IMMUNOMODELLING CHARACTERISTICS OF MATURE DENDRITIC CELLS STIMULATED BY COLON CANCER CELLS LYSATES*

SEBASTIAN RADEJ1, JACEK ROLIŃSKI2, KAROL RAWICZ-PRUSZYŃSKI3, PAWEŁ BURY4, GRZEGORZ BOROWSKI5, JACEK FURMAGA4, ANDRZEJ CHROŚCICKI5, GRZEGORZ WALLNER5, RYSZARD MACIEJEWSKI1

Department of Human Anatomy, Medical University in Lublin1
Kierownik: prof. dr hab. R. Maciejewski

Department of Clinical Immunology, Medical University in Lublin2
Kierownik: prof. dr hab. J. Roliński

PhD student, Department of Surgical Oncology, Medical University in Lublin3
Kierownik: prof. dr hab. W. Polkowski

Department of General and Transplant Surgery and Nutritional Treatment, Medical University in Lublin4
Kierownik: prof. dr hab. S. Rudzki

Department of General and Oncological Surgery, District Specialist Hospital in Lublin5
Kierownik: dr n. med. A. Chrościcki

Department of General and Gastroenterological Surgery and Surgical Oncology of the Alimentary Tract, Medical University in Lublin6
Kierownik: prof. dr hab. G. Wallner

Application of cells with high TAA (tumor associated antigen) presentation potential seems to be crucial in neoplasia immunotherapy. Such feature is distributed in dendritic cells, which present peptides from processed TAA – MHC molecules complex to the T cells of a host.

The aim of the study was to assess the influence of colon neoplasia tissue lysate on functioning of generated autologous DCs in the field of autologous CD4+ lymphocytes immunological response towards Th1/Th2 under in vitro environment together with comparison and assessment of DCs’ immunosuppressive properties acquired from patients with colon cancer.

Material and methods. The population of this study consisted of 16 healthy- controls, 36 colon cancer patients. Blood samples were collected 24h before planned surgery and preventive antibiotic therapy. Neoplastic tissue sample, was digested for cell lysates preparation. DC’s generation from PBMC was carried out in standard conditions and medium enriched with rhGM-CSF and rhIL-4. Mature DC’s and cocultured autologus CD4 lymphocytes immunophenotype assessment was analyzed with flow cytometer. Intracellular and culture medium cytokines concentration was analyzed with ELISA and FACS method.

Results. DC’s generated from colon cancer patients stimulated with lysates presented greater maturity, lower expression of CD206 antigen, significantly higher expression of HLA-DR, CD208 and CD209 and high intracellular expression of IL-12, compared to non-stimulated cells.

Conclusions. The neoplastic tissue in vivo produces a number of substances having an unfavorable effect on immune system, our results suggests using lysates as good dendritic cells stimulators that possibly could have application in colon cancer immunotherapy.

Key words: dendritic cells, colon cancer, cancer cells lysates, Th1, Th2

Although destruction of genetically impaired cells (possible source of neoplasm) is one of the numerous functions of human immune system, group of cells avoid the immunological barrier and step into serrated neoplasia pathway. Their impaired immunogenic
properties play a preventive role from destruction by immune cells. Application of cells with high TAA (tumor associated antigen) presentation potential seems to be crucial in neoplasia immunotherapy. Such feature is distributed in dendritic cells, which present peptides from processed TAA – MHC molecules complex to the T cells of a host. As a result, specific T cytotoxic (CD8+) lymphocytes are activated.

Recent studies on the immune system, especially involving T regulatory cells and Th17 lymphocytes, shed new light on the process of dendritic cells antigen presentation (1-4). It seems that mentioned cells modulate the action of DC’s (dendritic cells). Thus, in order to increase both, specificity and efficacy of immunotherapy, a method which would use the interaction of T regulatory cells, Th17 and dendritic cells, followed by presentation of antigen to cytotoxic cells capable of neoplastic cells extinction, should be devised.

The aim of the study was to assess the influence of colon neoplasia tissue lysate on functioning of generated autologous DC’s in the field of autologous CD4+ lymphocytes immunological response towards Th1/Th2 under in vitro environment together with comparison and assessment of DCs’ immunosuppressive properties acquired from patients with colon cancer and with free of disease blood donors, basing on phenotype study and ability of selected immunosuppressive cytokines production.

MATERIAL AND METHODS

Clinical characteristics of colon cancer patients

36 colon cancer patients treated surgically without preoperative chemotherapy were introduced to the study. Patients were hospitalized in the Medical University of Lublin I Chair and Department of General and Transplant Surgery, General Surgery Department, Cardinal Stefan Wyszyński District Specialist Hospital in Lublin; II Chair and Department of General and Gastrointestinal Surgery and Surgical Oncology of the Alimentary Tract. Patients age varied from 48 to 70 (average 59±11 years). The study group consisted of 17 females and 19 males. Samples for histopathological material for the study were obtained during the surgeries.

Characteristics of control group

16 patients were introduced to the study, from whom leukocyte-platelet supernatant was obtained. Age of the control group donors varied from 42 to 47 years (average 44.5± 2.5 years). 8 females and 8 males comprised the control group. All study participants showed no signs of infection, nor underwent treatment affecting the immune system and had no blood transfusion. Patients with a history of allergic diseases were excluded from the study. The study was carried out in line with the protocol accepted by the Medical University of Lublin Bioethics Committee (KE-0254/50/2010 from 25.02.2010)

Study material

Blood samples from cephalic vein were collected twenty four hours before planned surgery and preventive antibiotic therapy into filled with sodium heparin test-tubes (Polfa, Warsaw) in the amount of 20 U/ml of blood. Neoplastic tissue was placed in Ringer’s solution (Polfa Lublin, Poland). Obtained solid tissue and blood were processed immediately after the withdrawal. Mononuclear cells required for control studies were obtained from healthy donors from leukocyte-platelet specimen handed over by Lublin Regional Blood Donation and Blood Treatment Center.

Mononuclear cells isolation

Whole blood was diluted in 1:1 ratio with buffered Ringer’s solution – calcium- and magnesium-free phosphate-buffered saline (PBS) (Biochrome AG, Germany), piled on Gradiisol L (Aqua Medica, Poland) and centrifuged at 700xg for 20 minutes in the gradient density. Obtained mononuclear cell fraction washed twice in calcium-magnesium-free PBS and centrifuged at 700xg for 5 minutes. After washing process the cells were suspended in 1 ml calcium-magnesium-free PBS, counted in Neubauer chamber,
followed by life span assessment with trypan blue staining technique (0.4% Trypan Blue Solution, Sigma, Germany). Cells with a life span under 90% were disqualified from further procedure.

Magnetic separation of CD3+ cells with “Easy Sep” technique

Isolated mononuclear cells of circulatory blood were suspended in calcium-magnesium-free PBS complemented by 0.5% BSA and 2 mm EDTA in the quantity of 1x10^8 cells/ml. 100 µl/ml of antibody anti-CD3 coupled with dextrane (StemCell Technologies, USA) had been added to suspension and incubated for 15 minutes in room temperature. Subsequently, the solution was incubated with secondary antibody anti-dextran coupled with paramagnetic for 10 minutes in room temperature. After incubation period cells were placed in magnetic field and additionally incubated for 5 minutes. Negative fraction was effused, whereas positive fraction settled in test tubes. Fraction purity under 90% (assessed with flow cytometry) disqualified CD3+ lymphocytes from further studies. Amount of CD3+ cells in CD3- fraction over 10% disqualified them from further studies. Dendritic cells were generated from CD3- fraction, whereas CD3+ fraction banked until mature autologous dendritic cells were obtained.

Neoplastic cells isolation

Neoplastic tissue sample, free of necrotic area, 2 cm³ of a size, was fragmented with a surgical scalpel. Fragmented tissue suspended in 30 ml of RPMI 1640 (PanBiotech, Germany) with additional enzymes: 1 mg/ml type I collagenase (Biochrome AG, Germany) and 1 mg/ml type I DNase (Sigma, Germany). Commixture was placed in a 100 ml glass bottle and digested in sterile condition with a usage of magnetic stirrer (Kucharczyk, Poland) in a temperature of 37°C for 60 minutes afterwards. Digested suspension was filtered and centrifuged at 700xg for 5 minutes. Obtained cells were washed twice in unenriched RPMI 1640, counted and divided into two groups, where group I was banked and group II was used for cell lysates preparation.

Neoplastic cell lysates preparation

Neoplastic cell lysates were prepared through a five cycle rapid freeze-rapid thaw process (-80°C and 37°C consecutively) of a cell solution in 1 ml RPMI 1640 phenol red-free (PanBiotech, Germany). The suspension was centrifuged at 700xg for 5 minutes, subsequently the supernatant was collected and filtered through 0.2 µm filter. First part of the filter was freezed (until adding it to the dendritic cells culture), whereas protein concentration was estimated in the second part of the filter.

Protein level estimation in neoplastic cells lysates

In order to estimate the protein level, light absorbance at 280 nm and spectrophotometer DU®530 (Beckman, USA) were used. Moreover, in order to minimalize the influence of nucleic acids content, additional absorbance at 260 nm was measured, whereas protein concentration was measured with a following method:

\[
\text{protein concentration (mg/ml)} = 1.5 \times \text{absorbance at 280 nm} - 0.75 \times \text{absorbance at 260 nm}
\]

Freezing and thawing of cells

Isolated cells were freezed until autologous dendritic cells were prepared. The freezing process took place in ice-bath. 10-20x10⁶/ml of cells were soluted in RPMI 1640 with 10% human albumin and 10% DMSO (ICN PolfaRzeszów, Poland). Subsequently, the cells were rapidly thawed in a water bath (37°C), placed in a 10 ml test-tube, where RPMI 1640 with 10% human albumin was added every minute in a following scheme: 0.1 ml; 0.12 ml; 0.15 ml; 0.19 ml; 0.28 ml; 0.36 ml; 0.62 ml; 0.86 ml; 1.69 ml; 4.75 ml. The cells were centrifuged and suspended medium adequate to further protocol.

Immature dendritic cell generation

DC’s generation from circulating blood monocytes was conducted in culture bottles of 25 cm² area of adherence (Corning, USA), in 37°C temperature and atmosphere of 5%
CO₂. Culture medium constituted of RPMI 1640 with 2% human albumin and following antibiotics: 100 IU penicillin/ml, 50 µg streptomycin/ml, 100 µg neomycin/ml (Sigma, Germany). Separated CD3- negative fraction in amount of 5-7x10⁶/25 cm² suspended in 5 ml culture medium and conducted 90 minute incubation in standard conditions in order for monocytes adherence. Subsequently, non-adherent cells were washed in calcium-magnesium-free PBS. Adherent cells were cultured in 5 ml culture medium enriched with rhGM-CSF at a dosage of 1000 IU/ml (Genatour, Belgium) and rhIL-4 at a dosage of 500 IU/ml (Genatour, Belgia). Additionally, on the third and fifth day of the culture cytokines were added.

Stimulation of dendritic cells maturation process with lysates and irradiated neoplastic cells

On the sixth day of culture, to the bottle in which reactions of tumor cells lysates with dendritic cells were observed, prepared lysates in amount of 100 µg protein /ml culture medium were added. Into the second bottle, in which influence of irradiated tumor cells on dendritic cells maturation was studied, irradiated tumor cells in amount of 100 µg lysate protein /ml culture medium were added. In order to achieve additional induction of dendritic cells maturation, on the sixth day of culture rhTNF-α in amount of 50 ng/ml (Strathmann, Germany) was added. The cultures were harvested for 48 hours.

Culture completion

Supernatant from culture was isolated, centrifuged (for 5 min at 700xg) and kept in a -80°C temperature in order to determine the level of cytokines (IL-6, IL-10, IL-12, IFN-γ). Trypsin solution 0.25%/EDTA 0.02% in calcium-magnesium-free PBS (Biochrom AG, Germany) was poured into the bottle for 10 minutes in 37°C temperature and atmosphere of 5% CO₂. Harvested cells were matched with supernatant cells in calcium-magnesium-free PBS and centrifuged at 700xg for 5 minutes. Subsequently, the cells were suspended in medium adequate for further studies where their lifespan was assessed. The immature DC’s were collected on the sixth day of culture, whereas mature DC’s on the seventh.

Flow cytometry

In the conducted studies, cells immunophenotype assessment was realized with flow cytometer FACSCalibur (Becton Dickinson, USA), equipped in argon laser (emission of 488 nm). The analysis was carried out with CellQuest program (Becton Dickinson, USA) and the following parameters were covered: a) intensity of light crossing cells in laser beam extension – FSC (Forward Scatter) corresponding to the cell’s size, b) scattered light intensity on cell organelles – SSC (Side Scatter) dependent on intracellular structure (line scale) c) green fluorescence detection (FL-1), dependent on labeled with FITC (fluorescein isothiocyanate) monoclonal antibodies binding (logarithmic scale) d) orange fluorescence detection (FL-2), dependent on labeled with PE (phycoerythrin) monoclonal antibodies binding (logarithmic scale) e) red fluorescence detection (FL-3), dependent on labeled with CyChrome (PeCy5) monoclonal antibodies (logarithmic scale). Based on SSC and FSC parameters, a gate for analyzed cells was determined, excluding possible tincture of erythrocytes, platelets, necrotic cells and their fragments. Percentage of positive cells and antigen cell expression was assessed basing on mean fluorescence intensity (MFI)

Immunophenotype and dendritic cells antigen absorption assessment.

The assessment concerned immunophenotype of dendritic cells from the 5-day culture medium as non-stimulated by neoplastic antigens in comparison with immunophenotype of stimulated by 48 hour lysate culture. For assessment of immunophenotype generated DCs, following monoclonal antibodies against human antigens:

a) pure dendritic cell isotope control;

b) anti-CD45 FITC /anty-CD14 R-PE (isotype IgG₁/IgG₂a) (BD Pharmingen, USA) – in order to assess gate for generated DC’s subpopulation and to estimate the culture effectiveness. During maturation DC’s lose
CD14 marker, specific for monocytes/macrophages;
c) anti-CD83 FITC (isotype IgG1) (BD Pharmingen, USA) /anty-CD1a R-PE (isotype IgG2a) (BD Pharmingen, USA) /anti-HLA-DR PeCy-5 (isotype IgG2a) (BD Pharmingen, USA) – in order to differentiate mature and immature DC’s;
d) anti-CD86 FITC (isotype IgG1) (BD Pharmingen, USA) /anti-CD80 R-PE (isotype IgG2b) (BD Pharmingen, USA) /anti-HLA-DR PeCy-5 (isotype IgG2a) (BD Pharmingen, USA) – in order to assess costimulatory DC’s antigens;
e) anti-CD209 FITC (isotype IgG1) (BD Pharmingen, USA) /anty-B7-H2 R-PE (isotype IgG2b) (BD Pharmingen, USA) /anti-HLA-DR PeCy-5 (isotype IgG2a) (BD Pharmingen, USA) – in order to assess co-stimulatory DC’s antigens and adherence abilities;
f) anti-CD209 FITC (isotype IgG1) (BD Pharmingen, USA) /anti-CD206 R-PE (isotype IgG2b) (BD Pharmingen, USA) /anti-HLA-DR PeCy-5 (isotype IgG2a) (BD Pharmingen, USA) – in order to assess expression of C-Lectines family;
g) anti-CD83 FITC (isotype IgG1) (BD Pharmingen, USA) /anti-HLA-DR PeCy-5 (isotype IgG2a) (BD Pharmingen, USA) – in order to determine cut-off values for intracellular markers;
h) anti-CD83 FITC (isotype IgG1) (BD Pharmingen, USA) /anti-CD208 R-PE (isotype IgG2a) (BD Pharmingen, USA) /anti-HLA-DR PeCy-5 (isotype IgG2a) (BD Pharmingen, USA) – in order to determine cell’s ability to create antigen-MHC II molecule complexes;
i) anti-CD83 FITC (isotype IgG1) (BD Pharmingen, USA) /anti-IL-12 R-PE (isotype IgG2a) (BD Pharmingen, USA) /anti-HLA-DR PeCy-5 (isotype IgG2a) (BD Pharmingen, USA) – in order to determine IL-12 formation and ability to arouse Th1 response.

Generated DC’s were washed twice in calcium-magnesium-free PBS containing 0.5% bovine serum albumin (BSA) and 0.2 mM EDTA. In order to exclude nonspecific antibody binding, Fc receptor was inactivated by incubation of 1x10^7 cells from 20 μL of blocking reagent (FcR, MiltenyiBiotec, Germany) for 5 minutes in 22°C temperature. Subsequently, cells were labeled with mentioned above set of monoclonal antibodies by adding 10 μl of antibodies for every 1x10^7 assessed cells. After 30 minute incubation in temperature of 4°C, the cells were centrifuged at 700xg for 5 minutes and analyzed with flow cytometry. Dendritic cells immunophenotype was evaluated as 6 day culture by non-stimulated neoplastic antigens and compared with immunophenotype of stimulated dendritic cells (48 hour culture), lysates or irradiated neoplastic cells.

The analysis had begun from gate establishment for DC’s in linear coordinate system SSC/FSC. Selected DC’s in region R1 were analyzed with the following criteria: clarity of generated DC’s evaluated on the basis of cells with CD45+ immunophenotype. On CD45+ cells expression was determined by a marker characteristic for monocytes CD14, recognizing CD45+/CD14+ cells as immature monocyte-like dendritic cells. HLA-DR+ cells were analyzed. Degree of maturity was also assessed with following criteria: cells with CD83+/CD1a+ and CD83+/CD1a- phenotype were recognized as mature DC’s, whereas phenotype CD83-/CD1a+ designated immature DC’s. Antigen expression of C-Lectin family (CD209, CD206) antigens was also evaluated, recognizing immunophenotype CD209+/CD206+ as potentially phagocytic and capable of migrating to lymph nodes, immunophenotype CD209-/CD206+ as non-migrating and phagocytic, whereas immunophenotype CD209+/CD206- as migrating but non-phagocytic. Percentage of cells expressing co-stimulatory molecules CD80, CD86 and B7-H2 was also determined. The influence of neoplastic antigens stimulation on MHC II molecules expression was evaluated on the basis of MFI HLA-DR+ cells and intracellular antigen CD208(DC LAMP).

Statistical description of the study

Statistical analysis of obtained data was conducted with STATISTICA 7.1 PL (StatSoft Inc, USA) software. Results were presented as: median, arithmetic mean ± standard deviation (SD), smallest value of the statistical series (Min.) and largest value of the statistical series (Max.).

Distribution compliance of individual variables within groups with normal distribution was determined with Shapiro-Wilk (SW) test. Since the studied variables were not normally
distributed, further analysis was carried out with nonparametric tests:
a) comparison between paired groups variables was assessed with Wilcoxon T test;
b) comparison of differences between patient group and control group was assessed with Mann-Whitney U test;
c) the relationship between variables was assessed using Spearman rank correlation coefficient. Results of significance level at p≤0.05 were considered as statistically significant.

RESULTS

Evaluation of dendritic cells generation stimulated with colon cancer cells lysates

Dendritic cells stimulated with colon cancer cells lysates were assessed on the seventh day of the culture. We evaluated immunophenotype and the concentration of the cytokines in culture’s supernatant. The control group was made of dendritic cells generated from the healthy donors and stimulated with colon cancer cells lysates (the control group for DC’s generated from patients with colon cancer stimulated with colon cancer lysates). Evaluating the immunophenotype of generated dendritic cells stimulated with cancer cells lysates, after seven days of culture, statistically higher percentage of CD45+/CD14+ cells in CC (colon cancer patients) group (p<0.05) in comparison to the control group was stated. Evaluating CD1a and CD83 antigens on unstimulated dendritic cells, it was estimated that dendritic cells generated from patients with colon cancer characterized with statistically much lower CD1a+/CD83+ cells percentage (p<0.0001) compared to the control group. The results are shown in tab. 1. Assessing C-type Lectins family antigens on stimulated dendritic cells, statistically much higher (p<0.05) percentage of CD206+/CD209+ cells in CC group compared to the control group was estimated. The results are shown in tab. 2.

The assessment of CD208, HLA-DR antigens and costimulatory molecules CD80, CD86 and B7-H2 was performed after 48 hour incubation of the generated dendritic cells with cancer cells lysates. Evaluating intracellular antigen CD208 of the stimulated dendritic cells, we observed that dendritic cells generated from the patients suffering from colon cancer characterized with much higher percentage of CD83-/CD208+ (p<0.01) and CD83+/CD208+ (p<0.01) compared to the control group. Assessing CD80 and CD86 antigens on stimulated dendritic cells, it was estimated that dendritic cells generated from patients with colon cancer featured significantly higher (p<0.01) percentage of CD80+/CD86- compared to the control group. Assessing B7-H2 antigen on stimulated dendritic cells, it was observed that dendritic cells generated from patients with colon cancer characterized statistically

### Table 1.
Evaluation of dendritic cells maturity markers stimulated with neoplastic cell lysates generated from healthy donors (control group) and colon cancer patients (statistical significance as described in text)

<table>
<thead>
<tr>
<th>Percentage of cells (%)</th>
<th>Control group (colon cancer)</th>
<th>Colon cancer patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SD median min. max.</td>
<td>mean ± SD median min. max.</td>
</tr>
<tr>
<td>45+/14+</td>
<td>0,51±0,24 0,50 0,05 1,05</td>
<td>0,07±0,85 0,91 0,21 3,17</td>
</tr>
<tr>
<td>45+/14-</td>
<td>92,53±6,62 95,07 77,79 98,64</td>
<td>93,61±5,50 95,80 83,09 99,21</td>
</tr>
<tr>
<td>1a+/83-</td>
<td>15,82±8,80 15,28 1,77 37,53</td>
<td>23,20±4,99 18,18 4,55 48,98</td>
</tr>
<tr>
<td>1a+/83+</td>
<td>53,27±10,06 53,64 35,64 79,50</td>
<td>29,67±9,54 29,47 15,49 45,65</td>
</tr>
<tr>
<td>1a-/83+</td>
<td>20,55±12,75 18,70 2,82 59,96</td>
<td>32,56±17,59 31,90 5,13 61,50</td>
</tr>
</tbody>
</table>

### Table 2.
Assessment of C-Lectins family antigens on dendritic cells stimulated by neoplastic cells lysates generated from healthy donors (control group) and colon cancer patients (statistical significance as described in text)

<table>
<thead>
<tr>
<th>Percentage of cells (%)</th>
<th>Control group (colon cancer)</th>
<th>Colon cancer patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SD median min. max.</td>
<td>mean ± SD median min. max.</td>
</tr>
<tr>
<td>206+/209-</td>
<td>2,14±0,64 2,10 1,21 3,70</td>
<td>2,08±1,24 1,92 0,44 4,98</td>
</tr>
<tr>
<td>206+/209+</td>
<td>22,69±5,52 21,10 14,76 38,34</td>
<td>34,30±24,59 31,12 0,60 73,60</td>
</tr>
<tr>
<td>206-/209+</td>
<td>65,90±6,97 65,13 55,13 80,34</td>
<td>57,21±24,49 57,09 24,17 95,26</td>
</tr>
</tbody>
</table>
much higher (p<0.05) percent of B7-H2+/CD209+ in comparison with the control group. Examining the HLA-DR antigen expression on stimulated dendritic cells, it was estimated that dendritic cells generated from patients with colon cancer featured significantly higher HLA-DR antigen expression compared to the control group. The results are shown in tab. 3.

Assessment of IL-6, IL-10, IFN-γ, IL-12 concentration in stimulated dendritic cells culture supernatant and percentage of dendritic cells generating IL-12

In supernatants of dendritic cells cultures stimulated by neoplastic cells lysates concentration levels of IL-6, IL-10, IFN-γ and IL-12 were assessed with immunoenzymatic technique. Immunophenotype of IL-12+DC’s was analyzed with flow cytometry, marking dendritic cells with intracellular IL-12 detection method. DC’s generated from colon cancer patients showed significantly higher concentration of CD83-/IL-12+ cells (p<0.001) and CD83+/IL-12+ (p<0.0001) cells compared to control group. The results are shown in tab. 4.

There were no statistically significant differences in cytokines concentration between dendritic cells cultures generated from healthy donors, colon cancer patients. The results are shown in tab. 5.

Assessment of intracellular IL-2, IL-4, IL-10 and IFN-γ expression of lymphocytes incubated in presence of autologous dendritic cells

In the conducted study, we assessed the autologous lymphocytes intracellular cytok-

Table 3. Assessment of antigens involved in specific immunological response of dendritic cells stimulated by neoplastic cells lysates generated from healthy donors (control group) and colon cancer patients (statistical significance as described in text)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Control group (colon cancer)</th>
<th>Colon cancer patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SD</td>
<td>median</td>
</tr>
<tr>
<td>B6+/B0-</td>
<td>3.07±1.52</td>
<td>3.04</td>
</tr>
<tr>
<td>B6+/B0+</td>
<td>85.3±11.57</td>
<td>88.23</td>
</tr>
<tr>
<td>B6-/B0-</td>
<td>6.10±1.16</td>
<td>3.50</td>
</tr>
<tr>
<td>B7H2+/B0-</td>
<td>0.85±0.50</td>
<td>0.77</td>
</tr>
<tr>
<td>B7H2+/B0+</td>
<td>2.65±0.93</td>
<td>2.48</td>
</tr>
<tr>
<td>83-/B0-</td>
<td>5.79±2.86</td>
<td>5.45</td>
</tr>
<tr>
<td>83+/B0+</td>
<td>17.58±3.82</td>
<td>17.82</td>
</tr>
<tr>
<td>MFI HLA-DR</td>
<td>928.5±490.6</td>
<td>835.35</td>
</tr>
</tbody>
</table>

Table 4. Assessment of intracellular IL-12 expression of dendritic cells stimulated with neoplastic cells lysates generated from healthy donors (control group) and colon cancer patients (statistical significance as described in text)

<table>
<thead>
<tr>
<th>Percentage of cells (%)</th>
<th>Control group (colon cancer)</th>
<th>Colon cancer patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SD</td>
<td>median</td>
</tr>
<tr>
<td>IL-12+/B0-</td>
<td>0.27±0.24</td>
<td>0.25</td>
</tr>
<tr>
<td>IL-12+/B0+</td>
<td>0.49±0.14</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Table 5. Assessment of cytokines concentration in supernatants from dendritic cells cultures stimulated by neoplastic cells lysates generated from healthy donors (control group) and colon cancer patients (statistical significance as described in text)

<table>
<thead>
<tr>
<th>Cytokine concentration pg/ml</th>
<th>Control group (colon cancer)</th>
<th>Colon cancer patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SD</td>
<td>median</td>
</tr>
<tr>
<td>IL-6</td>
<td>419.5±63.69</td>
<td>411</td>
</tr>
<tr>
<td>IL-10</td>
<td>1.78±1.06</td>
<td>1.78</td>
</tr>
<tr>
<td>IL-12</td>
<td>4.66±0.87</td>
<td>4.63</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.01±0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Immunomodelling characteristics of mature dendritic cells stimulated by colon cancer cells lysates 77
ines expression with flow cytometry method. The autologous lymphocytes were cocultured with mature dendritic cells stimulated with lysates or irradiated neoplastic cells. In culture’s supernatant concentration of selected cytokines were determined with ELISA method. Autologous lymphocytes culture with dendritic cells generated from healthy donors and colon cancer patients stimulated with irradiated cancer cells, comprised the control group.

Assessment of autologous lymphocytes cytokines expression cocultured with DC’s stimulated by irradiated neoplastic cells

Autologous lymphocytes cocultured with colon cancer patient dendritic cells presented statistically significant higher percentage of CD4+/IL-2+ (p<0.001), CD4+/IL-4+ (p<0.01), CD4+/IL-10+ (p<0.01) and CD4+/IFN-γ+ (p<0.01) compared to the control group. The results are shown in fig. 1.

Assessment of correlation between immunophenotype of dendritic cells stimulated with neoplastic cells lysates and the cytokines concentration and the percentage of lymphocytes IL-2, IL-4, IL-10, IFN-γ positive

The percentage of CD1a+/CD83+ DC’s generated from colon cancer patients correlated positively with percentage of CD4+/IL-4+ lymphocytes (R=0.68; p<0.05). The results are shown in fig. 2.

There were no statistically significant differences in the control group of autologous lymphocytes cocultured with dendritic cells stimulated with irradiated colon cancer cells.

Assessment of cytokines generation in culture of autologous lymphocytes with dendritic cells stimulated with neoplastic cells lysates

Flow cytometry analysis of cytokines generation showed that autologous lymphocytes cultured with colon cancer patient dendritic cells demonstrated significantly higher percentage of CD4+/IL-2+ (p<0.001), CD4+/IL-10+ (p<0.01) and CD4+/IFN-γ+ (p<0.01), whereas significantly lower percentage of CD4+/IL-4+ (p<0.01) was observed compared to the control group. The results are shown in fig. 3.

Assessment of correlation between the immunophenotype of dendritic cells stimulated with neoplastic cells lysates with the cytokines concentration and the percentage of lymphocytes IL-2, IL-4, IL-10, IFN-γ positive

The percentage of CD1a+/CD83- cells from patients with colon cancer negatively correlated with the percentage of CD4+/IL-2+ (R=-0.66; p<0.05) and CD4+/IFNγ+ (R=-0.66; p<0.05) cells. CD4+/IL-2- and CD4+/IFN-γ+ lymphocytes negatively correlated with the percentage of B7-H2+/CD209+ cells, (R=-0.73; p<0.05) and (R=-0.73; p<0.05) respectively and
the proportion of CD206+/CD209- cells (R=-0.71; p<0.05) and (R=-0.71; p<0.05) respectively. In the control group of autologous lymphocytes cultured with dendritic cells stimulated with colon cancer cells lysates, there were no significant statistical differences.

**DISCUSSION**

Analysis of source materials provided little information regarding the usage of immune system cells in colon cancer immunotherapy in clinical trials. In Stoch et al. research the patients (over 100 people) were characterized by the highest degree of disease progression in all analysed clinical studies (5). What appears to be significant in other authors’ studies is that over 40% of patients who underwent such therapy showed fundamental response of immune system (6, 7). The source of antigen were neoplasm RNA (8) or HLA specific peptides (5, 9) subsequently processed by dendritic cells.

Evaluating the maturity degree of dendritic cells generated by patients with colon cancer, it was estimated that the percent of CD14+ cells was significantly higher after irradiated neoplastic cells stimulation. Whereas in DCs group from the healthy donors, higher percent of immature cells was observed after lysates stimulation, in the group of cells generated from the healthy donors higher expression of CD83+ antigen was showed while using radiated neoplastic cells. The percentage of cells obtained both from the healthy donors by Matsumoto et al. (10) as well as from the sick by Holt et al. (11) are higher than those obtained in our study. It can be assumed that the significant impact on the maturation process had the use of the additional maturation stimulators. Moreover, the type of immune response induced by dendritic cells depends on maturity state. The small percent of CD14+ cells is irrelevant. Arroyo et al. observed that DCs generated from the health donors do not induce other immune response with monocytes presence or absence in the culture (12). In favourable conditions in in vitro culture the percent of immature cells of CD1a+/CD83-phenotype should be minimal. The dendritic cells generated from the healthy donors after lysates stimulation showed higher percent of immature cells in comparison to the DCs stimulated by radiated neoplastic cells. Chen et al. research (13) on the stimulation of the DCs of patients suffering from colon cancer stimulated with lysates from culture line Colo320, SW480 and SW620 are consistent with the observations of our own. These authors indicated that the DCs stimulation with the above-mentioned lysates causes the DCs maturation and expression of CD1a, CD83 and CD86 markers in a significantly higher percentage in comparison to the control group.

Additionally, in our studies we estimated the percentage of autologous CD4 lymphocytes incubated with dendritic cells stimulated with neoplastic cells’ antigens. Intracellular ability to produce cytokines determining the immune response type was established. These cytokines were IL-2, IL-4, IL-10 and IFN-γ. In the conducted research the percentage of immature DCs generated from patients suffering from colon cancer and stimulated with lysates of neoplastic cells negatively correlated with the percentage of CD4+ lymphocytes producing IL-2 and INF-γ.

In their researches, Nonaka et al., generated DCs from healthy donors and incubated them with colon cancer cell line (14). They discovered that dendritic cells adhere to the neoplastic cells with the help of DC-SIGN. This reaction has an inhibitory effect on immature DCs and stimulates the dendritic cells to produce IL-6 and IL-10. In our own studies we observed significantly higher percent of CD206+/CD209+ cells among patients with colon cancer in comparison with the control group with neoplastic cells lysates stimulation.
Moreover the percent of CD206+ cells generated from patients with colon cancer negatively correlated with the percentage of autologous CD4+/IL-2+ and CD4+/IFN-γ+ lymphocytes. A significant factor in immunotherapy is the use of proper source of antigen. It can be expected that DCs’ stimulation used by Chen et al. in their studies (lysates of colon cancer line cells) is the essence of a stronger interaction between the antigen presented via DCs and cytotoxic lymphocytes against neoplastic cells. In our research we used autologous neoplastic cells which in course of neoplasm expansion developed specific mechanism escape from the control of the immune system.

Kichler-Lakoma et al. studies, who yet again confirmed that autologous dendritic cells generated from patients with neoplasm can induce tolerance (15). DCs generated by authors had much lower expression of CD80, CD83 and CD86 antigens and weaker ability to stimulate autologous lymphocytes compared to DC’s generated from healthy donors. Kichler-Lakoma et al. studies were not conducted among patients with advanced cancer, but among the group of woman in the early stages of breast cancer (15). The poor immunogenicity of colon cancer was also confirmed with Remmel et al. studies, who stated that dendritic cells cultured with colon cancer line cells have low CD80 and CD86 expression with high HLA-DR expression (16). Our studies confirm these results. DCs generated from patients with colon cancer, stimulated with irradiated colon cancer cells, characterized with significantly lower CD80+/CD86+ percentage and higher CD80 antigen expression in comparison with control group. The use of lysates caused the increase of double positive cells percentage. The attempts of immunosuppression made by Hunyadi et al. (17) with the use of autologous neoplastic cells lysates, despite the high percentage of CD86+ (which is in agreement with our results), prove this therapy to be well tolerated by patients.

The mature dendritic cells present the antigens by creating an immunological synapse. A Signac from MHC-antigen complex and co-stimulatory molecules activates a lymphocyte. The additional lymphocyte activation takes place due to cytokine secretion. The dendritic cells produce cytokines determining the type of immune response: IL-10 and IL-12. In presented study the intracellular expression of IL-12 in stimulated DCs was measured. The dendritic cells generated from patients with colon cancer had significantly higher percentage of IL-12+ cells compared to cells generated from healthy donors. These observations remain in agreement with Ghanekar et al. studies, who also observed higher expression of IL-12 in cells generated from patients with cancer (18). No statistical differences in the concentration of the above-mentioned cytokines between patients’ groups and control or comparing different DCs’ stimulants were observed. Pellagatta et al. (19) also observed high expression of IL-12 in immunotherapy with the use of DC’s stimulated with autologous glioblastoma cells lysates, which fully coincides with our data.

On the basis of our research results we can draw an assumption that dendritic cells generated from the patients and from the healthy donors that underwent lysates stimulation activate autologous T lymphocytes in Th1 immune response direction.

Galea-Lauri et al. suggest that the immunophenotype of mature DCs was independent from lysates or apoptotic neoplastic cells stimulation and has no influence on the immune response type (20). Lesterhuis et al. used vaccines of autologous mature dendritic cells in immunotherapy of patients with colon cancer (21). This condition guaranteed, among authors, the induction of specific immune response (increase of T lymphocytes and lymphocytes producing INF-γ and IL-2 percentage). However, in our studies the immunophenotype of mature dendritic cells was dependent on the type of stimulation. Using lysates as a source of antigen, the higher percentage of lymphocytes with IL-2 and INF-γ expression was observed among patients suffering from cancers compared to the control group. This may indicate the existence in the organism of the patients some immune response inhibitory agents.

Summing up the previous considerations, we can conclude that the generated immature dendritic cells did not significantly differ in the groups of patients and healthy control. The analysis of the immunophenotype of the stimulated dendritic cells showed differences in maturation markers expression, costimulatory molecules, secretion of IL-12 and MHC components. It is obvious the neoplastic tissue
in vivo produces a number of substances having an unfavorable effect on immune system, therefore conducted research suggests using lysates as safer dendritic cells stimulators that possibly could have application in colon cancer immunotherapy.

CONCLUSIONS

1. Dendritic Cells generated from colon cancer patients which underwent autologous neoplasia lysate stimulation presented greater maturity, lower expression of CD206 antigen, significantly higher expression of MHC II molecules (HLA-DR, CD208) and C-type Lectins, compared to non-stimulated cells.

2. High intracellular expression of IL-12 allows to presume that introduction of an antigen as a lysate of neoplastic cells, creates a potentially higher possibility to induce Th1 response than irradiated colon cancer cells stimulation.

REFERENCES


Received: 31.01.2015 r.
Address correspondence: 20-081 Lublin, ul. Staszica 16
e-mail: radejs@wp.pl