Correction of immune disorders in the course of chemotherapy
at generalized form skin melanoma patients

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Introduction

A skin melanoma (SM) is an extremely heterogeneous tumour consisting of a large number of cellular subclones with different geno- and phenotypical signs [1], that stipulates, on the one hand, its low sensitiveness to the chemotherapeutic agents [2], on other - its high immunogenicity [3]. However the last doesn’t influence on a clinical prognosis that for most of SM patients remains worst among all solid malignant new tumors. In opinion of many authors it is related to induction of local and system mechanisms of immunosuppression, resulting in the end in weakening of antitumoral immune defence of organism [3-5].

Immunosuppression of the SM patients is formed gradually on a background of numerous mechanisms, including the decline of expression of molecules of HLA class I on the surfaces of tumour cells, deficiency of co-stimulating signals for the generation of antitumor effector cells, production of immunosuppressive cytokines, induction of T-cells of anergy [6]. These processes find the reflection in a clinic at the estimation of immune status of patients on the stages of treatment [7].

The clinic-laboratory signs of violations in the immune system of patients are find out already on the early stages of SM as activating dysfunction with a subsequent increase in circulation of amount of regulatory T-cells (Treg), decline of indexes of proliferation and cytotoxic activity of peripheral blood lymphocytes (PBL) in vitro, pathognomonic for a metastatic regional lymph nodes. During generalization of tumour process these signs acquire the lines of system
immunosuppression, the morphological equivalent of which is progressive reduction of general amount of PBL with simultaneous reduction of most functionally significant populations - T-lymphocytes and natural killers [8]. Such changes in the immune system of most patients are not succeeded to level in the process of adjuvant therapy by interferon preparations, possessing a pleiotropic action [9] or by a chemotherapy, that grounds the necessity of including to the treatment regimens of SM patients immune preparations with the directed mechanism of action, able to recover the regulatory and effector functions of T-lymphocytes. Among preparations with such mechanism of action is the transfer factor (TF) the active component of that are low-molecular factors of transfer polypeptides of T-origins [10], initiator development of the cellular-mediated immune answer on Th1-scenario (due to induction γ-interferon, interleukin - 1 and -2), that assists renewal of amount and functional activity of T-lymphocytes.

However spared these aspects of treatment of SM patients due attention because of absence of consensus among specialists in relation to the role of immunocompetence of organism in the increase of efficiency of basic treatment, and also in connection with got before [11] unsatisfactory results of application of row of modifiers of biological answer with a pleiotropic action for patients with metastases.

Foregoing served founding for the study of influence of TF on the indexes of the immune system of patients with the generalized form of SM in the conditions of realization of standard chemotherapy.

Object and research methods

There are 20 patients with the inoperable generalized form of SM (9 men and 11 women, middle age (45,9 ± 3,5) of year) were included in research. All patients signed the informed consent to participating in research that was approved by Commission on questions of ethics of the National Cancer Institute. A research design is presented on a fig. 1.
Patients were randomized on two groups: control (10 patients) and basic (10 patients) (table). The standard monochemotherapy of dacarbazine (intravenously for 250 mg/m² during 5 days, with the repeated course in 3 weeks) was conducted the patients of control group; to the patients of basic group additionally to this treatment appointed preparation of TF (dializat of leucocytes of healthy donors - "Immodin" ("Sevapharm", Czech Republic)). Immodin entered hypodermic for 4,0 ml of the dialyzed leucocytes concentrate 1 time per a week, since a 3rd day after completion of 1st course of chemotherapy (3 doses); 4th dose - in 1 month after a 3rd dose.

Table. The characteristic of the surveyed patients

<table>
<thead>
<tr>
<th>The characteristic</th>
<th>Basic group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>6</td>
</tr>
<tr>
<td>Middle age, years</td>
<td>42,7 ± 5,3</td>
<td>49,0 ± 4,5</td>
</tr>
<tr>
<td>Melanoma metastasises in a skin, subcutaneous fat or regional lymph nodes</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Lung melanoma metastasises</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Metastasises in other internal, raised level LDH</td>
<td>9</td>
<td>5</td>
</tr>
</tbody>
</table>

The clinic-laboratory examination of patients was conducted in 4 stages: before treatment (1 stage), in 3 days (2 the stage) and 3,5 weeks (3 the stage) after completion of 1st course of chemotherapy and in 3,5 weeks after completion of 2nd course of chemotherapy (4 the stage). As a group of comparison it was inspected 60 practically healthy people (PHP) (groups are comparable on sex and age).

The study of phenotype of PBL was conducted by the method of flow cytometry with the use of monoclonal antibodies to CD3, CD4, CD8, CD16, CD19, HLA - DR, CD95, marked FITC ("Becton Dickinson", the USA), CD25,
marked PC- 5, CD127, marked RE ("Beckman Coulter", the USA), as described [12, 13]. Treg determined among the subpopulation of CD4+-lymphocytes on the presence of high expression of CD25 in combination with low or negative expression of CD127 (CD4$^{+25^{high}}$127$^{low/neg}$). An immunoregulatory index was expected as ratio of CD4+ / CD8+- lymphocytes. Functional activity of PBL was studied in the reaction of blasttransformation - lymphocytes (RBTL) and cytotoxic test. RBTL was realized by morphological method with the use of monoclonal antibodies to CD3 (anti- CD3, "МедБиоСпектр", Russia) in a concentration 3 $\mu$g/ml or phytohemagglutinin (PHA, "Sigma", Germany) in a concentration 10 $\mu$g/ml. Results expressed in the percents of blasttransformation cells (% BT). The estimation of the induced (anti- CD3 or PHA in the same concentrations) apoptosis of PBL was conducted by the method of flow cytometry [12] with the use of propidium iodide. Results expressed in the percents of lymphocytes being in a state of apoptosis.

Cytotoxic activity of PBL was determined by the method of flow cytometry with the use of cells-targets of line K- 562 [12]. Results expressed as cytotoxic index (CI) in percents:

$$CI = \frac{A-B}{C-B} \times 100\%,$$

where A – is an amount of dead cells-targets in experience;

B – is an amount of dead cells -targets in control;

C – is a common amount of cells -targets.

Account of results of cytotoxic activity, intensity of apoptosis and PBL typing conducted on flow cytometry FACScan ("Becton Dickinson", the USA) with the use of the program "Cell Quest".

The statistical analysis of the obtained data was conducted with the use of the programs of Excel (MS Office 2003, XP) and STATISTICA 6,0 (StatSoft Inc.,
the USA). For determination of authenticity of distinctions (p) of indexes in groups used a criterion Mann-Whitney. Results were presented as a arithmetic mean (M) and its standard error (m). Distinctions estimated as reliable at p < 0.05.

**Results and discussion**

In view of that the our work is devoted to the study of influence of immunotherapy on the indexes of the immune system for patients with the generalized form of SM in the conditions of realization of chemotherapy (without the estimation of its efficiency), we counted up possible to unite data control and basic groups patients of that got of the same type treatment at the 1- and 2nd stages of immunological research (fig. 2).
As be obvious from the data presented on a fig 2, to treatment and on 3rd day after a 1 course of chemotherapy the absolute amount of lymphocytes in peripheral blood of patients registers oneself on the low bound of norm ((1,58 ± 0,12) x 10⁹/L vs (1,77 ± 0,10) x 10⁹/L at PHP, p > 0,05). However in 3,5 weeks (stage 3) maintenance of circulatory lymphocytes goes ((1,24 ± 0,18) x 10⁹/L, p < 0,05) down for the patients of control group. This tendency is saved and upon completion of 2nd course of chemotherapy. Opposite, a chemioimmunotherapy with the use of TF for the patients of basic group is accompanied by maintenance at initial level of absolute amount of lymphocytes in these terms.

According to the results of population analysis of PBL presented on a fig. 2, development of lymphopenia on a background a chemotherapy for the patients of control group is conditioned by the deficiency of the most significant populations of lymphocytes (T- lymphocytes (CD3⁺) and natural killers (CD16⁺)), including, subpopulations of cytotoxic - lymphocytes (CD8⁺). Decline relative amounts over of CD8⁺ клеток in circulation brings to disproportion in subpopulation composition of T-л lymphocytes, what an immunoregulatory index size of that for the patients
of control group on 3 - and 4th stages of treatment substantially exceeds an index at PHP (accordingly \((2,31 \pm 0,12), (2,30 \pm 0,18)\) and \((1,55 \pm 0,10), p < 0,05\)). It is necessary to mark that for the patients of control group in 3,5 weeks after the 1st course of cytostatic therapy relative maintenance of natural killers is restored, their quantity is saved at physiological level and upon termination of treatment, and maintenance of B- lymphocytes \((CD19^+)\) sharply goes down after the 2nd course of chemotherapy \((p < 0,05)\).

Unlike it, application of TF and chemotherapy for the patients of basic group assists renewal of amount of circulatory T- lymphocytes due to the increase of quantity of CD8\(^+\)-cells. Immunoregulatory index after 1- and 2nd courses of chemotherapy does not differ from such at PHP accordingly \((1,42 \pm 0,29), (1,44 \pm 0,33)\) and \((1,55 \pm 0,10), p > 0,05\). It is important to underline that on a background a chemooimmunotherapy the relative amount of natural killers and B- lymphocytes in peripheral blood of patients remains depressed.

Consequently, use of TF and chemotherapy for patients with the generalized form of SM results in renewal of pool of circulating lymphocytes due to the population of T- lymphocytes, disfunction of that, in opinion of most authors [14, 15] is a basic in pathogeny of this disease. In particular, forming of T-cells anergy may be conditioned by an immunosuppression that is related to enhanceable maintenance of Treg [16]. Therefore the dynamics of maintenance of Treg in peripheral blood of patients was studied on the stages of treatment (fig. 3).
Fig. 3. Changes of Treg quantity (%) in peripheral blood of SM patients during treatment

The note. * – distinctions at comparison with a parameter at PHP are statistically significant (p < 0.05).

As be obvious from the data presented on fig.3, before treatment the relative amount of Treg in peripheral blood is substantially enhanceable ((3,79 ± 0,24) vs (2,75 ± 0,14) % at PHP, p < 0,05) and remain at high level for the patients of control group after the 1st course of chemotherapy. Opposite, for the patients of basic group application of TF causes a proof decline of circulatory Treg during the chemotherapy.

The analysis of activating markers on the surface of PBL showed their enhanceable expression during the chemotherapy (fig. 4).
Fig. 4. Changes of activated lymphocytes quantity (%) in peripheral blood of SM patients during treatment

The note. * – distinctions at comparison with a parameter at PHP are statistically significant (p < 0,05).

As be obvious from the data presented on a fig. 4, for patients to treatment the relative amount of lymphocytes is substantially enhanceable, expresive both early (CD25) and late (CD95) activating antigens (p < 0,05), that can testify to their polyclonal activating [8]. During the chemotherapy in the patients of control group the proportion of these lymphocytes in circulation remains at high level to the end of supervision. In addition expression of HLA-DR-antigen rises steadily (p < 0,05). Opposite, addition of TF treatment regimen of patients of basic group assists a decline in peripheral blood of relative amount of activated (CD25⁻, CD95⁻, HLA - DR⁻) lymphocytes to the level at PHP.

Acquirement by lymphocytes an activating phenotype assumes, change of their functional activity. For deeper analysis of consequences of PBL reorganisation the methodical approach, allowed to estimate reaction T-cells to various ways of activation in vitro has been used: through CD3-TCR- receptor (a classical way of activation) and by PHA (alternative) (fig. 5).
As be obvious from the presented data, for patients to treatment and on 3rd day after the 1st course of chemotherapy the level of proliferation of lymphocytes in reply to activating on a classic and alternative way does not differ. Thus to treatment, activating of Т-lymphocytes of patients on a classic way results in strengthening of their death, and on alternative – no. Another reaction is observed on 3rd day after the 1st course of chemotherapy: renewal of intensity of apoptosis of Т-lymphocytes at activating through CD3-TCR-receptor complex and decline at mitogen stimulation. The subsequent course of chemotherapy for the patients of control group results in suppressing of mitogen-induced proliferation of Т-lymphocytes, but does not influence on ability to answer proliferation on activating through CD3-TCR-receptor complex. Thus intensity of apoptosis of Т-lymphocytes after the 2nd course of chemotherapy without depending on the Anti-CD3-induced proliferation

PHA-induced proliferation

Anti-CD3-induced apoptosis

PHA-induced apoptosis

AntiCD3-индуцированный апоптоз

ФГА-индуцированный апоптоз
method of induction goes down. It is important to mark as a discussion, that, although some authors [6, 17] specify on development anergy of peripheral T-lymphocytes - in pathogeny of SM got by us data, testify to convertible character of these violations, as T-lymphocytes demonstrate the adequate answer of in vitro during activating on a classic way. An analogical conclusion is done on the basis of the successful activating and cloning of lymphocytes of SM patients in technology of adaptive cellular immunotherapy [18].

Addition of TF in the treatment regimen of patients of basic group results in strengthening (3 stage) of proliferation of T-lymphocytes during their activating both on classic and alternative way (p < 0,05) with subsequent normalization of its intensity after the 2nd course of chemotherapy. Thus the T-lymphocytes apoptosis index at the 4th stage of research is saved at normal level, unlike the patients of control group. In this connection it is possible to suppose that TF in the conditions of the lymphopenia induced by a chemotherapy, creates necessary conditions for homeostatic proliferation of cytotoxic T-lymphocytes (CD8⁺) [19], the increase of amount of that in circulation is observed during the chemo-immunotherapy for the patients of basic group (fig. 2).

On the other hand, positive dynamics of maintenance and functional activity of cells of adaptive immunity (T-lymphocytes), registered during the treatment of patients with the use of immunotherapy, does not correlate with activity of effectors of the system of innate immunity (fig. 5). Apparently, depressed cytotoxic activity of natural killers vs standard cells-targets of in vitro, registered for patients to the chemotherapy, remains low during all period of supervision (p < 0,05). In these conditions application of TF is not accompanied by renewal of cytotoxic activity of natural killers for patients the generalized form of SM. Going back to data of population composition of PBL, note should be taken on that the patients of control group have a positive dynamics of quantity of natural killers during the treatment, however this fact didn’t influence on cytotoxic activity in vitro.
Working out the total to the expounded material, it is possible to assert that dysfunction of the immune system that is aggravated during chemotherapy is characteristic for patients with the generalized form of SM. The clinic- laboratory criteria of diagnostics of these violations it is been: development of lymphopenia with the signs of T-cell immunodeficiency (due to the decline of maintenance of cytotoxic T-lymphocytes), substantial increase of proportion of Treg and activated lymphocytes in circulation, and also suppressing of mitogen-induction proliferation and cytotoxic activity of lymphocytes of in vitro.

In these conditions the TF, operating exceptionally on T-cells mechanisms of immune homeostasis prevents the development of deep violations in the immune system of patients during the chemotherapy. The important result of the combined chemo-immunotherapy is renewal of quantity of lymphocytes in peripheral blood, including subpopulations of cytotoxic T-lymphocytes, decline of Treg in circulation and maintenance of T-cells functional activity that can influence on efficiency of basic treatment of this category of patients. The follow-up of them will allow to confirm or refute this supposition.

Literature


Control group:

Basic group:
STAGES OF IMMUNOLOGICAL RESEARCH

Fig. 1. Design of research

Notes. ChT-1, -2 – 1-st, 2-nd chemotherapy courses accordingly, IT – immunotherapy.