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Department of Clinical Immunology, Medical University of Lublin
Pulmonary Department, Medical University of Lublin

AGATA SURDACKA, PAWEŁ KRAWCZYK, MAŁGORZATA DAŃKO, IWONA JASTRZĘBSKA, JANUSZ MILANOWSKI, JACEK ROLIŃSKI

The presence of dendritic cells in the malignant pleural effusion. The pilot study

Approximately half of all patients with metastatic cancer develop malignant pleural effusion. Pleural metastases occur in 7 to 15% of bronchogenic cancers. In these cases most malignant effusions are exudative and about one third are bloody. Pleural metastases cause a pleural inflammatory response that results in an increased capillary permeability leading to accumulation of fluid in pleural space. Bloody effusion may be formed when lung cancers are associated with invasion or occlusion of blood vessels or with capillary dilatation caused by the release of vasoactive substances (9).

Tumour cells are presented in the pleural fluid if the lung cancer involves the mesothelial surface. It is a main cause of the inflammatory development. Cancer antigens may be uptaken and processed by dendritic cells (DCs), which belong to the most effective antigen presenting cells. They can internalise antigens by macropinocytosis and endocytosis by at least two types of receptors for antigen uptake: Fc receptor (FcγRI and FcγRII) and mannose receptor. After this process, DCs leave peripheral tissues and migrate to lymphoid organs in order to present antigens to naïve T lymphocytes. This migration is associated with the downregulation of inflammatory chemokine receptor statement induced through DC maturation. In the lymph nodes, DCs form clusters with T lymphocytes through various co-stimulatory and adhesion molecules. The DC/T cell interaction is stabilised by the specific ligation of the T cell receptor by the MHC-peptide complex (4,10,19).

Two distinct lineages of DCs have been described: meyloid DC (DC1) and lymphoid (plasmocytoid) DC (DC2), according to their ability to induce naive T-cell differentiation to Th1 and Th2 lymphocytes, respectively. Immature DC1 expresses myeloid markers (CD13 and CD33) originating from myeloid bone marrow precursors. HLA-DR and CD11c antigens, and constitutively CD40 are upregulated on DC1 by several cytokines such as GM-CSF and TNF-α. CD40 stimulation upregulates co-stimulatory molecule statement on DC1 and induces them to produce p75 interleukin-12 (IL-12). This cytokine plays the key role in the emergence of the Th1 cellular response. Activated Th1 lymphocytes become CD40Ligand (CD40L) positive cells and deliver, in turn, a stimulation signal to CD40+ DC1. These DCs are than able to prime antigen-specific anti-tumour cytotoxic T lymphocytes (CTL) (2,19).

Accumulation of lymphocytes in the pleural fluid, which frequently occurs in neoplastic effusions, is characterised by very low percentage of natural killer cells and anti-tumour cytolytic activity. Several authors demonstrated that interleukin-10 (IL-10) level was more elevated in malignant pleural effusion than in peripheral blood. IL-10 production by the tumour cells has been shown to prevent DC1 accumulation and dominance of the Th2 pathway among effusion-

associated lymphocytes (EALs) (6). On the over hand, T-cell differentiation into Th2 lymphocytes is depended on lymphoid DC. DC2 lacks many of the myeloid-associate markers and is characterised by the presence of plasmocytoid antigens and α chain of pre-T-cell receptor. The lymphoid DCs appear to be interleukin-3-dependent and hence express the IL-3-receptor α chain (CD123). Precursors of DC2 isolated from the blood produce high amounts of type 1 interferons, but in course of maturation and upon CD40 ligation they are capable of secreting interleukin-8. Activation of Th2 response by DC2 may influence immunotolerance in course of metastatic lung cancer (2,15).

Occurence of malignant pleural effusion in terminally ill patients could present significant diagnostic and therapeutic challenges. Because of dissatisfaction with current treatment options, recent research has focused on new approaches to the management of malignant pleural effusions. Several authors made known to public that the patients with pleural effusion from non-small cell lung cancer (NSCLC), who have extremely poor prognosis, may be treated intrapleurally with human interferon alfa or beta. This therapy might clear a malignant pleural effusion of cancer cells, arrest fluid accumulation and prolong the survival of patients.

The role of interferon alfa (IFN-α) treatment could be partially explained by developing a chemical pleurodesis by a sclerosis agent but signification of interferon in anticancer immunotherapy is generally accepted. IFN-α (leukocyte IFN) and IFN-β (fibroblast IFN), the two types of type 1 antiviral interferons, are distinct from type 2 IFN-γ produced by effector T cells. The natural IFN-producing cells (IPCs), which released IFN-α in response to enveloped viruses, bacteria and tumour cells, express CD4 and MHC class II but lack hematopoietic-lineage markers. These cells differentiate into DC2 when cultured with IL-3 and CD40L. Type 2 dendritic cells precursors may produce 200 to 1000 times more IFN than other blood cells after microbial challenge (16).

Type 1 IFN has pleirotropic effects on the immune system, including upregulation of MHC class I on all cell types and activation of macrophages and NK cells. Peripheral blood mononuclear cells cultured with IFN-α and GM-CSF developed a dendritic morphology and expressed high levels of the MHC class I and II, co-stimulatory molecules, adhesion proteins and CD40. IFNs are also critical in the activation and survival of both CD4+ and CD8+ T lymphocytes. In this cause, IFN-α has been widely used for treating hepatitis B and C as well as various cancers. (13,16).

We made an attempt to explain the role of dendritic cells presence in pleural effusion during course of metastatic lung cancer and possible effectiveness of IFN-α treatment of malignant pleural effusion in connecting with dendritic cells occurrence. We hoped that our investigation will help in understanding the role of DC in antitumour immunologic response.

SUBJECTS AND METHODS

The examined group consisted of 5 male and 3 female patients with ipsilateral pleural effusion from non-small cells lung cancer (NSCLC). Patients were divided into two groups: patients who did not received immunotherapy with interferon alfa, and patients (75-years-old female patient with macrocellular cancer of the lung and 58 male patient with lung adenocarcinoma) who were treated with local application of Roferon A (interferon alfa-2a) in addition to other symptomatic treatment. After fluid drainage and lung re-expansion, Roferon A, 3 x 10^6 unit diluted in 1 ml of isotonic salt solution, was injected intrapleurally in 14-day- intervals, as long as the pleural accumulated, up to 8 times. [20]. Mean age of patients not treated with Roferon A was 62,7 ± 6,9. Patients did not received chemo- and radiotherapy during one month before examination.

Cell preparation. Mononuclear cells from peripheral blood and pleural effusion were separated by density gradient centrifugation on Gradisol-L (Aqua Medica, Poland) for 25 minutes at 400xg at room temperature. Interphase cells were removed and washed twice in phosphate

buffered saline (PBS) without Ca^{+2} and Mg^{+2}, containing 0.5% bovine serum albumine (BSA) and 2mM EDTA.

Phenotyping of the cells. The following monoclonal antibodies were used: mouse anti-human BDCA-1 FITC (Miltenyi-Biotec, Germany), BDCA-2-FITC (Miltenyi–Biotec, Germany), CD123-PE (Pharmingen, USA), CD19 CyChrome (Pharmingen, USA). Immunnofluorescent direct staining was prepared according to manufacturers' protocol. Cells were collected using a FACSCalibur flow cytometer equipped with 488-nm argon laser (Becton Dickinson). A total of 300 000 events were acquired and analysed using Cell Quest Software. The myeloid DCs were identified as a BDCA-1 positive and CD19 negative cells. The lymphoid DCs were identified as a double BDCA-2 and CD123 positive cells (8).

RESULTS

In three cases, the pleural fluid was bloody (including two patients treated with Roferon A). In all subjects, the Rivalty tests gave the positive results, and the mean glucose and protein levels were 108.75±27.92 mg% and 3.78±1.06 g%, respectively. In the pleural fluid there were more lymphocytes (70.2±25.5%) than macrophages (12.28±11.82%).

In patients not treated with Roferon A, the percentage of BDCA1+ cells in the pleural fluid was 0.41±0.28% and of BDCA-2+0.035 ± 0.033%. The BDCA-1/BDCA-2 ratio was 39.92± 36.23. The statistically significant ($p<0.05$) positive corellation ($R=0.82$) between BDCA-1/BDCA-2 ratio and the lymphocyte percentage in the pleural fluid was noticed.

In two Roferon A treated patients, in comparison to the other examined patients, there was more than tenfold increase in the percentage of BDCA-2+ cells (0.46% and 0.48%), and the BDCA-1/BDCA-2 ratios were 0.3 and 5.21, respectively (Fig. 1).

In the peripheral blood, no differences in the percentage of BDCA-1+ and BDCA-2+ cells between the group of inteferon treated patients (0.8±0.37% and 0.41±0.31%, respectively) and those not treated (0.48% and 1.44%, and 0.18% and 0.29%, respectively) were observed. The BDCA-1/BDCA-2 ratio in the peripheral blood of patients not treated with Roferon A was 3.19± 2.85, whereas the percentage of two DC subpopulations was significantly higher ($p<0.05$) in the peripheral blood comparing with the pleural fluid.

DISCUSSION

In the present study, we demonstrated that two major subsets of DCs appeared in different percentage in malignant pleural effusion. Myeloid DCs occurred in pleural fluid but lymphoid DCs were almost undetectable. In this cause, the DC1/DC2 ratio was extremely high. Moreover, percentage of both types of DCs in pleural fluid was lower than in peripheral blood. According to our knowledge it is the first report concerning the detection of mature DCs in malignant pleural effusion.

Suzuki et al. examined whether pleural macrophages in malignant pleural effusion could differentiate to immunostimulatory DCs *in vitro*. Conventional culture conditions with GM-CSF and IL-4 did not induce efficient numbers of DCs from mature macrophages, whereas the addition of TNF-α effectively contributed to generate DCs (18). Almand et al. paid attention to the fact that functionally competent DC can be generated only in the absence of tumour-derived factors from peripheral blood progenitor cells. This may result in a substantial decrease in the number of mature DCs in cancer patients. Moreover, the decrease in the presence of DCs in peripheral blood closely correlated with the stage and duration of the disease (1).

The investigators reported that several inhibitors of the DCs generation, such as IL-10 and vascular endothelial growth factor (VEGF), were present in malignant pleural effusion associated

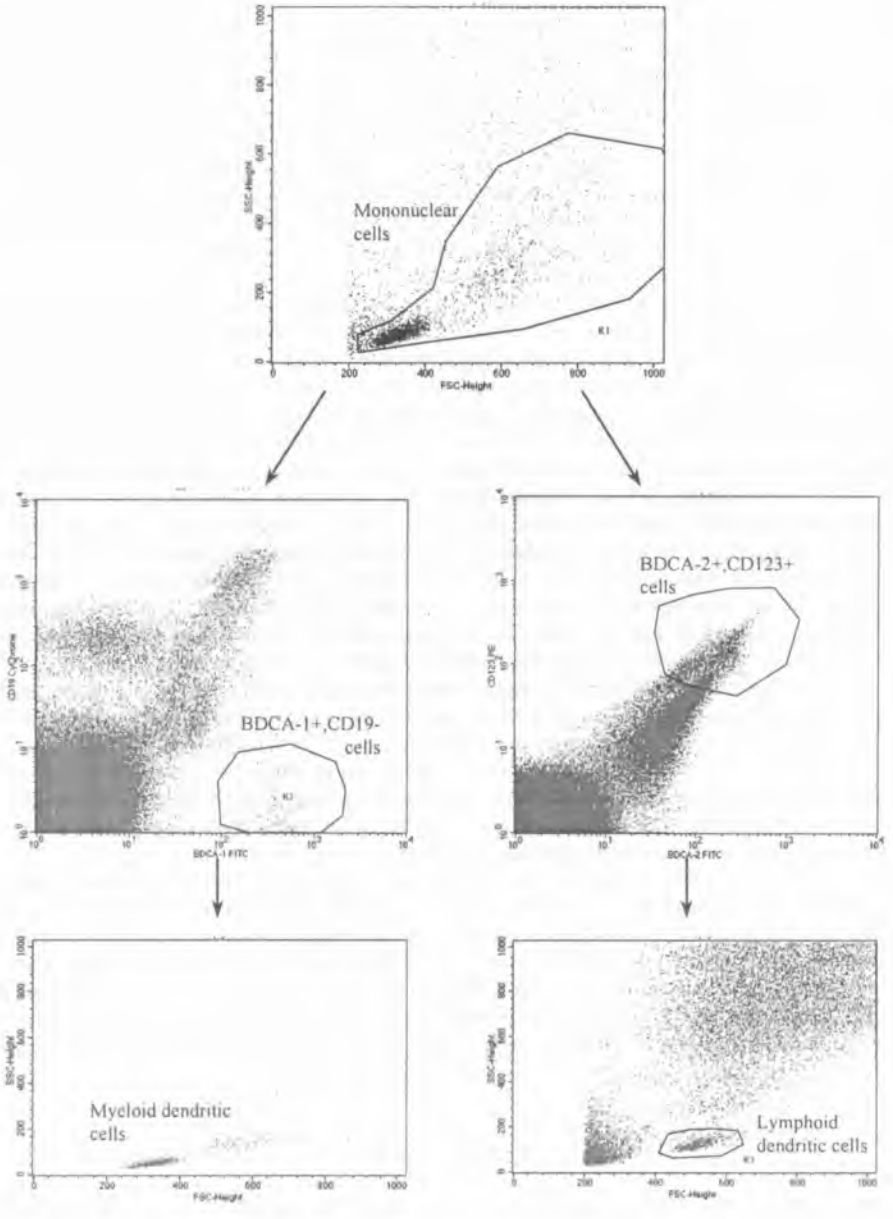

Fig. 1 Cytometric differentiation of myeloid and lymphoid dendritic cell from malignant pleural fluid of patients treated with Roferon A

with lung cancer (6). IL-10 is produced by Th2 clones, B cells, macrophages, mast cells and tumour cells. This cytokine inhibits antigens presentation through the downregulation of co-stimulatory molecules statement and suppression of inflammatory cytokines release, first of all IL-12 (3). Brossart et al. have shown that the addition of IL-10 to the cultures containing GM-CSF and IL-4 completely inhibited the generation of DCs from peripheral blood monocytes, which expressed high levels of IL-10 receptor. The simultaneous incubation of monocytes with IL-10 and TNF-α or soluble CD40L resulted in the generation of mature DCs and inhibition of IL-10R receptor upregulation. DCs grown in the presence of IL-10 and TNF-α or soluble CD40L elicited efficient CTL response against viral and tumour-associated peptide antigens (5). Kiertscher et al. noticed that not only IL-10 inhibits DCs' function. They used the tumour culture supernatant (TSN) for induce apoptosis maturing DC. The effect of TSN on DC apoptosis was not neutralised by antibodies against IL-10, VEGF, TGF-β or PGE_2 (12).

The activity of IL-10 in malignant pleural effusion should be attributed to the predominant DC2 function and Th2 cell response. Therefore, it was surprising, that inhibition of DCs maturation results in the almost complete disappearance of DC2 and half reduction of DC1 percentage in pleural fluid comparing to peripheral blood. It seems that state of antigen specific anergy in T cells, which was confirmed in cancers, is induced by the different than DC2 mechanism. On the other hand, the positive correlation between DC1/DC2 ratio and percentage of lymphocytes in pleural fluid may confirm the role of DC1 in pleuritis with effusion developing.

The observation that IFN-α treatment resulted in an increase in percentage of DC2 subsets and decrease DC1/DC2 ratio in pleural fluid may have a compliance in a new hypothesis concerning effectiveness of type 1 interferons. In the earlier study, the anticancer role of INF-α was associated with maturation of DCs (14). In the recent papers, authors suggested that type 1 interferons can act in an autocrine manner to activate only DC2. Huang et al. showed that type 1 interferons suppressed IL-12 production by dendritic cells in multiple sclerosis patients. They found that DCs derived from human blood monocytes, upon culture in the presence of IFN-β with GM-CSF and IL-4, differentiated into a population expressing receptor for IL-3 and other markers characteristic for lympoid DCs as well as co-stimulatory molecules and CD40. IFN-β treated DCs strongly suppressed IFN-γ production but enhanced IL-10 production by allogeneic blood mononuclear cells (11). McRae et al. have shown that IFN-α treated DCs secreted less IL-12 upon stimulation with bacterial antigens and that decrease correlated directly with their inability to support Th1 cells secretion of IFN-γ (17). For the same cause Vallin et al. used IFN-α therapy in patients with autoimmune disorders to produce immunotolerance(22).

On the basis of these data and our results, we hypothesised that IFN-α can induce the development of DC2, which can provide a permissive environment for Th2 differentiation in pleural effusion. It could have important immunotherapy implications because of two opposite ways in management with cases of malignant pleural effusion: first – interferon therapy towards pleurodesis and influence on expression of MHC class I and CD40 on DCs with simultaneous possible immunotolerance and second – activation of Th1 cells and cytolytic activity of EALs and downregualtion of IL-10 by IL2/IL12 therapy. Importance of this problem requires further studies. (7, 21).

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SUMMARY

The objective of the study was to evaluate the number of myeloid (DC1) and lymphoid (DC2) dendritic cells in malignant pleural effusion and peripheral blood of patients with lung cancer as well as to estimate changes in their presence during intrapleural interferon alpha treatment. The study comprised eight subjects including two patients treated with Roferon A. Isolated mononuclear cells were phenotyped with the following monoclonal antibodies: anti-BDCA-1 FITC and anti-BDCA-2 FITC, and analyzed using FACSCalibur flow cytometre. Very low percentage of DC2 and high BDCA-1/BDCA-2 ratio in malignant pleural effusion of patients not treated with IFN-α suggest intensive Th1 response probably responsible for inflammatory state maintenance and effusion constant development. The results of our study indicate that apart from the suggested antitumour activity, IFN-α therapy may result in unfavourable immunotolerance caused by increased DC2 activity.

Obecność komórek dendrytycznych w nowotworowym płynie z jamy opłucnej

Nowotworowy płyn wysiękowy w jamie opłucnej pojawia się w przypadkach przerzutów raka piersi, jelita grubego i innych nowotworów do płuc oraz w około 15% przypadków raka wywodzącego się z komórek nabłonka oskrzeli. Komórki dendrytyczne (ang. *dendritic cells* – DC), posiadające 100-krotnie większą zdolność prezentacji antygenu niż inne komórki prezentujące antygen, są prawdopodobnie odpowiedzialne za stymulację przeciwnowotworowej odpowiedzi komórkowej w trakcie reakcji zapalnej w jamie opłucnej, związanej z obecnością przerzutów. Celem pracy była ocena ilości komórek dendrytycznych linii mieloidalnej (DC1, z markerem BDCA-1 na powierzchni) i limfodalnej (DC2, z markerem BDCA-2 na powierzchni) w nowotworowym płynie z jamy opłucnej i we krwi obwodowej u pacjentów chorych na raka płuc oraz ocena zmian w ich występowaniu w trakcie doopłucnowego leczenia interferonem. Badaniami zostało objętych 8 chorych, w tym dwoje leczonych Roferonem A podawanym doopłucnowo. Komórki mononuklearne wyizolowano z płynu z jamy opłucnej oraz krwi obwodowej, następnie wykonano procedurę zewnątrzkomórkowego znakowania przeciwciałami monoklonalnymi (anty-BDCA-1 FITC i anty-BDCA-2 FITC). Analizę fenotypu przeprowadzono w cytometrze przepływowym FACSCalibur (Becton Dickinson). Odsetek komórek BDCA-1+ w płynie opłucnowym u pacjentów nieleczonych Roferonem A wynosił 0,41±0,28%, a komórek BDCA-2+ – 0,035±0,033%. Stosunek BDCA-1/BDCA-2 wynosił 39,92±36,23. U pacjentów leczonych Roferonem A zaobserwowano ponad 10-krotne podwyższenie odsetka komórek BDCA-2+, który wynosił 0,47%. We krwi obwodowej nie obserwowano różnic wartości odsetka komórek BDCA-1+ oraz BDCA-2+ pomiędzy grupami osób leczonych i nieleczonych interferonem. Natomiast odsetek obu subpopulacji DC był istotnie wyższy ($p<0,05$) we krwi obwodowej w stosunku do płynu z jamy opłucnej. Bardzo niski odsetek DC2 i wysoki stosunek BDCA-1/BDCA-2 występujący w nowotworowym płynie z jamy opłucnej pacjentów nieleczonych IFN-α sugeruje istnienie wzmożonej odpowiedzi typu Th1, która jest prawdopodobnie odpowiedzialna za utrzymywanie się stanu zapalnego i narastanie płynu. Wyniki naszych badań wskazują, że terapia IFN-α poza sugerowanym działaniem przeciwnowotworowym może wpływać na pojawienie się niekorzystnego zjawiska immunotolerancji, powstającej na skutek zwiększonej aktywności DC2.