DETERMINATION OF THE ACTIVITY OF CD134 (OX-40) AND CD137 (4-1BB) ADHESIVE MOLECULES BY MEANS OF FLOW CYTOMETRY IN PATIENTS WITH COLORECTAL CANCER METASTASES TO THE LIVER

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Colorectal carcinoma (CRC) is one of the most common reasons of mortality in patients diagnosed with neoplasms. In nearly 20% of patients with colorectal carcinoma metastatic lesions are diagnosed. In general, survival of patients with metastatic lesions to the liver and other organs is poor. Conventional therapy of colorectal carcinoma is based on the surgical excision of the tumor, chemotherapy, and radiotherapy.

The aim of the study was to determine the expression of CD134 and CD137 molecules inside the tumor, at the border of the tumor, in the healthy tissue, and peripheral blood, considering patients with colorectal carcinoma metastases to the liver.

Material and methods. The study group comprised 39 patients subject to surgical treatment at the Department of General and Gastroenterological Surgery, due to colorectal carcinoma with liver metastases. CD134 and CD137 adhesive molecule levels were determined inside the tumor, at the border of the tumor, and in the healthy margins of the surgical incision. Additionally, the authors evaluated the peripheral blood level of the above-mentioned molecules on the day of the surgical procedure, and 10 days, thereafter.

Results. The mean CD134 levels were the highest inside the tumor, significantly decreasing towards the direction of healthy tissues. The average peripheral blood molecule levels were four-fold higher on the day of the surgical procedure, as compared to values obtained on the tenth postoperative day. This dependency also concerned the remaining statistical measures. The mean CD137 levels showed no significant difference, regardless their location. The authors observed significant, peripheral blood, CD137 level differences, considering the day of the surgical procedure and tenth postoperative period. The mean CD137 peripheral blood level was several times higher on the day of the surgical procedure, as compared to the postoperative period.

Conclusions. The determination of the activity of CD134 and CD137 molecules might create opportunities to plan treatment and predict prognosis in case of colorectal carcinoma. Proper immunotherapeutic management which is based on the expression of the above-mentioned molecules might help determine the risk of metastases, preventing from their development. In advanced cases treatment of liver metastases might be possible.

Key words: colorectal carcinoma, liver metastases, adhesive molecules, CD134, CD137, flow cytometry

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Colorectal carcinoma (CRC) is the second most common (after lung cancer) tumor responsible for mortality. Nearly 20% of patients diagnosed with colorectal cancer present with metastases. The risk of metastatic lesions in case of colorectal carcinoma is connected with
the histological features of the tumor, its stage, and lymph node involvement. Although the most common reason for liver resections in highly-developed countries is the presence of colorectal cancer metastases, only 20% of the above-mentioned patients are subject to surgical intervention. The five-year survival in case of these patients ranges between 25 and 40%, despite concomitant adjuvant chemotherapy (2). Prognosis in case of colorectal cancer metastases to the liver and other organs is poor. Mean survival amounted to 37 months (1).

Conventional therapy of colorectal cancer consists in the surgical resection of the tumor, chemotherapy with the use of selectively toxic agents directed against the differentiation of neoplastic cells, and targeted gamma radiation (2). Many authors demonstrated that adjuvant chemotherapy may give very good results. After two years, only 10% of patients receiving adjuvant chemotherapy (HAI – Hepatic Arterial Infusion) were diagnosed with liver metastasis recurrence (2, 3, 4).

In recent years biological therapy has been successively introduced into treatment using agents inhibiting growth factors of malignant cells blocking tumor neovascularization (2). Amongst monoclonal antibodies (mAbs) which were introduced during tumor therapy, most exert an indirect activity leading towards minimal natural improvement and artificial destruction of cells (5, 6).

Despite the poor prognosis, better understanding of the biology of the tumor, together with the development of molecular biology, renders greater possibilities for the treatment of advanced colorectal cancer patients with the use of immunological methods (2).

Immunotherapy in colorectal cancer is promising, especially in case of metastases by means of the systemic stimulation of the anti-neoplastic response against the dissemination of host cancer cells (1). Investigations are aimed in the direction of the usefulness of neoplastic antigens in the diagnosis, monitoring, and treatment of cancer. Special attention is attributed to monoclonal antibodies (mAbs) (directed against neoplastic antigens) and vaccines, which mainly stimulate the cellular response (6).

Adhesive molecules (CD – cluster of differentiation) constitute a large group of molecules of differentiated structure and function, which play an important role in the immunological response during the course of many pathological conditions. In the past years CD134 and CD137 significance has been mentioned in the treatment of colorectal carcinoma (1, 3, 7-15).

CD134 (OX-40 receptor) and OX-40L (ligand) belong to the TNFR (tumor necrosis factor receptor) family. CD134 is a receptor located on the surface of active CD4+ cells. The molecule has receptors significantly influencing cellular activity, proliferation, and apoptosis. The corresponding OX-40L ligand is located on activated B cells, macrophages, dendritic and epithelial cells. The activated CD4+ cells (by the CD134 molecule) intensify their proliferation and cytokine production, and prolong the survival of specific memory T cells (2, 3, 8, 10, 11).

CD137 (4-1BB) is a receptor located on the surface of memory T and NK (natural killer) cells, while the 4-1BBL ligand is produced on the surface of B and dendritic cells, and macrophages. Lymphocytes B, macrophages, and dendritic cell expression is observed. CD137 and ligand stimulation induce T cells towards increased IL-2 production, which in turn stimulates the proliferation of T lymphocytes, development of NK and cytotoxic lymphocyte cells (1, 2, 9, 12-15).

The determination of the level of CD134 and CD137, considering different stages of the neoplastic disease, before and after the surgical procedure, as well as inside the tumor, at the border of the tumor, in the healthy tissue, and peripheral blood, might prove helpful when analyzing treatment results, and considering prognosis and possibility of local and distant recurrences. Analysis of the above-mentioned parameters might be of clinical significance, enabling to determine an effective adjuvant model in the treatment of colorectal cancer by means of immunotherapy.

The aim of the study was to determine the expression of CD134 and CD137 molecules inside the tumor, at the border of the tumor, in the healthy tissue, and peripheral blood, considering patients with colorectal carcinoma metastases to the liver.

MATERIAL AND METHODS

The study group comprised 39 patients subject to surgical treatment at the Department of General and Gastroenterological Surgery, Medical University in Białystok, due to
The study group comprised 15 female and 24 male patients. Mean age amounted to 62.5 years (±22.3). Eight patients were diagnosed with T2 intestinal wall infiltration, 16 with T3, and the remaining 15 with T4. All patients were diagnosed with local lymph node metastases. The study was approved by the Bioethics Committee.

The study was performed on the basis of data obtained during surgical procedures. CD134 and CD137 adhesive molecule expression was determined in the neoplastic tissue (N), at the border of the tumor (P), and healthy tissue margin. Additionally, the authors evaluated the peripheral blood expression of the above-mentioned molecules on the day of the surgical procedure (K1) and 10 days (K10), thereafter.

Laboratory investigations were performed at the Flow Cytometry Lab, Children’s Clinical Hospital, Medical University in Białystok. Lymphocytic cells were obtained by means of the mechanical isolation method. After double lavage these cells were counted in Burker’s chamber. Afterwards, a solution with a $10^6$ density comprising nuclear cells/ml (RPU/1640) was created. Following portions comprising CD134 and CD137 monoclonal antibodies were prepared. After 20 minutes of incubation at room temperature and proper stirring the samples were subject to quantitative analysis using the EPICS XL (Coulter) flow cytometer ($10^4$ of cells).

The obtained results were subject to statistical analysis. Mean, median, minimal, maximal, and standard deviation values were evaluated. Statistical analysis was performed by means of the Fisher test.

RESULTS

Table 1 presented statistical measures, considering the expression of CD134 inside the tumor, at the border of the tumor, in the healthy tissue, and in the peripheral blood on the day of the surgical procedure, and 10 days after surgery. Mean CD134 expression values were highest inside the tumor (20.92), consecutively diminishing in the direction of the healthy tissue (border of the tumor – 16.00, healthy tissue margin – 14.23). In the abovementioned case, p<0.05 was considered as a statistically significant difference. Similar dependencies were observed in case of minimal and median values. The maximal CD134 level was lower at the border of the tumor (25), as compared to the healthy tissue (30). Mean peripheral blood expression values of the above-mentioned molecules were four-fold higher on the day of the surgical procedure (9.56), as compared to values obtained 10 days after surgery (2.07) (p<0.05). The above-mentioned dependency also concerned the remaining statistical measures. Based on the obtained results the standard deviation of CD134 levels on the day of the surgical procedure was significantly higher (8.46), as compared to values obtained 10 days after the operation (1.50).

Table 2 presented the above-mentioned parameters, considering CD137 parameters. Mean CD137 expression values showed no differences, considering the tumor (3.55), border of the tumor (2.93), and healthy tissue (3.07). No statistically significant differences, considering the remaining measures were observed. In case of peripheral blood, significant differences considering CD137 expression values were observed, between the day of the surgical procedure, and 10 days after surgery. The mean CD137 expression value in the peripheral blood on the day of the surgical procedure was several times higher (7.38), as compared to day 10 after the operation (1.68) (p<0.05). The standard deviation was as follows: on the day of the surgical procedure 10.62, and 10 days after the operation 1.44.

Table 1. CD134 expression levels

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor (G)</td>
<td>20.92</td>
<td>18</td>
<td>11</td>
<td>41</td>
<td>8.5</td>
</tr>
<tr>
<td>Border of tumor (P)</td>
<td>16</td>
<td>16</td>
<td>7</td>
<td>25</td>
<td>4.75</td>
</tr>
<tr>
<td>Healthy tissue (Z)</td>
<td>14.23</td>
<td>13</td>
<td>3</td>
<td>30</td>
<td>7.54</td>
</tr>
<tr>
<td>Peripheral blood before surgery (K 1)</td>
<td>9.56</td>
<td>6.7</td>
<td>1.6</td>
<td>31</td>
<td>8.46</td>
</tr>
<tr>
<td>Peripheral blood after surgery (K 10)</td>
<td>2.07</td>
<td>1.8</td>
<td>0.1</td>
<td>4.9</td>
<td>1.5</td>
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</table>
Figure 1 presented the mean expression values of the above-mentioned adhesive molecules in the tumor and healthy tissue. One may observe significant differences in case of CD134 values (p<0.05), and no significant differences considering CD137 molecules (p-ns).

Figure 2 presented the mean CD134 and CD137 expression values, considering peripheral blood. One may observe a significant higher value of both molecules on the day of the surgical procedure, as compared to day 10 after the operation (p<0.01).

Table 2. CD137 expression levels

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<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Median</th>
<th>Min</th>
<th>Max</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor (G)</td>
<td>3,55</td>
<td>3</td>
<td>0,9</td>
<td>10</td>
<td>2,8</td>
</tr>
<tr>
<td>Border of tumor (P)</td>
<td>2,93</td>
<td>2,4</td>
<td>0,1</td>
<td>7</td>
<td>2,43</td>
</tr>
<tr>
<td>Healthy tissue (Z)</td>
<td>3,07</td>
<td>1,7</td>
<td>0,1</td>
<td>10</td>
<td>3,04</td>
</tr>
<tr>
<td>Peripheral blood before surgery (K 1)</td>
<td>7,38</td>
<td>2</td>
<td>0,4</td>
<td>34,2</td>
<td>10,62</td>
</tr>
<tr>
<td>Peripheral blood after surgery (K 10)</td>
<td>1,68</td>
<td>1,2</td>
<td>0,2</td>
<td>5,5</td>
<td>1,44</td>
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</table>

**DISCUSSION**

Tumor development is a multi-stage process. Carcinogen factors exert no direct effect on the development of cancer. However, they activate indirect factors, which may lead towards the development of cancer. If the activity of anti-carcinogenic factors is insufficient one may observe DNA damage. The immunological system is inactive at this stage. Immunity processes are initiated at the moment of neoplastic cell development, when antitumor mechanisms are inactive. Most neoplastic antigens are non-specific. Nevertheless, the above-mentioned may be used in the detection, monitoring and treatment of tumors. Patients with tumors present with attenuation of the cellular immune response. Some immunological processes in patients with tumors are antineoplastic inactive, and favor the development and tumor growth (2, 6).

Tumor treatment methods which involve the modification of the immunological system often complement conventional neoplastic therapy methods. Considering the different forms of immunotherapy the following should be distinguished: active (intensification of the patients’ immunological reactivity), passive (antibody administration), and adoptive (intravenous or local administration of immune system cells).

A different classification of immunotherapy methods is as follows: specific methods (antineoplastic vaccines, antibodies, and lymphocyte adoptive therapy), and non-specific methods (immunostimulating agents, cytokines, cellular adoptive therapy). The choice of the immunotherapeutic method in case of tumor presence is extremely difficult. The specific influence of the disease on the immunological system is required. Only the precise evaluation of immunological parameters characteristic of a given tumor, in comparison to the patients physiological condition, enable to determine standard immunotherapeutic management guidelines (5, 16, 17, 18).
In patients with diagnosed colorectal carcinoma one may frequently observe the development of distant metastases, especially in the liver. Patients with colorectal cancer liver metastases cannot always be subject to radical surgical procedures, consisting in the excision of part of the liver. Even if such a procedure is performed one often may observe disease recurrence, and survival of these patients is rather short. In many cases adjuvant chemotherapy and radiotherapy is insufficient (1-4).

Modern immunological methods demonstrated the decisive role of the dysfunction of the immunological system in the development of the neoplastic disease. Diagnosis and evaluation of immunological parameters responsible for the progression of the disease seem to be most important for contemporary investigators. Their value and influence on the immunity system is essential when planning immunotherapy methods.

Available literature data described treatment results considering mainly animal models (1, 8, 9, 11, 12, 14), with only few human models (3, 13, 15).

In our study we determined the expression of human tissue CD134 and CD137 adhesive molecules. Significant reduction of peripheral blood adhesive molecule values was observed between the first and tenth day after the operation. The production of interleukins was significantly reduced. The production of IgE, NK cells, and cytotoxic lymphocytes was also reduced. The evaluation of CD134 and CD137 adhesive molecule expression in the tumor, border of the tumor and healthy tissue show their consecutive decrease in the direction of the healthy tissue. This might be evidence of the weakened immunological response at the border of the tumor and in the healthy tissue margin. Results suggest the use of immunotherapy increasing the activity of CD134 and CD137 receptors, to a favorable modulation of the immunological system. Petty et al. determined the level of CD134 molecules in patients with colorectal cancer (3). They evaluated the expression of CD134 in the tumor, at the border of the tumor, in the healthy tissue, lymph nodes, and liver metastatic lesions. The authors observed a lower expression of OX-40 in healthy tissues, as compared to neoplastic tissue (3).

Until recently, CD137 level evaluation was only conducted on animal models. American authors who investigated the liver of mice with colorectal cancer metastases noted a positive CD137 application result. The results suggested a possible synergism between congenital and acquired immunity, considering therapy of metastatic lesions (1, 10). Our results were similar to those obtained by Dimberga et al. (13). The above-mentioned noted a significantly higher CD137 expression in the tumor, as compared to healthy tissue, considering patients with rectal carcinoma. In case of patients with colon cancer these differences were statistically insignificant (13). Mazzolini et al. believe that these results are a logical consequence of the neoplastic disease, which in turn is responsible for the fact that the activity of T lymphocytes and NK (natural killer) cells is insufficient considering the fight with the neoplastic disease (2).

CONCLUSIONS

The determination of the activity of CD134 and CD137 molecules might create opportunities to plan treatment and predict prognosis in case of colorectal carcinoma. Proper immunotherapeutic management which is based on the expression of the above-mentioned molecules might help determine the risk of metastases, preventing from their development. In advanced cases treatment of liver metastases might be possible.

REFERENCES

Activity adhesive molecules in patients with colorectal cancer metastases to the liver


