surgical treatment for patients with non-small cell lung cancer stage IB-IIA ». This study determined the efficacy of inclusion of cancer vaccine based on DCs in the treatment of NSCLC patients in the adjuvant setting mode and established the features of cell-mediated immune response in patients under the influence of anticancer immunotherapy.

The study found that the use of antitumor vaccines based on DCs in the treatment of NSCLC patients contributed significantly to increase their 5-year overall and disease-free survival, lengthening the time to disease progression, and increase in a survival median [28]. In particular, the rate of the overall 5-year survival of NSCLC patients increased by 25 % p < 0.001; HR = 0.22 (95 % CI: 0.13-0.39).

At present, it is important to determine in clinical trials DCs’ characteristics that have therapeutic effectiveness. Accordingly, the purpose of the work was to investigate the phenotypic and functional properties of DCs of monocyte origin, as a basis in anticancer immunotherapy of patients with non-small cell lung cancer.

Dendritic cells culture. DCs were generated from the monocytes of 10 healthy people aged 23 to 45 years (34.9 ± 5.4) and 40 patients with NSCLC IB-IIA stages aged 42 to 82 years (53.9 ± 3.4). Peripheral blood mononuclear cells were obtained using Histopaque-1077 (Sigma, USA) separation technique, after which the cells were resuspended in RPMI-1640 medium (Sigma, USA) supplemented with 2 µM L-Gly, 100 µg/mL streptomycin and 100 units/mL penicillin (Darmtsa, Ukraine) and incubated in a culture flask at 37 °C, 5 % CO2 atmosphere for 2-3 hours. After non-adherent cells were removed by gentle washing, the concentration of adherent cells was adjusted to 0.5×10^6/mL by culture medium, added with 1 % autologous plasma and 100 ng/ml of human GM-CSF Leucmax (Novartis, India), 20 ng/mL IL-4 (Sigma, USA) and cultured for 8 days in CO2 incubator at 37 °C and 5 % CO2. Growth factors were added to DCs again on the 3rd day of cultivation. On the 6th day of cultivation, as a source of tumor-associated antigens, we used autologous lyophilized tumor cells at a concentration of 0.05 mg/ml culture medium. On the 7th day of maturation we added 100 ng/ml LPS (Sigma, USA) and 10,000 units/mL IFN-2b «Leterabor» (Biopharma, Ukraine). All manipulations were carried out under aseptic conditions.

In NSCLC patients, autologous DCs-based immunotherapy was administered in an adjuvant mode, after primary treatment. Immunotherapy started 10-14 days after surgery. DCs were injected intravenously at a dose 3.0-10.0×10^7 cells per injection. All patients underwent 4 treatment stages at intervals of one per month. The written informed consent was obtained from all patients. The research was conducted according to ethical standards adopted by Ukrainian legislation. In NSCLC patients they were conducted under phase III of clinical trials approved by Central Ethics Committee of the Ministry of Health of Ukraine (No. 5.12-1201/KE, 05.11.2009).

Immunological methods. Analysis of DCs’ phenotypic characteristics was performed by flow cytometry using FITC–conjugated monoclonal antibodies to CD83, CD86, and phcoerythrin-labelled antibodies to HLA-DR (Beckman Coulter, USA). Analysis of samples was performed with BD FACS Calibur flow cytometer (Becton Dickinson, USA) using CellQuest PRO software (Becton Dickinson, USA).

Quantitative real time polymerase chain reaction (qPCR) analysis. The total RNA was isolated from DCs using «Ribo-sol-A» (Amplisens, Russia) according to manufacturer’s instructions. For the reverse transcription reaction, we used PCR test kit «Reverta-L-100» (Amplisens, Russia).avage of gene expression level of cytokines was evaluated by the 7500 Real-Time CT method (Ex =10-1/slope < 0.1) [20]. The effectiveness of PCR reactions was similar (Ex =10-1/slope < 0.1) [20]. The of gene expression level of cytokines was evaluated using ΔΔCT method with normalization according to expression control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Determination of cytokine IL-12p70 concentration in culture medium of differentiated DCs was performed using IL-12(p70) ELISA Kit Human (Thermo Scientific, USA).

Statistical methods. Statistical analysis was conducted using the Statistica 10.0 software (Statsoft Inc., USA). To compare the data in two groups we used Student’s t-test and Mann-Whitney test. The difference was considered statistically significant at p < 0.05.
RESULTS AND DISCUSSION

DCs quality control included evaluation of phenotypic characteristics at the final stage of DCs vaccine production just before its administration. DCs phenotype indicate the readiness of cells to perform certain functions: interaction with T-cells, contact with tissue microenvironment, and secretion of cytokines. The main DCs markers that characterize the degree of their maturity and functional capacity are surface molecules CD80, CD86, CD40, CD83 and HLA-DR, and others. [29-31]. DCs phenotype depends on the stage of maturation and activation.

Studies have shown that DC of the patients with NSCLC before immunotherapy were partially mature (Fig. 1). Thus, the level of simultaneous expression of CD86+ and HLA-DR+ was 63.50 ± 2.15 %, immunotherapy were partially mature (β). Thus, the level of phenotype depends on the stage of maturation and activation.

Fig. 1. Phenotypic characteristics of DCs of NSCLC patients at stages of immunotherapy (n = 34). HV – healthy volunteers (n = 10).

Note: * – p < 0.05 compared with the values before the immunotherapy.

After the four stage of immunotherapy, the difference in levels of mRNA expression of CCR7 in DCs of NSCLC patients and healthy people became statistically significant.

Along with the ability to induce activation of immunocompetent cells, DCs may suppress the immune response. Tolerogenic regulatory properties of DCs are due to different mechanisms, including the ability to express co-inhibitory molecules and receptors (B7-H1, ILT-2, ILT-3, ILT-4, CD209, CD200R and HLA-G), producing immunosuppressive cytokines (IL-10, IDO, TGF-β) and induce generation of CD4+CD25+ Treg [34-36].

Tolerogenic effects of DCs are accompanied by the initiation or increasing expression of certain molecules, which requires activation of genes. According to the results of studies, mRNA expression of immunosuppressive molecules in DCs of NSCLC patients is increased compared to DCs of healthy volunteers – p < 0.05 for TGF-β (Fig. 3).

By stage four of immunotherapy, the level of TGF-β mRNA expression decreased to the level of DCs of healthy volunteers. The level of IDO mRNA expression also significantly reduced at stages III-IV immunotherapy, its level was significantly lower, compared to that in DCs before the beginning of immunotherapy and levels in DCs of healthy individuals.

The main cytokine that determines Th1-polarizing properties of DCs is IL-12. Bioactive IL-12p70 is a heterodimeric complex composed of two subunits p35 and p40. Our research showed that the expression of these cytokines in DCs of NSCLC patients (the beginning of immunotherapy) and in DCs of healthy people do not have significant differences (Fig. 4).

Slightly lower level of mRNA IL-12p35 and immunosuppressive IL-10 was registered in DCs of healthy volunteers.

Measurement of anti-apoptotic Bcl-2 protein using flow cytometry revealed that its level in DCs in NSCLC patients is different from the level in healthy volunteers, and almost unchanged at all stages of immunotherapy. Thus, the percentage of cells expressing Bcl-2 and intensity of expression (mean fluorescence intensity – MFI) in DCs of NSCLC patients amount to 97.51 ± 0.73 % and 1001.16 ± 247.17 MFI units vs 98.93 ± 1.15 % and 1123.36 ± 215.16 MFI units in healthy people respectively.

Realization of DCs function is largely determined by their localization in the tissue and the ability to migration. DCs migration is regulated by the interaction of chemokines with their receptors, various proteases and their respective receptors, such as urokinase plasminogen activator-system (uPA)/uPAR. The main chemokine receptor responsible for DCs migration is CCR7. Chemokines CCL19 (ELC) and CCR21 (SLC), recognized by the receptor CCR7, provide the migration to the T-cell zone of the lymph nodes. These chemokines are secreted by stromal cells in lymph nodes.

According to the results of our studies, activity of CCR7 gene in DCs from NSCLC patients is almost unaffected, the level of mRNA gene expression is even slightly higher than in healthy volunteers’ DCs (Fig. 2).

Fig. 2. The level of mRNA CCR7 expression in DCs of NSCLC patients with at stages of immunotherapy (n = 16). HV – healthy volunteers (n = 10).

Note: * – p < 0.05 compared with the values before the immunotherapy.
At the same time, study of bioactive IL-12 amount in DCs culture medium found violations of its synthesis in NSCLC patients. Thus, the amount of this cytokine in DCs culture medium of NSCLC patients was significantly lower than in healthy volunteers (Fig. 5). The data indicate the need for further optimization of technologies for obtaining DCs in NSCLC patients, with emphasis on the stimulation of Th1-polarizing properties by increasing cytokine secretory potential.

The use of the integrated approach to the creation of autologous cancer vaccines using modern high-tech methods, based on biological characteristics of DCs, will help improve and develop the most effective protocols of making and rational use of DCs vaccines. This will ensure greater confidence of both professionals and patients in anticancer vaccinotherapy and open new opportunities for the treatment of patients with cancer.

**CONCLUSIONS**

1. Dendritic cells populations in non-small cell lung cancer patients are characterized by increase of partially mature cells percent, indicating a violation of their differentiation.
2. The level of CD83 expression, which determines the degree of DCs maturity, increases during cancer immunotherapy.
3. CCR7 gene activity in DCs of NSCLC patients with is almost unaffected, which may indicate a preservation of their migratory properties.
4. The level of expression anti-apoptotic protein Bcl-2 in DCs of NSCLC patients is different from the level in DCs of non-cancer people, and unchanged at the stages of immunotherapy.
5. The level of TGF-β and IDO mRNA expression in DCs of NSCLC patients significantly increased in comparison with DCs of healthy volunteers. Their level significantly reduces during cancer immunotherapy.
6. In DCs of NSCLC patients, the synthesis of bioactive IL-12 is violated, indicating the need for further optimization of technologies for obtaining Th1-polarizing DCs in NSCLC patients.

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23. The authors indicate no potential conflicts of interest.

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