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Immune status in patients differing in the stage of choroidal melanoma

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Purpose: *To perform a pre-treatment comparison of patients with different stages of choroidal melanoma (CM) for humoral and cell-mediated immune status.*

Material and Methods: *This immune status study included 35 patients with T1 CM (group 1) and 31 patients with T1 to T3 CM (group 2) who were treated at the State Institution "The Filatov Institute of Eye Diseases and Tissue Therapy of the National Academy of Medical Sciences of Ukraine". These patients were compared with 44 practically healthy individuals (group 3 or controls) for immune status. Immunologic evaluation of peripheral blood included absolute counts and percentages of leukocytes, lymphocytes, CD3+ T cells, CD4+ T helpers, CD8+ T suppressors, CD19+ B cells, CD16+ natural killer (NK) cells, phagocytic neutrophil activity (PNA), and imminoglobulins (Ig) A, M and G.*

Results: *Pre-treated patients in groups 1 and 2 had significantly increased absolute counts of leukocytes (p1-3 = 0.005 and p2-3 = 0.0003, respectively), lymphocytes (p1-3 = 0.04 and p2-3 = 0.0002, respectively), and cytotoxic CD8+ T suppressors (p1-3 = 0.0002 and p2-3 = 0.001, respectively), and CD4+/CD8+ ratio (p1-3 = 0.00002 and p2-3 = 0.0000, respectively), and PNA (p1-3 = 0.0004 and p2-3 = 0.0001, respectively), IgA (p1-3 = 0.003 and p2-3 = 0.009, respectively), and IgM (p1-3 = 0.0007 and p2-3 = 0.0000, respectively). There was, however, no significant difference in other parameters between patients and controls. Compared to patients in group 1 and controls, patients in group 2 had significantly increased absolute counts of CD4+ T helpers (p1-2 = 0.02 and p2-3 = 0.0000, respectively), CD16+ NK cells (p1-2 = 0.03 and p2-3 = 0.003, respectively), and IgM levels (p1-2 = 0.02 and p2-3 = 0.0000, respectively), and significantly decreased IgG levels (p1-2 = 0.006 and p2-3 = 0.003, respectively).*

Conclusion: *In pre-treatment patients differing in the stage of CM, the tumor process responds to immune changes that are expressed by activation of humoral and cell-mediated immunity (an increase in the activity of CD3+ T cells, CD4+ T-helpers, CD8+ T-suppressors, CD16+ NK cells, and IgM and IgA levels), which may have a substantial impact on treatment effect.*

Keywords:

choroidal melanoma, immune status, choroid, ocular oncology, immunology

Introduction

Immune response to treatment is rather important. Several authors demonstrated the role of body resistance to tumor growth in the mechanisms of immune response to tumor photocoagulation and brachytherapy (BT) in a patient with uveal melanoma (UM), and feasibility of immune modulating therapy that improves the efficacy of the above techniques [1-11]. In addition, the status of the immune system in a patient with small (T1) choroidal melanoma (CM; measuring ≤ 3 mm in prominence and ≤ 12 mm in basal dimension) was determined; this is important to know when initiating an eye-preserving treatment for CM [12-15]. Given that, BT combined with transpupillary thermotherapy (TTT) has been a leading method of treatment for CM in recent decades, it is feasible to determine the role of the immune system in patients with CM for assessing its involvement in the elimination of tumor cell break-up products exposed to different treatment factors.

The purpose of the study was to perform a pre-treatment comparison of patients with different stages of CM for humoral and cell-mediated immune status.

Material and Methods

This immune status study included 35 patients with T1 CM (group 1) and 31 patients with T1 to T3 CM (group 2) who were treated at State Institution "The Filatov Institute of Eye Diseases and Tissue Therapy of the National Academy of Medical Sciences of Ukraine".

Group 1 consisted of 26 women (74.3%) and 9 men (25.7%) with a mean age (standard deviation (SD)) of 53.9

(12.1) years. Group 2 consisted of 19 women (61.3%) and 12 men (38.7%) with a mean age (SD) of 50.9 (16.0) years. The control group (group 3) consisted of 44 practically healthy individuals, and was similar to groups 1 and 2 with respect to gender and age.

Patients were thoroughly examined by the physician and received a complete blood count, urianalysis, blood glucose test, coagulation examination, Wasserman test, chest X-ray, electrocardiography, and abdominal ultrasonography for potential detection of CM metastasis. By the initiation of treatment, there was no ultrasound evidence of regional or remote (N0) lymph-node involvement or metastasis (M0) in any patient. In addition, no patient exhibited clinical or ultrasound evidence of epibulbar or retrobulbar involvement by the presentation to an ophthalmologist. The tumor, node and metastasis (TNM) classification scheme was used for staging of CM on the basis of tumor dimensions (ultrasound measurements of tumor thickness (mm) and the largest basal diameter (mm)) (Table 1) [16].

The immunological examination was conducted at the immunology laboratory of the institute using conventional methods and included monoclonal antibody-based determination of the following peripheral blood parameters:

- absolute white blood cell (WBC) and lymphocyte counts
- absolute CD3+ T cell count and percentage
- absolute CD4+ T cell count and percentage
- absolute CD8+ T cell count and percentage
- absolute CD19+ B cell count and percentage
- absolute CD16+ natural killer (NK) cell count and percentage
- phagocytic neutrophil activity, and
- levels of immunoglobulins (Ig) A, G and M [17-19].

A 5-ml fasting blood sample was taken from the antecubital vein into a sterile tube filled with 1 mL of a heparin sodium 100 units/mL. Peripheral blood mononuclear cells were obtained from peripheral blood by ficoll-verografin (1.076 to 1.078 g/cm3) density gradient centrifugation. The obtained blood slides were fixed in 10% formalin vapor, placed horizontally in a humidity chamber, and 20 to 50 ml monoclonal antibody for the target gene was applied to particular sites. Incubation was performed at room temperature (18-20 ºC) for 1 h. Thereafter, antibodies of the first layer were removed, the slides were washed 3 times in phosphate-buffered saline, and the cell-free areas of the slide were dried, taking care not to contact reaction sites. This was followed by the application of 20-50 µl of conjugated rabbit serum anti-mouse Ig antibody in excess. Slides with rabbit serum anti-mouse Ig conjugated with biotin were incubated for 30 minutes in a humidity chamber at room temperature. Thereafter, antibodies of the second layer were removed, the slides were washed, and excessive moisture was removed with filter paper. Peroxidase-antiperoxidase (PAP) complex was applied for better cell visualization, and slides were incubated in a humidity chamber at room temperature. Cell nuclei were counterstained with methyl green or Mayer hematoxylin and air dried. Oil-immersion light microscopy was employed to calculate the percentage of lymphocytes expressing monoclonal antibodies. The value obtained was multiplied by 100 to calculate the absolute lymphocyte count (cell/µl) [18].

Statistical analysis

An MS Access data base was developed to facilitate straightforward accession, retrieval and analysis of information. Mean and SD values were calculated. Differences were assessed using the Student t-test for unpaired data and non-parametric Wilkoxon test. For comparisons involving quantitative parameters in more than two groups, one-way analysis of variance with post-hoc Fischer test or Newman-Keuls tests were used. Non-parametric Mann-Whitney test was used for comparisons between two independent groups. P values ≤ 0.05 were considered significant. Data were analyzed using JASP (The JASP Team, Amsterdam, the Netherlands) [20].

This paper is part of the research project "To Examine the Pathogenetic Mechanisms of the Clinical Effect of (Response to) Combination Treatment for Medium and Large Uveal Melanomas and Malignant Lesions of the Palpebral Conjunctiva, Semilunar Fold and Caruncle" (state registration number, 01224U00149). This study

Table 1. Choroidal melanoma T stages in different tumor thicknesses and diameters

Tumor thickness (mm)	Tumor T stage						
> 15.0					4	4	4
12.1-15.0				3	3	4	4
9.1-12.0		3	3	3	3	3	4
6.1-9.0	2	2	2	2	3	3	4
3.1-6.0	1	1	1	2	2	3	4
≤3.0	1	1	1	1	2	2	4
Tumor diameter (мм)	≤3.0	3.1-6.0	6.1-9.0	9.1-12.0	12.1-15.0	15.1-18.0	>18.0

involved human subjects and followed ethical standards as outlined in the 1964 Declaration of Helsinki of the World Medical Association with its further amendments and the European Convention on Human Rights and Biomedicine, and relevant laws of Ukraine. The study was approved by the bioethics committee of SI "The Filatov Institute of Eye Diseases and Tissue Therapy of the National Academy of Medical Sciences of Ukraine" (committee minutes dated April 12, 2024), and informed consent was obtained from subjects.

Results

Table 2 compares groups of patients with different stages of CM versus controls for the parameters of humoral and cell-mediated immunity.

It was found that, in patients with T1 CM (group 1) and T1 to T3 CM (group 2), the parameters of humoral and cell-mediated immunity were impaired compared to healthy controls. In groups 1 and 2 of patients with CM, the following peripheral blood parameters were found to be statistically significantly increased compared with controls:

- absolute WBC counts (p1-3 = 0.005 and p2-3 = 0.0003, respectively)
- absolute lymphocyte counts (p1-3 = 0.04 and p2-3 = 0.0002 respectively)
- absolute counts of cytotoxic CD8+ T cells (p1-3 = 0.0002 and p2-3 = 0.001, respectively);
- CD4+ to CD8+ ratio (p1-3 = 0.00002 and p2-3 = 0.0000, respectively)
- phagocytic neutrophil activity (p1-3 = 0.0004 and p2-3 = 0.0001, respectively)
- IgA (p1-3 = 0.003 and p2-3 = 0.009, respectively), and
- IgM (p1-3 = 0.0007 and p2-3 = 0.0000, respectively).

No significant difference was observed in other parameters between patients of both melanoma groups and controls.

In patients with T1 to T3 CM (group 2), the following peripheral blood parameters were found to be statistically significantly increased compared with group 1 and/or healthy controls:

- CD3+ T cells (p2-3 = 0.009)

Table 2. Comparing groups of patients with pre-treatment stage 1 choroidal melanoma (group 1) and stage 1 to 3 choroidal melanoma (group 2) versus controls (group 3) for immunity parameters

Immunity parameters	Group 1, n = 35	Group 2, n = 31	Group 3, n = 44	p
1	2	3	4	5
White blood cell count (thousands of cells/ µl)	6.5 (1.8) ↑	6.6 (1.3) ↑	5.5 (1.2)	p_{1-2}=0.79 p_{1-3}=0.005 p_{2-3}=0.0003
Lymphocyte count (thousands of cells/ µl)	1.9 (0.6)↑	2.2 (0.7)↑	1.6 (0.6)	p_{1-2}=0.07 p_{1-3}=0.04 p_{2-3}=0.0002
Lymphocyte percentage	30.1 (7.3)↑	33.4 (9.0)↑	27.9 (6.9)	p_{1-2}=0.11 p_{1-3}=0.18 p_{2-3}=0.004
CD3+ T-cell count (thousands of cells/ µl)	1248.7 (467.6)↑	1492.3 (653.4)↑	1116.1 (558.3)	p_{1-2}=0.08 p_{1-3}=0.27 p_{2-3}=0.009
Percentage of CD3+ T-cells	64.6 (10.7)↓	66.2 (11.9)↓	69.7 (10.5)	p_{1-2}=0.57 p_{1-3}=0.04 p_{2-3}=0.18
CD4+ T-helper count (thousands of cells/ µl)	887.6 (405.4)↑	1157.3 (513.2)↑	806.1 (454.6)	p_{1-2}=0.02 p_{1-3}=0.41 p_{2-3}=0.003
Percentage of CD4+ T-helper cells	46.0 (12.3)↓	50.1 (12.6)↑	49.0 (12.8)	p_{1-2}=0.19 p_{1-3}=0.31 p_{2-3}=0.71

Note: m, number of patients; p, level of significance by the Newman-Keuls test; ↑, the mean parameter value is higher than in controls; ↓, the mean parameter value is lower than in controls

Table 2 (continued) Comparing groups of patients with pre-treatment stage 1 choroidal melanoma (group 1) and stage 1 to 3 choroidal melanoma (group 2) versus controls (group 3) for immunity parameters

Показники імунітету	**Основна група I (1), n=35**	**Основна група II (2), n=31**	**Контрольна група (3), n=44**	**p**
1	2	3	4	5
Cytotoxic $CD8^+$ T-cell count (thousands of cells/ μl)	306.6 (120.7)↑	320.0 (195.2)↑	194.2 (132.5)	p_{1-2}=0.73 p_{1-3}=0.0002 p_{2-3}=0.001
Percentage of cytotoxic CD8+ T-cells	16.4 (5.3)	15.4 (8.5)↓	16.5 (4.6)	p_{1-2}=0.56 p_{1-3}=0.95 p_{2-3}=0.47
CD4+/CD8+ ratio	3.1 (1.4)↑	3.9 (1.2)↑	1.7 (1.3)	p_{1-2}=0.02 p_{1-3}=0.00002 p_{2-3}=0.0000
CD19+ B cell count (thousands of cells/ μl)	283.1 (134.9)↑	277.1(123.6) ↑	233.9 (110.7)	p_{1-2}=0.85 p_{1-3}=0.08 p_{2-3}=0.12
Percentage of CD19+ B cells	15.2 (6.3)↑	12.9 (4.6)↓	14.5 (4.7)	p_{1-2}=0.09 p_{1-3}=0.57 p_{2-3}=0.57
Phagocytic neutrophil activity (thousands of cells/ μl)	3015.1 (1178.3)↑	3013.9 (913.0)↑	2060.9 (1028.0)	p_{1-2}=0.99 p_{1-3}=0.0004 p_{2-3}=0.0001
Percentage of phagocytic neutrophil activity	66.3 (14.6)↑	73.1 (14.5)↑	54.6 (21.3)	p_{1-2}=0.06 p_{1-3}=0.007 p_{2-3}=0.0001
CD16+ NK cell count (thousands of cells/ μl)	187.8(87.0)↑	260.0(162.4)↑	171.5 (87.2)	p_{1-2}=0.03 p_{1-3}=0.41 p_{2-3}=0.003
Percentage of CD16+ NK cells	10.0 (2.5)↓	11.5 (4.6)	11.4 (4.2)	p_{1-2}=0.09 p_{1-3}=0.08 p_{2-3}=0.92
IgA (0)	2.7 (1.1)↑	2.6 (1.0)↑	2.1 (0.6)	p_{1-2}=0.7 p_{1-3}=0.003 p_{2-3}=0.009
IgM (0)	1.0 (0.3)↑	1.2 (0.4)↑	0.8 (0.2)	p_{1-2}=0.02 p_{1-3}=0.0007 p_{2-3}=0.0000
IgG (0)	13.4 (2.9)	11.4 (2.8)↓	13.4 (2.9)	p_{1-2}=0.006 p_{1-3}=1.00 p_{2-3}=0.003

Note: m, number of patients; p, level of significance by the Newman-Keuls test; ↑, the mean parameter value is higher than in controls; ↓, the mean parameter value is lower than in controls

- CD4+ T helpers compared to group 1 (p1-2 = 0.02) and healthy controls (p2-3 = 0.0000)
- CD16+ NK cells (p1-2 = 0.03 and p2-3 = 0.003)
- IgM (p1-2 = 0.02 and p2-3 = 0.0000).

In patients of group 2, peripheral blood IgG level was significantly decreased compared to group 1 and controls (p1-2 = 0.006 and p2-3 = 0.003).

Therefore, imbalance in immunity, with a significant increase in the activity of T cells (CD3+, CD4+, CD8+), NK cells (CD16+) and IgM and IgA, was found in patients with small (T1) CM and medium to large (T1-T3) CM.

Discussion

Current immunotherapy for CM is of low efficacy due to general immunosuppression, low immunogenicity

of immunotherapy, and immune privilege of tumor cells. Therefore, integrating immunotherapy into an eye-preserving treatment for CM requires understanding the mechanisms of interactions between immunocompetent and tumor cells. This will contribute to more adequate and effective immunocorrection, which in turn will allow a more accurate determination of the role of immunocorrection in an eye-preserving treatment for CM.

Immune response to tumor cells may contribute not only to tumor rejection, but also to tumor growth and progression [21]. In addition, some molecular mechanisms provide immunosuppression as well as immune tolerance of tumor cells [17].

Histomorphological studies revealed signs of cell-mediated immune response to CM, ranging from mild diffuse infiltration to massive focal agglomerates of lymphoplasma cellular infiltrations and macrophage infiltrations in the tumor parenchyma and adjacent choroidal and scleral compartments, with high functional activities of these infiltrates indicated by the presence of lymphoblasts with a DNA content as high as 30.0%. The changes found in lymphocytes and plasma cells are accompanied by a well-developed endoplasmic reticulum whose cisternae are dilated and filled with a homogenous material of moderate electron density [22].

Leukocyte infiltration that develops in the tumor should theoretically result in elimination of tumor cells before the clinical diagnosis. This, however, does not occur, which is likely associated with local suppression of immunity in the tumor microenvironment [23].

Our findings confirm those of others that tumor process results in a statistically significant increase in the activity ($p < 0.05$) of T cells (CD3+, CD4+, CD8+), NK cells (CD16+), IgM and IgA and imbalance in other characteristics [1-5, 7-10, 12, 14].

Conclusion

In pre-treatment patients differing in the stage of CM, the tumor process responds to immune changes that are expressed by activation of humoral and cell-mediated immunity (an increase in the activity of CD3+ T cells, CD4+ T-helpers, CD8+ T-suppressors, CD16+ NK cells, and IgM and IgA levels), which may have a substantial impact on treatment effect.

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Author Contribution: *The author developed a database of clinical and immune data of the examined patients which were processed and reported in this paper. The author is responsible for the content of the entire manuscript.*

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Ethical statement: *The study involved human subjects and followed ethical standards as outlined in the European Convention on Human Rights and Biomedicine and the 1964 Declaration of Helsinki of the World Medical Association (and its later amendments including the amendments of 2000), complied with existing regulations of Ukraine, and was approved by the Bioethics committee of SI "The Filatov Institute of Eye Diseases and Tissue Therapy of the National Academy of Medical Sciences of Ukraine" (committee meeting minutes of April 12, 2021). Informed consent was obtained from all study subjects.*

Data Availability Declaration: *All the data obtained or examined during this study has been incorporated into this published article.*

Abbreviations: *BT, brachytherapy; CM, choroidal melanoma; N, nodulus; M, metastasis; PNA, phagocytic neutrophil activity; T, tumor; TTT, transpupillary thermotherapy*