EVALUATION OF IMMATURE MONOCYTE-DERIVED DENDRITIC CELLS GENERATED FROM PATIENTS WITH COLORECTAL CANCER*

RYSZARD MACIEJEWSKI1, SEBASTIAN RADEJ1, JACEK FURMAGA2, ANDRZEJ CHROŚCICKI2, SLAWOMIR RUDZKI2, JACEK ROLIŃSKI4, GRZEGORZ WALLNER5

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

Department of General and Transplant Surgery and Nutritional Treatment, Medical University in Lublin2
Kierownik: prof. dr hab. S. Rudzki

Department of General and Oncological Surgery, District Specialist Hospital in Lublin3
Ordynator: dr n. med. A. Chrościcki

Department of Clinical Immunology, Medical University in Lublin4
Kierownik: prof. dr hab. J. Roliński

Department of General and Gastroenterological Surgery and Surgical Oncology of the Alimentary Tract, Medical University in Lublin5
Kierownik: prof. dr hab. G. Wallner

Dendritic cells are heterogeneous population of the leukocytes and most potent APC in activation of naive T lymphocytes. Therefore the DCs generated in vitro are under research for their application in anti-tumor immunotherapy.

The aim of the study was generation of the immature dendritic cells from peripheral blood monocytes collected from colorectal cancer patients and comparison of their ability to endocytosis, cytokine production and immunophenotype to DCs generated from healthy donors.

Material and methods. 16 adenocarcinoma stage II patients were included in the study. Dendritic cells were generated in the presence of rhGM-CSF and IL-4. PBMC were isolated from the blood of patients and 16 healthy donors – control group. Immunophenotype, ability of endocytosis of Dextran-FITC as well as intracellular IL-12 expression of the generated dendritic cells was measured using flow cytometry. The cytokines (IL-6, IL-10, IL-12p70, IFN-γ) concentration in the supernatants of DCs culture was measured by ELISA.

Results. The percentage of the immature dendritic cells and expression of CD206 and CD209 antigens was significantly higher in patients group (p <0.05 and p <0.001 respectively). Significantly (p <0.001) higher expression of the antigens which initiate the Th2 immune response (CD80-/CD86 + and B7-H2 + / CD209 +) was in the patients group. There were no differences in endocytosis ability and the cytokines (IL-6, IL-10, IL-12p70, IFN-γ) concentration between investigated groups.

Conclusions. High immature markers expression on the generated dendritic cells together with identical endocytosis ability in patients group is advantageous in antitumor autologous cells immunotherapy planning. However there is one troubling fact – high expression of markers, which may induce tolerance to particular antigen. It seems to be more reasonable to use the autologous DCs in the anti-tumor immunotherapy, especially due to the incompatibility in allogenic cells in the context of HLA complex.

Key words: dendritic cells, colon cancer, immunotherapy

* Funded from scientific funds for years 2010-2013 as a research project number NN 403 393 039.
Colorectal cancer accounts for about 10.9% of neoplasms in females and 11.3% in males, according to the 2009 National Cancer Registry. After lung cancer, it is the second most common cancer in the Polish population (1). Long-term predictions by Didkowska et al. suggest that until 2025, despite predicted decrease in male mortality to 188/100,000, number of deaths will reach 7900. In females, mortality is supposed to remain unchanged, however, as in males, death rate will go up, reaching 5900 (2).

Pathogenesis of colorectal cancer has not been fully explained, with genetic background and comorbidities which are supposed to be key factors. Two pathways of cancerogenesis are under research: one is induced by a cascade of mutations of oncogenes and suppressor genes (3), the other results from mutations of genes of DNA repair proteins, leading to formation of microsatellite instability phenotype tumors (4). Familial background is observed in over 10% of colorectal cancers (3).

As majority of colorectal cancer develops from primary lesions—epithelial dysplasia, adenomatous polyps should also be included among risk factors (3). Similarly, inflammatory bowel disease increases the cancer risk (5). Morbidity peak occurs about the 8th decade of life. Furthermore, dietary factors are important: morbidity increases in animal fat-rich, highly processed, low fibre, calcium and vitamin diet (6). Insufficient physical activity, smoking and smoke exposure, as well as recurrent constipations are important risk factors as well (7).

Neoplastic cells present multiple mechanisms of protection against the immune system reaction (8). Intracellular location of neoplastic antigens, and their release after the cell lysis, as well as their poor antigenic potential, combined with lack of Major Histocompatibility Complex (MHC), make neoplastic cells „invisible” for the immune system (8). Some authors show, that tolerance to neoplastic antigens, induced by dendritic cells, significantly increases dynamics of the disease (9). Furthermore, neoplasm may induce immunosuppression of dendritic cells, which inhibits the immune reaction against neoplastic cells (10). Most of dendritic cells which infiltrate the neoplasm are immature and remain at this stage, which leads to ineffective antigen presentation (10). Neoplastic tissue forms a „trap” for dendritic cells, which lose their migratory capabilities, and finally die by apoptosis induced by FasL (CD95L/Apo1) and TRAIL (Tumor Necrosis Factor Released Apoptosis Including Ligand).

Neoplastic cells release multiple factors, like CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CCL20 (MIP-3α), which increase dendritic cells tumor taxis, and in their close neighborhood multiple immunosuppressive factors are observed, including TGF-β, IL-4, IL-6, IL-10, VEGF, PGE₂, H₂O₂, NO, soluble IL-12 receptor, complement system inhibitors, proteases, gangliosides, hexosamine, α-fetoprotein, fibronectin and phosphatidylinerine (10).

In recent years, therapeutic improvement in cancer treatment included new surgical procedures, as well as selective radio- and chemotherapy. Simultaneously, new approaches are under extensive research (11), one of which is immunotherapy. In this field, dendritic cells are of particular interest, as multiple studies show a leading role of dendritic cells in antineoplastic response. Dendritic cell immunotherapy aims at induction of specific and effective immune reaction against neoplastic antigens. It is important that the vaccine should also be safe for the patient. Effective vaccine requires numerous aspects connected with dendritic cells and cancer biology to be included as the immunotherapy success may depend on that.

Searching for an optimal, clinically applicable method of generating dendritic cells, use of autologic immature dendritic cells from blood monocytes of colorectal cancer patients was compared with dendritic cells generated from blood monocytes of healthy donors.

MATERIAL AND METHODS

Study group (CC) included 16 patients operated due to grade II adenocarcinoma, not treated with pre-operative chemotherapy. Patients were hospitalized in I Chair and Department of General and Transplant Surgery and Nutritional Treatment of Medical University of Lublin and in the Department of General and Oncological Surgery of District Specialist Hospital in Lublin. Patients’ age ranged from 48 to 70 years (average 59 ± 11 years). Study group consisted of 7 women and 9 men.
Control group (H) consisted of leuko-platelet concentrates from 16 donors from Regional Blood Donation Center in Lublin. Donors age ranged from 42 to 47 years (average 44.5 ± 2.5 years). Control group consisted of 8 women and 8 men.

Within one month prior and during the study none of the people had infections, took medications affecting immune system. They also did not receive blood transfusions. Patients with the history of allergic diseases were excluded from the study. Study was done in accordance with protocol accepted by local Bioethical Commission (decision no. KE-0254/381/2004).

Isolation and generation of dendritic cells: Peripheral Blood Mononuclear Cells (PBMC) were isolated from the peripheral blood by centrifugation of peripheral blood in density gradient, diluted at 1:1 ratio by saline (PBS, Biochrome AG, Germany over Gradisol L (Aqua Medica, Poland). PBMC were washed twice in PBS. After the washing, number and viability were assessed. Viability of below 90% excluded the cells from further analysis.

Generation of Monocyte Derived Dendritic Cells (MoDCs) was performed in a culture medium composed of RPMI 1640 (Biochrome AG, Germany), 2% human albumin and antibiotics (100 IU penicillin/ml, 50 µg streptomycin/ml, 100 µg neomycin/ml – Sigma, Germany). PBMC was suspended in the culture medium, and after 90 min. incubation, non-adherent cells were washed out. Adherent cells were suspended in the culture medium, after 90 minutes of incubation, nonadherent cells were washed out. rhGM-CSF (Gentaur, Belgium) and rhIL-4 (Gentaur, Belgium) – enriched medium was added to nonadherent cells. Further doses of cytokines were added on 3rd and 5th day.

Growth termination: Supernatant was centrifuged on the 6th day, and stored in -80°C. Immature MoDCs were removed from the medium and added to supernatant, washed and resuspended. Cells were counted and their viability was assessed before further steps of the procedure.

Assessment of Dextran-FITC phagocytosis: Endocytosis was assessed at 4th and 24th hour of MoDC incubation with Dextran-FITC particles (fast and slow endocytosis, respectively). 40 000 kDa Dextran-FITC (Sigma, Germany) was added to the cellular suspension and incubated at 37°C for test sample and 4°C for control sample. Endocytosis was terminated at 4th/24th hour by placing the sample on ice. Immediately after washing, cytometric analysis was performed using FASC Calibur (BD, USA) flow cytometer. The results were presented as MFI, calculated by deducting the control sample results from the study sample results. Cells were analyzed in the LSM-5 Pascal (Carl Zeiss, Germany) confocal microscope, immediately after the specimen was prepared.

MoDC immunophenotyping: Immunophenotype of the generated MoDCs was assessed with following human monoclonal antibodies: anti-CD45 FITC, anti-CD14 R-PE, anti-CD83 FITC, anti-CD1a R-PE, anti-HLA-DR PeCy-5, anti-CD86 FITC, anti-CD80 R-PE, anti-CD209 FITC, anti-B7-H2 R-PE, anti-CD209 FITC anti-CD206 R-PE, anti-CD208 R-PE, anti-IL-12 R-PE (BD Pharmingen, USA); negative control were pure DCs or FMO (Fluorescence-Minus-One) control was used. MoDC immunophenotyping was performed with FACSCalibur (Becton Dickinson, USA) flow cytometer and analyzed with CellQuest software (Becton Dickinson, USA).

IL-6, IL-10, IL-12 and IFN-γ measurement in DC culture supernatant: IL-6, IL-10, IL-12 and IFN-γ concentration in MoDCs supernatants was measured by following ELISA (enzyme linked immunoabsorbent assay) tests: Human IL-6, Human IL-10, Human IL-12p70, Human IFN-γ (Bender MedSystems, Austria). Results were read with automatic ELISA reader ELx 800 (Bio-tek Instruments, USA).

Statistical analysis: Statistical analysis was performed with STATISTICA 7.1 PL (StatSoft Inc, USA) package. Normal distribution was evaluated with Shapiro-Wilk test. As the normal distribution was rejected, nonparametric tests were used in the further analysis. Statistical significance level p≤0.05 was used.

RESULTS

Maturity of the generated DCs: A significantly higher percentage of CD45+/CD14+ cells was observed on the 5th day of culturing in patients with colorectal cancer (CC) than in control group (H). Dendritic cells generated in colorectal cancer patients had also significantly lower CD1a+/CD83+ (p<0.00001) and
CD1a-/CD83+ (p<0.01) as compared with the control group. No significant differences were observed for CD1a+/CD83- markers. Results are presented in tab. 1.

C-type lectin family: In CC group, significantly higher cell percentage was observed for CD206+/CD209+ antigens (p<0.001) and significantly lower percentage of CD206-/CD209+ (p<0.05) as compared with the control group. Results are presented in tab. 2.

Antigens of specific immune reaction: DCs generated in colorectal cancer patients were characterized by significantly higher CD83-/CD208+ (p<0.01) percentage, and significantly lower CD83+/CD208+ (p<0.01) percentage than control group. Results are presented in tab. 3. DCs generated in colorectal cancer patients were characterized by significantly higher CD80-/CD86+ (p<0.001) percentage, and significantly lower CD80+/CD86+ (p<0.01) percentage than control group. Furthermore, MoDCs generated in colorectal cancer group were characterized by significantly higher (p<0.01) B7-H2+/CD209+ cell percentage and significantly higher (p<0.05) HLA-DR expression than the control group. Results are presented in tab. 3.

Dextran-FITC absorption by generated DCs: No significant differences of Dextran-FITC endocytosis between MoDCs generated in healthy donors and CC group for 4hrs and 24hrs incubation.

Concentration of IL-6, IL-10, IFN-γ and IL-12 in supernatant and IL-12 producing DC percentage: DCs generated in colorectal cancer group were characterized by significantly higher CD83-/IL-12+ (p<0.01) and CD83+/IL-12+ (p<0.0001) percentage than the control

Table 1. Maturity markers expression of dendritic cells generated from the healthy donors (control group, H), and colorectal patients (group CC), * = p<0.05

<table>
<thead>
<tr>
<th>Cell percentage (%)</th>
<th>Control group: H</th>
<th>Colorectal cancer: CC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SD</td>
<td>median</td>
</tr>
<tr>
<td>45+/14+ *</td>
<td>1.29 ±2.54</td>
<td>0.48</td>
</tr>
<tr>
<td>45+/14-</td>
<td>91.84 ±6.94</td>
<td>92.94</td>
</tr>
<tr>
<td>1a+/83-</td>
<td>58.07 ±14.06</td>
<td>58.53</td>
</tr>
<tr>
<td>1a+/83+ *</td>
<td>17.90 ±7.66</td>
<td>16.55</td>
</tr>
<tr>
<td>1a-/83+ *</td>
<td>9.56 ±7.28</td>
<td>8.29</td>
</tr>
</tbody>
</table>

Table 2. C-lectin family antigens expression of dendritic cells generated from the healthy donors (control group, H), and colorectal patients (group CC), * = p<0.05

<table>
<thead>
<tr>
<th>Cell percentage (%)</th>
<th>Control group: H</th>
<th>Colorectal cancer: CC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SD</td>
<td>median</td>
</tr>
<tr>
<td>206+/209-</td>
<td>1.68 ±0.58</td>
<td>1.61</td>
</tr>
<tr>
<td>206+/209+ *</td>
<td>46.69 ±12.55</td>
<td>44.96</td>
</tr>
<tr>
<td>206-/209+ *</td>
<td>42.09 ±12.25</td>
<td>42.28</td>
</tr>
</tbody>
</table>

Table 3. Specific immunological response antigenexpression of dendritic cells generated from the healthy donors (control group, H), and colorectal patients (group CC), * = p<0.05

<table>
<thead>
<tr>
<th>Cell percentage (%)</th>
<th>Control group: H</th>
<th>Colorectal cancer: CC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SD</td>
<td>median</td>
</tr>
<tr>
<td>86+/80- *</td>
<td>6.92 ±2.77</td>
<td>6.65</td>
</tr>
<tr>
<td>86+/80+ *</td>
<td>65.24 ±17.36</td>
<td>64.88</td>
</tr>
<tr>
<td>86-/80+</td>
<td>20.38 ±18.22</td>
<td>14.95</td>
</tr>
<tr>
<td>B7-H2+/209-</td>
<td>0.40 ±0.16</td>
<td>0.37</td>
</tr>
<tr>
<td>B7-H2+/209+ *</td>
<td>1.07 ±0.74</td>
<td>0.97</td>
</tr>
<tr>
<td>83-/208+ *</td>
<td>59.92 ±12.14</td>
<td>59.62</td>
</tr>
<tr>
<td>83+/208+ *</td>
<td>13.02 ±7.08</td>
<td>12.74</td>
</tr>
<tr>
<td>MFI HLA-DR *</td>
<td>589.86 ±234.3</td>
<td>554.90</td>
</tr>
</tbody>
</table>
group. No significant difference of IL-6, IL-10, IL-12p70, IFN-γ concentrations in supernatants of DC cultures from colorectal patients and healthy donors was observed. Results are presented in tab. 4.

**DISCUSSION**

Production of effective DC antineoplastic vaccine must adhere to several aspects of DC biology to be taken into account, as the immunotherapy success may depend on that. The vaccine must induce antineoplastic response, while remaining safe for the patient (11). Immunotherapy research is focused on autologous system, even though some authors suggest dysfunction of DCs in cancer patients (12, 13). Peripheral blood monocytes of colorectal cancer patients lose CD14 antigen within 5 days of culturing in IL-4 and GM-CSF medium, simultaneously CD1a expression – marker of immature DCs increases (14). CD14 expression is the first benchmark of DCs maturity. CD14+ cells percentage was significantly higher among MoDCs generated in colorectal cancer group, than in healthy donors. According to Conti et al., presence of small percentage of CD14+ cells should not be considered a failure, as CD14 enters the protein phagocytosis process, which is beneficial for antineoplastic vaccine (15).

CD84 is one of key markers of DC, and its expression increases with maturity and activation of DCs. Current literature lacks the information on the CD83 antigen expression on immature MoDCs generated from patients with colorectal cancer. Kim et al. as well as Matsumoto et al. reported high percentage of CD83+ monocytes generated from renal cell carcinoma patients (16, 17). High percentage of CD83+ MoDCs was observed in our study, and it was significantly higher for CD1a+/CD83+ and CD1a-/CD83+ in comparison with healthy donors. More mature MoDCs generated in patients could be unfavorable for antineoplasticimmunotherapy. Mature DCs loose their ability of phagocytosis, and would not initiate the immune response against the neoplastic antigens in a patient.

C-type lectin family antigens, which include CD205, CD206, CD207 and CD209 are typical for immature DCs. Main function of MMR (CD206) molecule is to phagocyte mannose and galactose antigens and transport into early endosomes (18). DC-SIGN mediated phagocytosis promotes a tolerance against particular antigen (19). Giordano et al. stated, that CD209 expression is greater in DCs generated from monocytes, as opposed to CD206, for which expression decreases with their maturation, according to Kato et al. (20, 21). In the own study, in concomitance with Giordano et al., and Kato et al., a higher percentage of CD206-/CD209+ cells was observed in control group than in colorectal cancer group (CC). Percentage of cells with stronger phagocytic and adhesive/tolerogenic properties (CD206+/CD209+) was higher in CC patients than in control group. The conducted analysis allows to deduct, that dendritic cells generated from patients, have more tolerogenic potential than ones generated from healthy donors.

Endocytosis is one of key properties of immature DCs, which „sample” their environment while residing in the tissues (11). Strong endocytic activity of DCs is related with C-lectin family receptors. In the own study, Dextran-FITC was used to measure phagocytic potential of DCs generated in vitro. Literature data shows, that Dextran-FITC particle phagocytosis is related with CD206 expression and is stronger in DCs generated from peripheral blood monocytes than in DCs generated from CD34+ cells (22). Dextran-FITC phagocytosis is typical for immature DCs. Von Euw et al. studied the Dextran-FITC phagocytosis by DCs generated from healthy donors, before and after stimulation by apoptotic and necrotic melanoma cells (19).

To our knowledge, noliterature from experimental studies is available on comparison

<table>
<thead>
<tr>
<th>Cell percentage (%)</th>
<th>Control group: H</th>
<th>Colorectal cancer: CC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SD</td>
<td>median</td>
</tr>
<tr>
<td>IL-12+/83- *</td>
<td>1,20 ±0,59</td>
<td>1,15</td>
</tr>
<tr>
<td>IL-12+/83+ *</td>
<td>0,42 ±0,28</td>
<td>0,34</td>
</tr>
</tbody>
</table>
of Dextran-FITC phagocytosis by DCs generated from the neoplastic and healthy donors. Most of authors just analyze the absorption of Dextran-FITC by DCs, which is supposed to prove the preserved absorption of particles by DCs. In the own study we did not observe significant differences between DCs generated by patients and controls. It is confirmed by the observation, that no difference of CD206+ cells percentage was observed between the groups. We did not observe a significant correlation of Dextran-FICT absorption and CD206+ percentage, which suggests more coexisting mechanisms of Dextran-FITC molecules.

Expression of B7 family costimulatory molecules, i.e. CD80, CD86 and B7-H2 is important for assessment of the generated DCs, as they provide the “second signal”, required for activation of the naive T-lymphocytes. Diliglou et al. in their research on the role of CD80 and CD86 antigens in MoDCs show that simultaneous blockade of both molecules results with decreased activation of T-lymphocytes (22). Costimulating signal of CD80 – CD154 receptor-complex induces Th1 type reaction, and CD86-CD28 complex induces Th1 reaction. In the own study, a significantly higher percentage of CD80+/CD86+ MoDCs was discovered in healthy donors than in CC patients. Potentially strongest ability to induce Th1 reaction was present in DCs generated from healthy donors, as CD80+ MoCDs were represented at the highest percentage in this group.

B7-H2 antigen was first identified by Wang et al. as a molecule of immature DCs, which acts via ICOS to stimulate lymphocytes to release IL-10, which results with its tolerogenic potential (23). Own analysis of B7-H2 expressing cells percentage shows, that a stronger expression occurs in immature DCs generated from monocytes of colorectal cancer patients than in control group. Lee et al. show, that high B7-H2 expression is significantly correlated with more aggressive character of tumor in colorectal cancer patients (24).

DC-LAMP (CD208) belongs to lysosomal proteins, and participates in formation of antigen complexes processed in lysosomes with MHC molecules (25). Bonehill et al. and Giordano et al. analyzed results of DCs generation from monocytes of healthy donors and showed, that intracellular expression of CD208 increases with DCs maturation, and is closely related to high expression of HLA-DR, CD83, CD80 and CD86 (20, 26). In the own study we observed a significantly higher percentage of CD208+/CD83+ in healthy donors group than in CC group. Interestingly, CD208+/CD83- was significantly higher in CC patients, which, accompanied by stronger expression of HLA-DR, B7-H2 and CD209 on DCs generated in CC patients, and weaker expression of CD83, costimulating molecules (CD80 in particular) suggests, that MoDCs generated fromneoplastic patients have stronger tolerogenic properties.

DCs release multiple cytokines, which are considered „third signal” for T lymphocyte stimulation (27). Depending on the degree of maturity, kind of antigen, and microenvironmental conditions, DCs release IL-12, inducing Th1 response, or IL-10 inducing Th2 response. Ghanekar et al. did non observe differences of intracellular expression of IL-12 between DCs generated in patients with advanced lung, breast and GI cancers, and healthy donors (28). The abovementioned results are different from our results, as both CD83- and CD83+ cells generated from colorectal cancer patients show significantly higher IL-12 expression in comparison with healthy donors. In the own study, we did not observe differences of IL-6, IL-10, IL-12 and IFN-γ in supernatants of generated DC cultures.

The results suggest, that allogenic immunotherapy with MoDCs might be highly useful because of the less tolerogenic features of the cells acquired. It seems however, that HLA incompatibility in case of allogenic MoDCs favors the use of the autologic system.

**CONCLUSIONS**

A higher percentage of immature DCs was observed in colorectal cancer patients than in control group. A higher expression of costimulating molecules of tolerogenic properties were observed in colorectal cancer patients than in control group. Dextran-FITC phagocytosis was comparable in the groups, however, C-lectin family antigens proteins expression was significantly higher in colorectal patients than in the study group. A significantly larger intracellular expression of IL-12 was observed in CC group than in the control group, however, no significant differences of IL-6, IL-10, IL-12 and IFN-γ release were observed between the study groups.
REFERENCES


Received: 18.10.2013 r.
Adress correspondence: 20-090 Lublin, ul. Jaczewskiego 4
e-mail: radejs@wp.pl