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ВЛИЯНИЕ ИММУНОТЕРАПИИ НА ОСНОВЕ АНТИГЕН-ПРАЙМИРОВАННЫХ ДЕНДРИТНЫХ КЛЕТОК НА ПРОТИВООПУХОЛЕВЫЙ КЛЕТОЧНЫЙ ИММУННЫЙ ОТВЕТ У БОЛЬНЫХ КОЛОРЕКТАЛЬНЫМ РАКОМ

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Резюме. Проблема лечения онкологических заболеваний является одной из актуальных для современной медицины. Существующие подходы лечении основаны на хирургическом, лучевом, химиотерапевтическом подходе и также использовании метода иммунотерапии, направленного на маркеры и/или специфические антигены опухолей.

Подходы, основанные на механизмах клеточной и молекулярной регуляции специфического противоопухолевого иммунного ответа, показали свою высокую эффективность (например, антитела против HER2 при раке молочной железы), но эти подходы имеют ряд побочных и нежелательных эффектов, которые ограничивают их применение. Учитывая центральную роль механизмов распознавания опухолевых антигенов и их презентации цитотоксическим клеткам в эффективной элиминации опухоли, является важным поиск и разработка подходов восстановления этих механизмов при онкологической патологии. В связи с тем, что при онкологических заболеваниях нарушается созревание, дифференцировка дендритных клеток и страдает их основная функция, ведутся научные исследования по получению зрелых дендритных клеток и восстановление естественного пути презентации антигена эффекторным клеткам.

В работе были проведены ограниченные клинические исследования (13 больных колоректальным раком), ранее разработанного протокола получения антиген-праймированных дендритных клеток больных колоректальным раком и их совместной культуры с аутологичными мононуклеарными клетками в условиях *in vitro*. Из периферической крови онкобольных были получены дендритные клетки, праймированные аутологичными опухолевыми антигенам (лизат опухолевых клеток), которые сокультивировали с собственными мононуклеарными клетками в присутствии иммунорегуляторных цитокинов (IL-12 и IL-18). Полученные клеточные суспензии очищались от культуральной среды и цитокинов и использовались для проведения курса иммунотерапии (еженедельно по 20-30 млн клеток

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внутривенно, капельно), состоящего из 3-5 введений. На разных сроках проведения иммунотерапии (до начала курса иммунотерапии, через 3 месяца и через 6 месяцев после окончания иммунотерапии) оценивали в периферической крови больных иммунологические показатели (иммунограмма ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, CD19, $CD16^+CD56^+$-клетки), относительное содержание Т-регуляторных клеток ($CD4^+CD25^+FoxP3^+$-клетки), миелоидных супрессорных клеток ($CD14^+HLA\text{-}DR^-$-клетки)) и цитотоксическую активность мононуклеарных клеток периферической крови больных против клеток опухолевой линии колоректального рака человека (Colo-320).

Полученные данные показали, что у онкологических больных на фоне проводимой иммунотерапии достоверно возрастает показатель прямого цитотоксического теста, что позволяет судить об эффективной стимуляции противоопухолевого клеточного иммунного ответа. На это также указывает возрастание относительного количества $CD16^+CD56^+$-клеток (NK-клетки) через 3 месяца после иммунотерапии. Изучение иммуносупрессорных клеток в крови онкобольных показало отсутствие значимых изменений $CD14^+HLA\text{-}DR^-$-клеток и Т-регуляторных клеток.

Таким образом, проведенные ограниченные клинические исследования иммунотерапии больных колоректальным раком на основе аутологичных дендритных клеток, праймированных лизатом аутологичных опухолевых клеток, продемонстрировали повышение противоопухолевого цитотоксического иммунного ответа.

Ключевые слова: иммунотерапия, дендритные клетки, опухолевые антигены, клеточная цитотоксичность, колоректальный рак

EFFECT OF ANTIGEN-PRIMED DENDRITIC CELL-BASED IMMUNOTHERAPY ON ANTITUMOR CELLULAR IMMUNE RESPONSE IN PATIENTS WITH COLORECTAL CANCER

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Abstract. The problem of treatment of oncological diseases is one of the most urgent for modern medicine. Existing treatment approaches are based on a surgical, radiation, chemotherapeutic approach, and the use of immunotherapy methods aimed at markers and / or specific antigens of tumors.

Approaches based on the mechanisms of cellular and molecular regulation of a specific antitumor immune response have shown their high efficiency (for example, antibodies against HER2 in breast cancer), but these approaches have a number of side and undesirable effects that limit their application. Considering the central role of the mechanisms of recognition of tumor antigens and their presentation to cytotoxic cells in effective tumor elimination, it is important to search for and develop approaches to restore these mechanisms in cancer pathology. Because maturation, differentiation of dendritic cells and their main function are impaired in oncological diseases, scientific research is underway to obtain mature dendritic cells and restore the natural way of antigen presentation to effector cells.

The work carried out limited clinical studies (13 patients with colorectal cancer), a previously developed protocol for obtaining antigen-primed dendritic cells of patients with colorectal cancer and their joint culture with autologous mononuclear cells *in vitro*. From the peripheral blood of cancer patients, dendritic cells primed with autologous tumor antigens (tumor cell lysate), which were co-cultured with their own mononuclear cells in the presence of immunoregulatory cytokines (IL-12 and IL-18). The resulting cell suspensions were purified from the culture medium and cytokines and used for a course of immunotherapy (weekly, 20-30 million cells intravenously, dropwise), consisting of 3-5 injections. At different periods of immunotherapy (before the start of the course of immunotherapy, 3 months and 6 months after the end of immunotherapy), immunological parameters were assessed in the peripheral blood of patients (immunogram ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$,

CD19, $CD16^+CD56^+$-cells), the relative content of T-regulatory cells ($CD4^+CD25^+FoxP3^+$-cells), myeloid suppressor cells ($CD14^+HLA\text{-}DR^-$ cells)) and assessed the cytotoxic activity of peripheral blood mononuclear cells of patients against cells of the tumor line of human colorectal cancer (Colo-320).

The data obtained showed that in cancer patients, against the background of ongoing immunotherapy, the indicator of the direct cytotoxic test significantly increases, which makes it possible to judge the effective stimulation of the antitumor cellular immune response. This is also indicated by an increase in the relative number of $CD16^+CD56^+$-cells (NK-cells) 3 months after immunotherapy. The study of immunosuppressive cells in the blood of cancer patients showed the absence of significant changes in $CD14^+HLA\text{-}DR^-$-cells and T-regulatory cells.

Thus, limited clinical studies of immunotherapy of patients with colorectal cancer based on autologous dendritic cells primed with lysate of autologous tumor cells demonstrated an increase in the antitumor cytotoxic immune response.

Keywords: immunotherapy, dendritic cells, tumor antigens, cellular cytotoxicity, colorectal cancer

Introduction

The incidence of cancer in the population in many countries worldwide shows a steady trend to increase. In Russia, colorectal cancer ranks the second in the pattern of malignant neoplasm incidence (11,3%) and related mortality (13,1%). Every year in Russia this nosology is diagnosed in more than 50 thousand patients. According to the histological classification of the World Health Organization, there are 4 main forms of colorectal cancer: adenocarcinoma, mucous adenocarcinoma, cricoid and squamous cell carcinoma. Adenocarcinoma accounts for 75-80% of colorectal cancer cases [5].

Surgical intervention, chemotherapy and radiation therapy are considered classical methods of treating patients with colorectal cancer, but the results of these methods, in general, remain ineffective [9]. Currently, along with other methods of conservative therapy for malignant neoplasms, specific immunotherapy is a modern and promising approach, the main purpose of which is to induce and maintain a long-term immune response aimed at recognizing and eliminating tumor cells.

The effectiveness of modern immunotherapy for oncological diseases relies on correcting impaired antigen presentation and generation of antigen-specific cytotoxic T-lymphocytes. Dendritic cells (DCs), professional antigen-presenting cells, play a key role in these processes [7, 10]. Dendritic cells are considered the most powerful stimulators of the body immune responses, able to recognize and present antigens to T- and B-lymphocytes in the context with MHCI and class II molecules, which are expressed in large quantities on the cell surface along with co-stimulatory molecules (CD80, CD86) [6]. Therefore, mature DCs demonstrate a high ability to present tumor-associated antigens *in vitro* and *in vivo* [2, 9]. Primed $CD4^+$ and $CD8^+$T-lymphocytes secrete cytokines such as interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), which promote CTL proliferation, destruction of tumor tissue and potential control and even elimination of tumor cells [8]. However, the functional activity of DCs in cancer patients is significantly reduced mainly due to impaired DC maturation to functionally active counterparts [1, 6]. In this regard, the production of mature functionally active DCs *in vitro* and their activation with tumor-associated antigens to stimulate a cytotoxic response is promising for developing dendritic cell-based antitumor vaccines and allows mobilizing the patient own defense systems using natural ways of recognizing tumor antigens and theirs subsequent elimination.

There are many methods for the production of DCs *in vitro* that stimulate the antitumor response, including loading of cells with proteins of tumor-associated antigens, as well as the introduction of DNA or mRNA encoding such antigens [3]. The loading of dendritic cells with tumor lysate allows for the presentation of the entire spectrum of patient-specific tumor antigens. The possibility of antigen-specific activation of dendritic cells with the formation of antitumor cytotoxic immune response is currently considered as one of the promising methods to fight cancer.

Thus, **the aim of the study** was to study the effect of cellular immunotherapy of patients with colorectal cancer based on autologous antigen-primed dendritic cells and mononuclear cells “trained” by them to stimulate antitumor cytotoxic immune response.

Materials and methods

The studies were carried out within the limited clinical protocol NCT0321493 (clinicaltrials.gov), dedicated to investigate clinical and laboratory effectiveness of immunotherapy based on autologous antigen-activated dendritic cells in the treatment of patients with colorectal cancer. The study was

approved by the local ethics committee at the RIFCI. The selection of patients (13 patients with colorectal cancer) was carried out according to the inclusion and exclusion criteria.

The study consisted of the following main stages:

1. Stage of clinical examination (selection of patients with colorectal cancer who meet the research criteria, and their clinical examination, review and providing signed up informed consent).

2. The stage of obtaining biological material (collection of peripheral venous blood of a cancer patient, a histological sample of a malignant tumor (colorectal cancer) during planned surgical treatment).

3. Laboratory stage for preparing cell suspension for immunotherapy.

The immunotherapy is based on the use of *in vitro*-induced autologous dendritic cells, primed with their own tumor antigens, and their co-culture with autologous mononuclear cells under *in vitro* conditions to "train" effector cells and induce an antitumor cytotoxic immune response [12]. The maturation of the induced dendritic cells and their viability were monitored by flow cytometry (BD FACS Verse). Co-culture of antigen-primed dendritic cells and MNCs was performed in the presence of recombinant IL-12 and IL-18 to obtain specifically activated lymphocytes. The final cell suspensions were cryopreserved.

4. Stage of immunotherapy.

Immunotherapy was carried out as planned (3-4 weeks after surgery; in the period between chemotherapy courses). Injections of the cell suspension were carried out weekly by using 20-30 million cells dissolved in 100 ml of 0.9% NaCl (physical solution) administered via IV drip in the hospital of the RIFCI immunopathology clinic. The number of injections (a course of cellular immunotherapy) depended on the final number of cells obtained during the laboratory stage upon preparing cell suspension for immunotherapy and ranged from 3 to 5 injections.

3 and 6 months after the end of the full course of cellular immunotherapy), repeated blood sampling was performed to assess the immunological parameters.

5. Stage of evaluating the effectiveness of the conducted immunotherapy.

For patients with IIA, IIB, IIIA, IIIB stages of colorectal cancer, it is planned to assess the quality of life, as well as overall and disease-free survival within 3 years after completing all stages of treatment.

Assessment of peripheral blood immunological parameters during immunotherapy included parameters of cellular immunity ($CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$, $CD19^+$, HLA-DR expression on monocytes), assessment percentage of T-regulatory cells ($CD4^+CD25^+FoxP3^+$), analyzed by flow cytometry. Monitoring of immunological parameters was carried out before vaccine administration, 3, and 6 months after the last administration. In addition, as a direct indicator of antitumor cytotoxic activity, we assessed the ability of MNCs at different periods of immunotherapy (before vaccine administration and 3 and 6 months after the last administration) to cause the death of tumor cells (human colorectal cancer cell line Colo-320) by using "CytoTox 96 Non-Radioactive" kit (Promega). The change in the level of intracellular enzyme (lactate dehydrogenase) in the conditioned environment of the co-culture between tumor cells and the cell "preparation" was used to estimate the percentage of tumor cell death in the cytotoxic test and, thereby, an effect of immunotherapy on inducing antitumor immune response.

Results and discussion

Evaluating cytotoxic test of peripheral blood mononuclear cells 6 months after immunotherapy showed significant increase in the tumor cell death (human colorectal cancer cell line Colo-320) compared to that observed before immunotherapy and 3 months after immunotherapy (Figure 1).

When assessing the parameters of the cellular immune response, a significant increase in percentage of $CD16^+CD56^+$-cells was revealed 3 months after the end of immunotherapy (Figure 2). The relative quantity of $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$-cells in the peripheral blood of cancer patients at different timepoints before and after immunotherapy did not significantly differ.

Percentage of T-regulatory cells ($CD4^+CD25^+FoxP3^+$) in the blood of patients with colorectal cancer decreased during immunotherapy, but showed no significant differences between the groups (data not shown).

When assessing the level of HLA-DR expression on monocytes, in particular, the density of HLA-DR-negative $CD14^+$ monocytes (myeloid suppressor cells), no significant changes in were revealed (data not shown).

Thus, while applying ongoing cellular immunotherapy after co-culture of autologous antigen-primed dendritic cells and mononuclear cells, it resulted in increased anti-tumor cellular activity as shown in *in vitro* cytotoxic test and increased amount of $CD16^+CD56^+$-cells serving as an indicator of general stimulation in effector cells.

The percentage of suppressor cells (T-regulatory cells and myeloid suppressor cells) in the peripheral blood of patients did not change significantly.

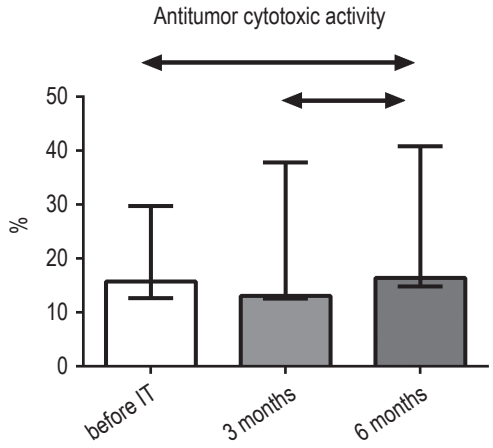

Figure 1. Antitumor cytotoxic activity of mononuclear cells in patients with colorectal cancer at different periods of immunotherapy (before immunotherapy (IT), 3 and 6 months after the end of immunotherapy)

Note. The graph shows the percentage of dead tumor cells of the Colo-320 cell line when co-cultured with mononuclear cells of patients with colorectal cancer *in vitro*. Data are presented as median and upper and lower quartiles, arrows indicate statistically significant differences between groups (n= 13).

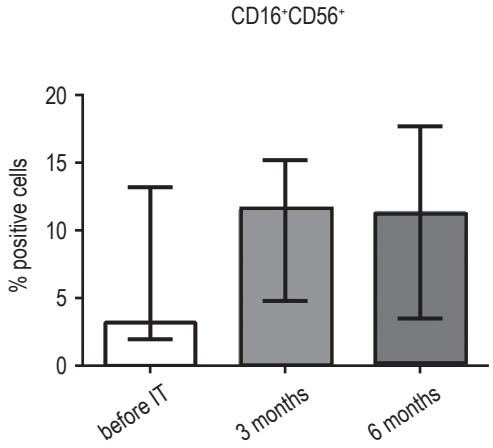

Figure 2. Relative content of $CD16^+CD56^+$-cells in the peripheral blood of patients with colorectal cancer at different periods of immunotherapy (before immunotherapy (IT), 3 and 6 months after the end of immunotherapy)

Note. Data are presented as median and upper and lower quartiles; the arrow indicates statistically significant differences between groups (n = 13).

Conclusion

The data obtained demonstrate that in patients with colorectal cancer receiving ongoing immunotherapy, the indicator of the direct cytotoxic test was significantly increased allowing to conclude about effective stimulation of the antitumor cellular immune response. This is also indicated by increased percentage of $CD16^+CD56^+$-cells (NK-cells) 3 months after immunotherapy. Assessing frequency of immunosuppressive cells in the blood of cancer patients showed a tendency towards a decrease in the number of T-regulatory cells at 3 and 6 months after the end of immunotherapy and no significant changes in the number of $CD14^+HLA\text{-}DR^-$-cells.

Thus, limited clinical studies of immunotherapy of patients with colorectal cancer based on autologous dendritic cells primed with lysate of autologous tumor cells demonstrated enhanced antitumor cytotoxic immune response.

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