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Phenotype of dendritic cells generated in the presence of non-small cell lung cancer antigens – preliminary report

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Abstract: Therapeutic outcomes of definitively treated non-small-cell lung cancer (NSCLC) are unacceptably poor. It has been proposed that the manipulation of dendritic cells (DCs) as a "natural" vaccine adjuvant may prove to be a particularly effective way to stimulate antitumor immunity. Presently, there is no standardized methodology for preparing vaccines and many questions concerning the optimal source and type of antigens as well as maturation state and activity of DCs are still unsolved. The study population comprised of ten patients with histologically confirmed NSCLC (mean age: 67.63 ± 6.15 years). Resected small tumor pieces were placed in tissue culture dishes containing different growth factors in order to obtain pure cancer cells. Seven days after the operation, the PBMC were collected and monocytes were purified by the adherence to culture dishes. Monocytes were cultured in RPMI 1640 medium supplemented with 10% of autologous plasma in the presence of rhIL-4 and rhGM-CSF to generate immature autologous (DCs). TNF-α with or without tumor cells' lysate were added to maturation of DCs. After 7 days of culture, DCs were harvested and the expression of CD1a, CD83, CD80, CD86 and HLA-DR antigens were analyzed by flow cytometry. We discovered higher (p=0.07) percentage of semi-mature DCs in tumor cell lysate culture in comparison with TNF-α culture (21.22 ± 16.82% versus 11.27 ± 11.64%). The expression of co-stimulatory and maturation markers (CD86, CD83 and HLA-DR) was higher on DCs from the culture with tumor cell lysate compared with TNF-α culture as a control. Specimen of NSCLC's culture prepared in this way could generate differences in DCs phenotype, which may have an influence on the therapeutic and protective antitumor immunity of the vaccine. Our research seems to be the next step in the development of DC-based vaccine. We are going to continue the investigation to start the preparation of a pattern of immunological vaccine against lung cancer.

Key words: dendritic cells, non-small cell lung cancer, vaccine

Introduction

In the 20th century, the incidence and mortality due to lung cancer dramatically increased in most European countries. Despite recent advances in the treatment of non-small cell lung cancer (NSCLC), clinical outcomes remain poor. For several decades effective immune-based anti-tumor therapy has been sought. There is a strong evidence that the immune system, when properly stimulated, can eradicate cancer cells.

Dendritic cells are potent antigen-presenting cells that have been investigated as components of a tumor vaccine. DCs functions depend not only on the subset division (the phenotype) but also on the environmental stimuli (such as cytokines and immunogens) and their maturation status. The DCs process captures proteins into peptides that are loaded onto major histocompatibility complex class I and II (MHC I and II) molecules, and these peptide-MHC complexes (pMHC I and II) are transported to the cell surface for the recognition of antigen-specific T cells. (pMHC I and pMHC II are recognized by CD8+ and CD4+ T cells, respectively [1]. Maturation is a terminal differentiation process that transforms DCs from cells specialized in antigen capture into cells specialized in T-cell stimulation. This process is induced by components of pathogens or by host molecules associated with inflammation or tissue injury. Maturation is characterized by the reduction of phagocytic activity, the development of cytoplasmic extensions or "veils", migration to lymphoid tissues, and enhancement of T-cell activation potential [2,3].

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The DCs phenotype varies, depending on the stages of maturation and differentiation. CD1a is preferentially expressed on human immature myeloid DCs, whereas CD83 is typically upregulated in response to the activation stimuli such as TNF-α, TLR, LPS etc. DCs change their antigen presentation properties during maturation. It is well established that immature DCs are characterized by the intermediate surface expression of an MHC class II and low level co-stimulatory molecules CD80 and CD86 [4]. CD80 and CD86 were expressed at high levels on DCs, particularly after maturation. These antigens functioned to costimulate T cell activation, especially high level production of IL-2 that typifies T cell stimulation by DCs. The function of CD80 and CD86 on DCs *in vivo* should be studied directly.

Immunotherapy of cancer is a field of intense research, especially the induction of active immunity with either therapeutic or adjuvant purpose. Tumor vaccines may have an adjuvant role in surgically resectable and unresectable NSCLC by consolidating responses to the conventional therapy. Presently, there is no standardized method for preparing vaccines and many questions concerning the optimal source and type of antigens as well as maturation state and activity of dendritic cells are still unsolved [5-8]. Therefore, the aim of our study was to investigate the influence of autologous tumor cell lysate on the phenotype of dendritic cells generated from peripheral blood of NSCLC patients.

Materials and methods

Patients. The study population comprised of ten patients with histologically confirmed NSCLC (mean age: 67.63 ± 6.15 years). We used laboratory tests, chest-X ray, computer tomography, ultrasonography and bronchoscopy for the evaluation of lung cancer stage and the type of disease manifestation.

Generation of dendritic cells from adherent monocytes. The blood product, generally 40 ml, was collected aseptically from the patients and diluted 1:1 with sterile magnesium and calcium free phosphate buffered saline (PBS). The diluted blood was placed in 15 ml centrifuge tubes and underplayed with Lymphoprep (Nycomed, Norwegia). The tubes were centrifuged for 20 min. at 400 × g. The buffy coat was removed under the laminar flow hood, transferred to new tubes and washed twice in full volume PBS. The harvested PBMC were split into 6-well dishes. The culture medium consisted of RPMI 1640, 10% autologous plasma and 1% v/v of antibiotics (Penicilin, Streptomycin). The mononuclear cells were incubated in the culture medium for 1.5 h at 37°C in humidified 5% CO_2 atmosphere. After 1,5 h incubation, non-adherent cells were removed. Adherent cells were then cultured in the presence of rhGM-CSF (1000 IU/ml, Gentaur, Belgium) and rhIL-4 (500 IU/ml, Gentaur, Belgium). The cytokines were added on 3rd and 5th days of the culture. Maturation of dendritic cells was induced on 6th day using tumor cell lysates and TNF-α (1000 IU/ml, Strathmann, Niemcy). In the control culture, the maturation of DCs was induced only by the stimulation with TNF-α.

Preparation of tumor cell lysates. After surgical resection, small tumor pieces were placed in tissue culture dishes in Medium 199 containing different growth factors: (*i.a.*, Medium 199 supplemented with 10% fetal bovine serum, EGF, transferrin, insulin, hydrocortisone, glutamine and antibiotics. The culture plates were maintained in an appropriate culture microenvironment at 37°C in a humidified 5% CO_2 atmosphere. The medium was changed every 48 hours. After two weeks, confluent cultures of NSCLC cells were incubated with Trypsin/EDTA-solution for 10 min, carefully detached with a cell scraper, washed twice in Medium 199, and centrifuged for 5 min. at 300 × g. The cell suspensions at a density 106 cells/ml of Medium 199 were homogenized in four repeated cycles of freezing at -80°C and subsequent thawing. The lysates were centrifuged and supernatants were passed through a 0.2 μm millipore filter. Lysate, prepared on this way, was added to the dendritic cells culture of DCs.

Flow cytometric analysis. On day 7, at the end of the culture, supernatants were collected and adherent cells were harvested by the incubation with Trypsin/EDTA solution. After 15 minutes of incubation, the floating cells were blended with supernatants, washed twice in PBS without Ca^{2+} and Mg^{2+} and resuspended prior to immunophenotyping. To characterise the immunophenotype of generated dendritic cells, panel of FITC-, PE- or TC-conjugated monoclonal antibodies (Becton Dickinson or Caltag, USA) were used: mouse anti-human IgG1/IgG2a/IgG2a (negative control), mouse anti-human CD45/CD14, mouse anti-human CD83/CD1a/HLA-DR, mouse anti-human CD80/CD86/HLA-DR.

The cells were incubated for 20 minutes in dark and afterwards washed in PBS without Ca^{2+} and Mg^{2+}. The phenotypic characterization of DCs was carried out using a FACS Calibur flow cytometer (Becton Dickinson, USA) and CellQuest software. Cytometer was calibrated and mean fluorescent intensity was calculated.

Statistical analysis. Statistical analysis was performed using the non-parametric *U* Mann-Whitney rang test and the Wilcoxon matched pair test in the Statistica 6.0 software.

Results

After eight days of DCs cultures using flow cytometry, cells were harvested and analyzed. According to the expression of CD1a antigen, we recognized: *semi-mature* dendritic cells as CD83+/CD1a+/HLA-DR+ cells; *mature* dendritic cells as CD83+/CD1a-/HLA-DR+ cell; *immature* dendritic cells as CD83-/CD1a+/HLA-DR+ cells.

Higher (p=0.07) percentage of semi-mature DCs was discovered in tumor cell lysate culture in comparison with TNF-α culture (21.22 ± 16.82% versus 11.27 ± 11.64% respectively). The expression of co-stimulatory and maturation markers (CD86, CD83 and HLA-DR) was higher on DCs from the culture with tumor cell lysate compared with TNF-α culture. In this study we observed a significantly higher (p=0.05) expression of CD83 on CD1a-/HLA-DR+ cells in tumor cell lysate culture in comparison with TNF-α culture (275.8 ± 97.08 MFI versus 238.9 ± 64.21 MFI respectively). We also found a significantly higher (p=0,02) expression of HLA-DR on CD83+/CD1a- cells in tumor cell lysate culture in comparison with TNF-α culture (252.16 ± 113.24 MFI versus 203.2 ± 93.03 MFI, respectively) (Fig. 1).

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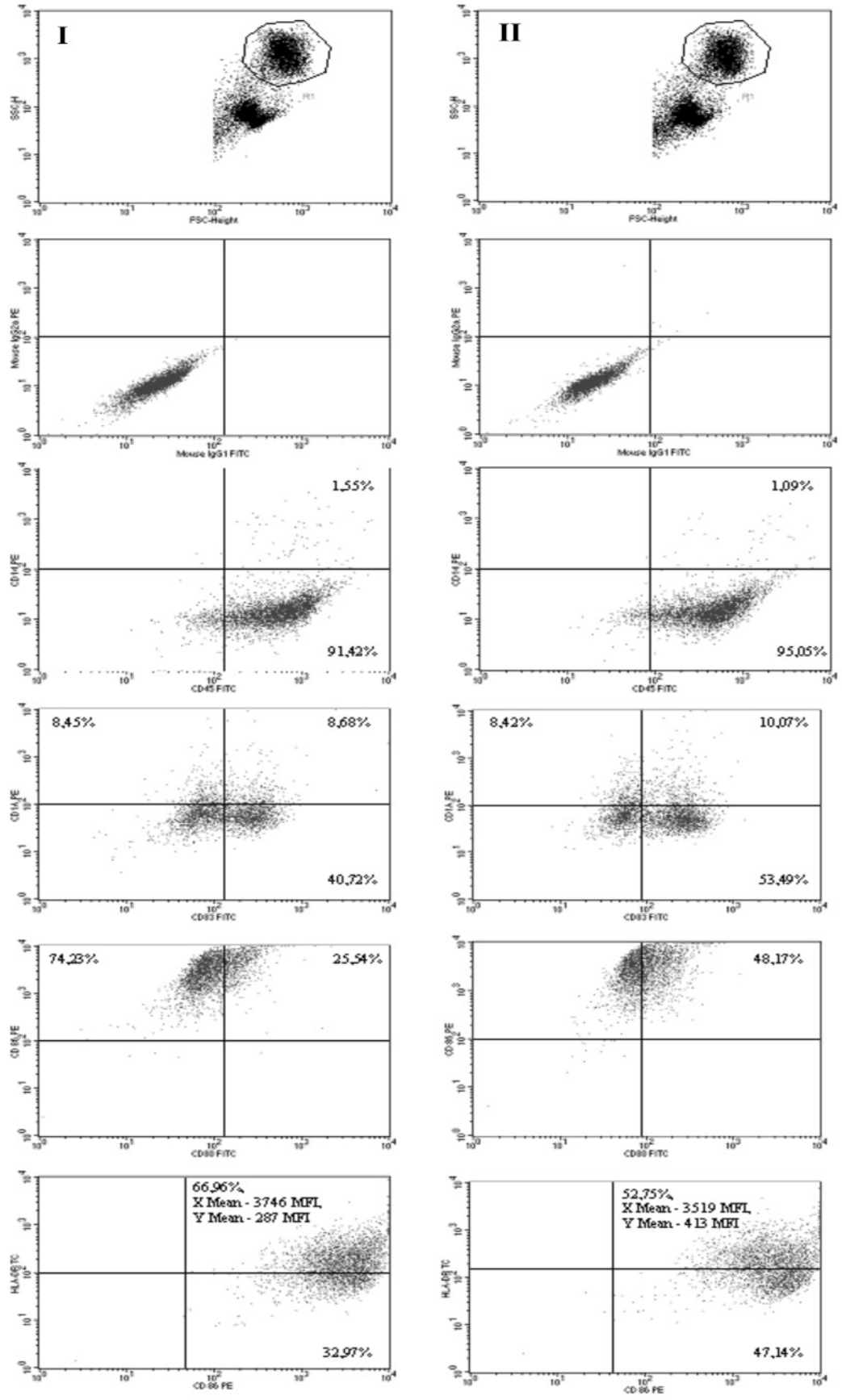

Fig. 1. Exemplary immunophenotype analyze of dendritic cells generated in cultures with TNF-α (I) and TNF-α plus tumor cells lysate (II) in NSCLC patient.

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Table. 1. The percentage of subpopulations of dendritic cells in different cultures (*p=0.07).

	Immature DCs (%)	Semimature DCs (%)	Mature DCs (%)
TNF-α culture	24.54 ± 15.44	11.27 ± 11.64*	24.81 ± 13.58
TNF-α and tumor cell lysate culture	25.37 ± 20.38	21.22 ± 16.82*	28.92 ± 16.10

More than 95% of cells expressed CD86 antigen. However, the expression of CD80 was detected only in less than 15% of cells. The percentage of CD80+/CD86+ was lower in the culture with tumor cell lysate compared with TNF-α culture. The population of CD80-/CD86+ cells was characterized by higher (p=0.09) expression of CD86 co-stimulatory molecule in the culture with tumor cell lysate compared with TNF-α culture (3079.23 ± 964.36 MFI versus 2627.69 ± 920.25 MFI, respectively). The CD80-/CD86+ cells were also characterized by significantly higher (p=0.05) expression of HLA-DR in the culture with tumor cell lysate compared with the culture stimulated only with TNF-α (269.24 ± 159.76 MFI vs. 239.44 ± 138.88 MFI, respectively) (Table 1 and 2, Fig. 1).

Our research reveals that DCs-pulsed with tumor cell lysate has significantly different expressions of maturation markers and co-stimulatory molecules compared with DCs generated in the medium containing only TNF-α.

Discussion

Immature DCs possess high endocytic and phagocytic capacity permitting Ag capture, but express low levels of MHC class II molecules and co-stimulatory molecules on their surface. After microbial infection and tissue damage, DCs acquire a "mature" phenotype and the maturation process is associated with several coordinated events such as: loss of endocytic and phagocytic receptors, high level of expression of MHC class II on the cell surface and the increased production of co-stimulatory molecules, including CD80, CD86, CD40 as well as changes in morphology [9-11].

Our research showed, that autologous tumor cell lysate were sufficient to maturate autologous DCs. High expression of CD83 antigen indicates the change in maturation state of DCs. Interestingly, in this study we found that about 95% of cells expressed CD86 antigen, but CD80 antigen was detected only in less than 15% of cells on Day 9 culture. It seems that tumor cell lysate or cytokines cause the lack of CD80 antigen on "exhausted" DCs. It was possible that CD80 antigen could be undetectable by flow cytometry technique because of its very low expression. In the other hand, it is possible that CD80 antigens would undergo fragmentation and desquamation.

We have cultured DCs at the presence of GM-CSF, IL-4 and TNF-α. GM-CSF stimulates normal hematopoietic progenitor cells to differentiate *in vitro* and form colonies containing granulocytes and macrophages. Ryncarz and Anasetti have tested the hypothesis that CD34+/CD86+ cells are the common precursors of both macrophages and dendritic cells. CD34+/CD86+ marrow cells cultured in GM-CSF generated macrophages, but in the presence of GM-CSF plus TNF-α generated almost exclusively CD1a+/CD83+ dendritic cells. In contrast, CD34+/CD86- cells cultured in GM-CSF generated a predominant population of granulocytes, but in the presence of GM-CSF plus TNF-α they generated a variety of cell types. This study suggests that the expression of CD86 on hematopoietic progenitor cells is regulated by TNF-α and denotes differentiation towards the macrophages or dendritic cell lineages. These results reveal that the stimulation with TNF-α induces CD86 expression on original CD34+/CD86- cells. This population of TNF-α-induced CD86+ cells differentiated into CD1a+ dendritic cells in response to GM-CSF, in contrast to CD34+ cells with constitutive expression of CD86+, which differentiated into macrophages in response to GM-CSF. TNF-α, depending on immunophenotypes of cells and times of culture, could have different effects [12].

Dilioglous *et al.* induced human umbilical cord blood CD14+ monocytes and CD34+ stem cells to differentiate into dendritic cells using GM-CSF, IL-4 and TNF-α. RT PCR revealed that differentiating monocytes initially expressed CD86 mRNA while CD80

Table 2. The expressions of maturation markers and co-stimulatory molecules in different cultures (*p=0.09, **p<0.05).

	Expression of CD83 antigen on CD1a-/HLA-DR+ cells (MFI)	Expression of CD80 antigen on CD86+/HLA-DR+ cells (MFI)	Expression of CD86 antigen on CD80-/HLA-DR+ cells (MFI)	Expression of HLA-DR antigen on CD80-/CD86+ cells (MFI)	Expression of HLA-DR antigen on CD83-/CD1a- cells (MFI)
TNF-α culture	238.93 ± 64.21**	167.86 ± 54.27	2627.69 ± 920.05*	239.44 ± 138.88**	203.2 ± 93.03**
TNF-α and tumor cell lysate culture	275.87 ± 97.08**	188.81 ± 48.04	3079.23 ± 964.36*	269.24 ± 159.76**	252.16 ± 113.24**

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mRNA appeared on Day 2. CD14- and CD34-derived DCs prior to the functional assay were stimulated with LPS. A decrease in stimulation, as depicted by decreased T-cell activation, was significant when blocking antibodies against both CD80 and CD86 were added into the culture. However, the authors showed that this effect is detected on stem cell-derived DCs using only blocking antibodies to CD86 antigen [13].

There were many reports suggest that DCs with high expression of co-stimulatory molecules could be able to activate cytotoxic T lymphocytes and may be a promising alternative for cancer immunotherapy but the number of reports concerning lung cancer is limited.

Ishikawa *et al.* researched safety and feasibility of DC-vaccine and showed clinical response in patients with advanced non-small cell lung cancer or recurrent lung cancer received i.v. injections of αGalCer-pulsed DCs. Immature monocytes-derived DCs expressed HLA-DR, CD80, CD86, in all preparations. The expression of CD83 was marginal. Administration of αGalCer-pulsed DC was well tolerated and this therapy could be used safely even in patients with advanced disease [14].

Immature DCs were also investigated in the study by Chang G.C. *et al.* Autologous DCs were generated by cells co-cultured with autologous necrotic tumor cells which were derived from pleural effusion specimens to allow internalization of tumor antigens. It as shown that the proportion of cells expressing CD83 was higher in DCs co-cultured with TNF-α and with control PBMC lysate (42.5%) than in those co-cultured with TNF-α and with tumor lysate from a patient with lung carcinoma (23.8%). Contrary to our study, this result suggests that co-culture with the tumor lysate may inhibit the maturation of DCs. Minor to moderate increases in T-cell responses against tumor antigens were observed after DC vaccination in six of eight patients. One patient had minor tumor response and two patients had a stable disease [15].

In the study by Hirschowitz, DCs vaccines were generated from CD14+ precursors, pulsed with apoptotic bodies of an allogeneic NSCLC cell line that overexpressed Her2/neu, CEA, WT1, Mage2. After that, dendritic cell/T cell-derived maturation factor (DCTCMF) from culture supernatants was added to the culture. DCs were partially matured with DCTCMF that induced surface molecule expression but minimal cytokine production. Finally vaccine products were CD14-negative and the cell population expressed MHC class I (100%) and II (90%), coexpressed CD80 and CD86 (60% to 75%) and the maturation marker CD83 (15% to 30%). Six of 16 patients showed an antigen specific response, but immune responses were independent of stage and prior therapy. The authors therefore hypothesize that after the injection partially mature DCs interact with host inflammatory and resident cells to complete their maturation *in vivo* and subsequently induce the immune responses mentioned in this study [16].

After their first investigation, Hirschowitz *et al.* reported the results of a continuation study with similar inclusion criteria, immunization protocol, and analysis, using an immature DCs vaccine. The cell surface phenotype of the final vaccine product was as follows: CD83 about 5%, CD80/86 about 33%. Additional comparison of DCs phenotype revealed similar percentage of cells with maturation markers CD80 and CD83 pre- and post antigen pulsing. Immune responses were observed in 4/7 stage III unresectable, and 6/7 stage I/II surgically resected patients. Results indicate that immature DCs pulsed with apoptotic tumor cells have similar biological activity to DCs stimulated with DCTCMF [17].

Itoh *et al.* performed a clinical study of a vaccine using autologous DCs pulsed with CEA652 and cytokines. Generated DCs showed an immature phenotype with high expression of HLA-DR and CD86, no expression of CD14 and low expression of CD80 and CD83 [18]. Also Babatz *et al.* conducted a phase I/II clinical trial to determine the capability of DCs differentiated from monocytes and pulsed with a carcinoembyonic antigen-derived altered peptide (CEAalt) to induce specific CD8+ T cells in cancer patients. DCs population showed high percentage of cells expressing the marker of maturation: CD83 (86%) and co-stimulatory molecules CD80 and CD86 (93%). Vaccines prepared in this way efficiently expand peptide-specific CD8+ T lymphocytes *in vivo* and may be a promising, safe and feasible alternative for cancer immunotherapy [19].

Yoshida *et al.* analyzed the *in vitro*-responses to cells from metastatic brain tumor of lung adenocarcinoma using DCs from the peripheral blood. They found that the matured DCs, stimulated with autologous tumor lysates, displayed the typical surface phenotype of CD3+, CD45+, CD80+, CD86+ and developed a strong cytotoxic T lymphocyte activity [20].

The present study suggests that autologous DCs loaded with tumor antigens are fully competent and could present cancer antigens to T lymphocytes. A vaccine prepared in this way could serve as a novel immunotherapy method in lung cancer treatment. Presently, there is no standardized methodology for preparing vaccines and many questions concerning the optimal source and type of antigens as well as maturation state and activity of dendritic cells (DCs) are still unsolved. Our research seems to be the next step in the development of DC-based vaccine. We are going to continue the investigation to elaborate a pattern of immunological vaccine against lung cancer.

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doi: 10.2478/v10042-008-0078-4

References

[1] Guermonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol.* 2002;20:621-667.

[2] O'Neill DW, Adams S, Bhardwaj N. Manipulating dendritic cell biology for the active immunotherapy of cancer. *Blood.* 2004;104(8):2235-2245.

[3] Banchereau J, Palucka A K. Dendritic cells as therapeutic vaccines against cancer. *Immunology.* 2005;5:296-304.

[4] Kuo-Ching Sheng, Pietersz GA, Mark D. Dendritic cell: Activation and maturation – applications for cancer immunotherapy. *Curr Med Chem.* 2005;12:1783-1800.

[5] Cranmer LD, Trevor KT, Hersh EM. Clinical applications of dendritic cell vaccination in the treatment of cancer. *Cancer Immunol Immunother.* 2004;53:275-306.

[6] Steinman R. Some interfaces of dendritic cell biology. *APMIS.* 2003;111(7-8):675-97.

[7] Ardavin C, Amigorena S, Sousa CR. Dendritic cells: Immunobiology and cancer immunotherapy. *Immunity*. 2004; 20:17-23.

[8] Raez LE, Fein S, Podack ER. Lung cancer immunotherapy. *Clin Med Res.* 3(4):221-228.

[9] Sato K, Fujita S. Dendritic cells-nature and classification. Allergol Int. 2007;56:183-191.

[10] Watanabe S, Kagamu H, Yoshizawa H *et al.* The duration of signaling through CD40 directs biological ability of dendritic cells to induce antitumor immunity. *J Immunol.* 2003;171: 5828-36.

[11] Nouri-Shirazi M, Banchereau J, Fay J, Palucka K. Dendritic cell based tumor vaccines. *Immunology Letters*. 2000;74:5-10.

[12] Ryncarz RE, Anasetti C. Expression of CD86 on human marrow CD34+ cells identifies immunocompetent committed precursors of macrophages and dendritic cells. *Blood.* 1998; 91(10):3892-3900.

[13] Dilioglou S, Cruse JM, Lewis RE. Function of CD80 and CD86 on monocytes- and stem cell-derived dendritic cells. *Exp Mol Pathol.* 2003;75:217-227.

[14] Ishikawa A, Motohashi S, Ishikawa E, Fuchida H, Higashino K, Otsuji M et. al. A Phase I study of A-Galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res.* 2005;1(11):1910-17.

[15] Chang GC, Lan HC, Juang SH, Wu YC, Lee HC, Hung MY. A pilot clinical trial of vaccination with dendritic cells pulsed with autologous tumor cells derived from malignant pleural effusion in patients with late-stage lung carcinoma. *Cancer.* 2005;103(4):763-771.

[16] Hirschowitz EA, Foody T, Kryscio R, Dickson L, Sturgill J, Yanneli J. Autologous dendritic cell vaccines for non-small cell lung cancer. *J Clinl Oncol.* 2004;22(14):2808-2815.

[17] Hirschowitz E, Foody T, Hidalgo G, Yannelli J. Immunization of NSCLC patients with antigen-pulsed immature autologous dendritic cells. *Lung Cancer.* 2007;57(3):365-372.

[18] Itoh T, Ueda Y, Kawashima I, Nukaya I, Fujiwara H, Fuji N et. al. Immunotherapy of solid cancer using dendritic cells pulsed with the HLA-A24-restricted peptide of carcinoembryonic antigen. *Cancer Immunol Immunother.* 2002;51:99-106.

[19] Babatz J, Rollig Ch, Lobel B, Folprecht G, Hoack M, Gunther H et. al. Induction of cellular immune responses against carcinoembryonic antigen in patients with metastatic tumors after vaccination with altered peptide ligand-loaded dendritic cells. *Cancer Immunol Immunother.* 2006;55:268-276.

[20] Yoshida S, Morii K, Watanabe M, Saito T, Yamamoto T, Tanaka R. The generation of anti-tumoral cells using dendritic cells from the peripheral blood of patients with malignant brain tumors. *Cancer Immunol Immunother*. 2001;6(50):321-7.

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