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Research Article

New Pathogenetic Approaches to Inhibit the Growth of Glioblastoma Relapses

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Abstract

Currently, many researchers support the view, that if tumor microenvironment is normalized by suppressing tumor-associated inflammation (TAI), tumor stem cells, which only 3 - 5% contains in the tissues of primary glioblastomas, can lose their malignant properties. In the work we showed the possibility of TAI II stage normalizing by blocking the activity of NMDA - dependent calcium channels with low concentrations of verapamil - hydrochloride, that, as a result, led to a significant increase in the life expectancy of patients with glioblastomas. The patients were investigated in the distant postoperative period and treated with such concentrations of verapamil - hydrochloride, that minimize the level of blood cells aggregation in II stage of the inflammatory process. 12 patients received treatment (I group), of these 9 patients died, and 3 patients continued treatment with verapamil. 37 patients did not received treatment (II group) with calcium blocker using verapamil - hydrochloride examples in pills. For the objectification of TAI, that present's in the patient's body, the definition of blood cells aggregation was examined on the "Plasmon" sensor. Peripheral blood cells were collected from patients to determine the indicators of blood cells aggregation with the addition of various aqueous dilutions of verapamil - hydrochloride (from 1:10 to 1:100,000 times). Patients took drugs at the lowest level of blood cells aggregation doses. Drug dose was performed by decreasing its concentration by a factor of 10, 000. From the 49 patients, admitted to the clinic, only 3 patients did not undergo chemotherapy at will. After the surgical removal of glioblastoma and the postoperative irradiation course, they only took verapamil - hydrochloride at low concentration daily without interruption for life). All 3 patients continue to live for 20, 26 and 29 months, respectively. In the I group of 9 patients, undergoing combined treatment courses and taking verapamil - hydrochloride, the average life expectancy was 20.4 ± 6.8 months and 9.11 ± 1.5 months in the group of 37 patients (Cox's F - test p = 0,00063). The paper presents for the first time the results of highly malignant glioblastoma treatment with low concentrations of the NMDA-dependent calcium blocker verapamil - hydrochloride. The results indicate a high antitumor activity of the drug, both when used together with traditional methods (9 patients) of treatment or separately (3 patients).

Keywords: *Glioblastoma Relapses; Tumor-Associated Inflammation; Blood Cells Aggregation, Surface Plasmon Resonance, Low Concentrations of Verapamil*

Introduction

Brain gliomas are one of the most malignant human tumors with an unfavorable prognosis and a short period of the life in the postoperative period, averaging about 9 months [1-3]. Breach of relationship between the mechanisms of reparation (the predominance of the inflammatory process) and regeneration (replacement of the defect with stem cells) can lead to the development of a tumor. Tumor growth begins, when the transition of repair to regeneration is incomplete and the process of stem cell development is adversely affected by cells and factors of inflammatory genesis over a fairly long time. In the body, there are mechanisms of protection against such effects on stem cells as apoptosis and the epithelial-mesenchymal transition (EMT) [4,5].

The study of the inflammatory process, that accompanies the growth of malignant gliomas, called tumor-associated inflammation (TAI), becomes relevant [6-9]. In our previous studies, a relationship was found between the increased inflammatory component and the growth of glioma malignancy degree [10].

However, the mechanism of the influence of TAI on the growth and progression of glioblastoma remains unclear, which causes interest in this problem. Some authors argue that the magnitude of the transmembrane potential (TMP) is a decisive factor determining the possibility of cells proliferation [11]. Since the occurrence of TAI is associated with a decrease in the TMP of blood cells, one can associate the level of TMP with the proliferation of peripheral blood lymphocytes and their transformation into lymphoblasts *in vitro* in patients with glioblastomas. It seems possible to solve this problem by studying the relationship between changes in the level of aggregation of blood cells and lymphocytes blast transformation. Therefore, the use of methods for inhibiting TAI in the postoperative period acquires considerable interest in order to prevent possible continued gliomas growth. Most anti-inflammatory drugs have a number of side effects and are not recommended for long-term use in patients with malignant tumors. Suppression of TAI can be accomplished by reducing the activity of ionotropic receptors, such as NMDA - receptors [12,13].

The structure of NMDA - receptors includes calcium channels. Calcium ions are essential in the development of the inflammatory process in many pathological processes, including tumors. The action of ketamine (selecting blocker of NMDA-receptors) in experiments was more effective than verapamil, but long-term use of ketamine in the clinic can be dangerous. This indicates a link between the mechanisms of blocking calcium channels by verapamil and a decrease in the activity of ionotropic NMDA - receptors, due to the structural feature of the NMDA- receptor, containing the built-in calcium channel. The TAI inhibition, using NMDA-dependent calcium blocker verapamil, can help to slow the growth of glioblastomas, without causing of toxic effects on the body with prolonged use.

Aim of the Study

The aim was to investigate the life expectancy of patients with glioblastomas in the distant postoperative period, who were treated with low concentrations of verapamil - hydrochloride, and the relationship between changes in the level of blood cells aggregation and lymphocytes blast transformation in the experiments *in vitro*.

Materials and Methods

49 patients were divided into 2 groups, that received (group I) or who did not receive treatment (group II) with a calcium blocker, using the example of verapamil - hydrochloride in pills ("Pharmak"). For the objectification of TAI presence in the patient's body, definition of blood cells aggregation was examined on the instrument of "Plasmon" sensor [14]. Under physiological conditions, NMDA-receptors, are known, to be activated by millimolar concentrations of glutamate, which is present in the synaptic cleft for several milliseconds. In pathological impulses, receptors are activated by micromolar concentrations, but for a significantly longer time [15]. We took the same pattern as a basis for the effects of millimolar and micromolar concentrations of verapamil - hydrochloride on the level of blood cells aggregation, given the location of the calcium channel in the structure of the NMDA - receptors of lymphocytes and which function is NMDA-dependent [15,16]. Selection of optimal concentrations of verapamil - hydrochloride was carried out with the aim of treating patients with glioblastomas in the postoperative period.

Peripheral blood cells were collected from patients to determine the data of blood cells aggregation with the addition of various aqueous dilutions of verapamil- hydrochloride (from 1:10 to 1: 100,000 times). Patients took drugs at a dosage at which the level of aggregation of blood cells was the lowest. Dosing of the drug was performed by reducing its concentration by a factor of 10,000.

From the 49 patients, admitted to the clinic, only 3 patients did not undergo chemotherapy. After the surgical removal of glioblastoma and the postoperative irradiation course, they only took verapamil - hydrochloride at low concentrations daily. Histological studies of the bioptic material were carried out according to standard methods, the sections were stained with hematoxylin-eosin and pikrofuksin.

Lymphocyte proliferation activity determination

The primary peripheral blood cell cultures from 28 patients with glioblastomas were investigated. The degree of dilution of phytohemagglutinin (PHA) affects the level of aggregation of blood cells [10]. Modification of lymphocytes blast transformation reaction (LBTR) was realized *in vitro* by application of different concentrations of PHA (Sigma, 1 mg/ml H_20). Solutions make ready in subsidiary dilutions from 10^{-1} to 10^{-5} times immediately before 72 hours blood cells cultivation in RPMI medium. 2 ml of RPMI medium, 600 mcl of blood cells without plasma, 20 mcl of antibiotic and 60 mcl of PHA solution alone was put into each 2-cm Petri dishes.

Trans membrane potential data by the blood cells aggregation determination

TMP level model on blood cells membrane mediates by blood cells aggregation level indices. New method for blood cells aggregation level was determined at malignant gliomas by use of ultrasensitive instruments based on surface plasmon resonance phenomenon (SPR) [14]. Application of the new method it become possibility to determine objective data without use of buffer systems or salt solutions, that can influence on blood cells aggregation levels. Highest possible SPR signal was taken on blood cells without plasma. SPR unit is laser angle of deviation, that measured in relative numbers and converting in percents.

Statistical treatment of findings was realized by "Statistica - 10v" package. Standardize of different indexes was realized by using of: Xn - X∑/σ, where Xn- individual meaning; X∑ - average value; σ - standard deviation.

Results and Discussion

Clinical results

In I group of patients, undergoing combined treatment courses and taking verapamil, the average life expectancy was 18.6 ± 1.82 months. In II group of patients, undergoing combined treatment courses but without taking verapamil, the average life expectancy was 8.47 ± 1.02 months (Table 1 and Figure 1). Three patients without chemotherapy in the postoperative period received daily treatment with verapamil - hydrochloride without interruption. All 3 patients continue to live for 19, 25 and 29 months, respectively.

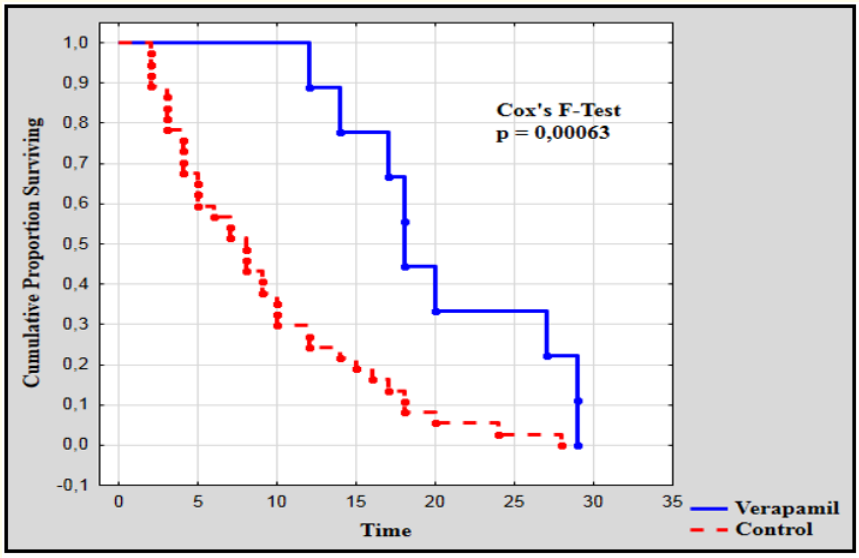

Figure 1: *I (9 patients) and II (37 patients) groups of patients, who received and did not receive verapamil treatment, are significant by the Cox's F- test. The significance level is 0.00063.*

Group	Number of patients	Mean (months)	Standard deviation (months)	Median life expected (months)
Patients, treated without verapamil - hydrochloride	37	9,11	1,5	7,5
Patients, treated with verapamil - hydrochloride	9	20,44	6,81	18

Table 1: *The life expectancy of patients, receiving a course of treatment with verapamil - hydrochloride with a combined treatment of glioblastomas in the late postoperative period.*

The effectiveness of such an approach to the treatment of glioblastoma can be compared with the data of other authors, in which with early diagnosis of primary glioblastoma and new medical tactics, the life expectancy of patients averaged only 15.3 months in postoperative period [17].

The number of patients, taking verapamil along with chemotherapy and radiation, who lived more than a year after surgery, was 100%.

Studies of the level of blood cells aggregation were expressed in SPR data, which quantitatively reflected the level of the blood cells aggregation of patients, since nanoscale intercellular distances (of the order of 200 - 300 nm) in the flow cell between blood cells located on a glass plate, covered with a thin layer were determined with high accuracy gold.

Baseline indicators of SPR before treatment with verapamil - hydrochloride in both groups of patients differ slightly, which indicates the adequacy of the selection of groups. In order to select the optimal concentration of verapamil - hydrochloride for treating patients, aqueous dilutions of verapamil - hydrochloride (from 1:10 to 1: 100.000 times) were added to the blood *in vitro* beforehand. Dilutions of verapamil tenfold led to an increase in the aggregation level, and large dilutions of verapamil (10,000 times), on the contrary, contributed to a decrease in the blood cells aggregation. The concentration of verapamil - hydrochloride, under the action of which noted the greatest decrease in the level of blood cells aggregation, was then used in the treatment of patients with glioblastoma in the postoperative period. Patients took a solution of the drug 3 times a day, constantly, without interruption, for life. Indicators of SPR in patients of groups I and II are presented in figure 2.

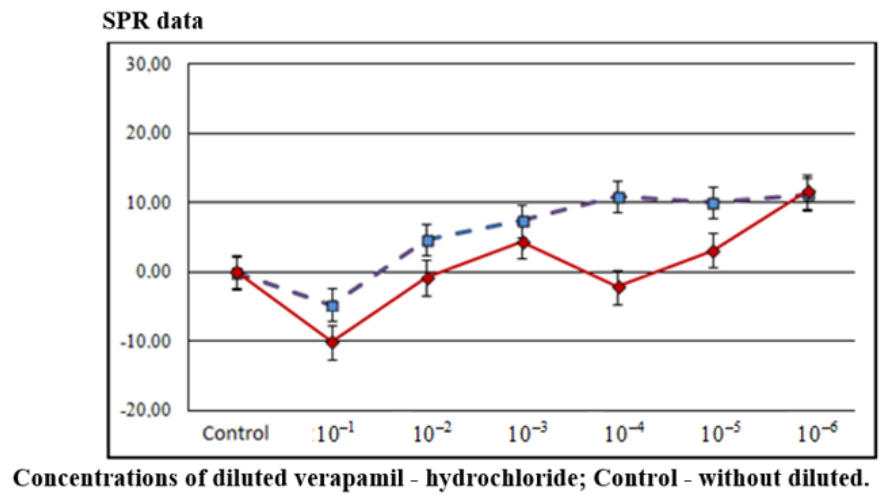

Figure 2: *The ratio of indicators of blood cells aggregation of under the action of verapamil dilutions in patients with glioblastomas, treated and not treated with verapamil (line with a stroke). A comparison was made of SPR indices in relation to the indices of blood dilution with water (20 µl of water and 200 µl of blood, with no hemolysis in any of these dilutions).*

As can be seen from figure 2, the decrease in the SPR curve under the action of verapamil-hydrochloride, when diluted with water is 10,000 times the same as postoperative remission in patients with glioblastoma, as evidenced by visualization methods performed. An increase in the SPR curve was observed in patients with recurrent glioblastoma.

The dependence of the level of blood cells aggregation of on the presence of a tumor in the brain is well expressed when verapamil is added to the blood at a dilution of 10,000 times (concentration 10-4), which leads to a decrease in SPR. This is characteristic of benign or conditionally benign tumors, such as meningiomas or grade II gliomas, which were shown in our previous studies [18].

Preliminary experiments to determine the level of blood cells aggregation in patients with glioblastomas under the action of verapamil and ketamine, which is a selective blocker of NMDA - receptors, as well as in the treatment of experimental rats with a transplantable glioma of 101.8 strain (human glioblastoma analog) showed coincidence of data on the effect of verapamil and ketamine both on the level of patient cell aggregation in glioblastoma and on the life expectancy of experimental animals [10].

The action of ketamine was more effective than verapamil, but long-term use of ketamine in the clinic can be dangerous. This indicates a link between the mechanisms of blocking calcium channels by verapamil and a decrease in the activity of ionotropic NMDA - receptors, due to the structural feature of the NMDA- receptor containing the built-in calcium channel. Movalis, a nonsteroidal anti-inflammatory pharmacological drug, is a selective blocker of COX-2 (cyclooxygenase-2) and at a dilution of 10,000 times did not have an antitumor effect on rat glioma 101.8.

In the patient, who did not take chemotherapy courses, upon re-operation in the case of continued tumor growth (relapse at the 12th month) histopathological changes were observed while taking verapamil, which corresponded to the symptoms of medical pathomorphosis (Figure 3). Calcium deposits indicate that low concentrations of verapamil prevent calcium from entering the cells due to the blockage of calcium channels.

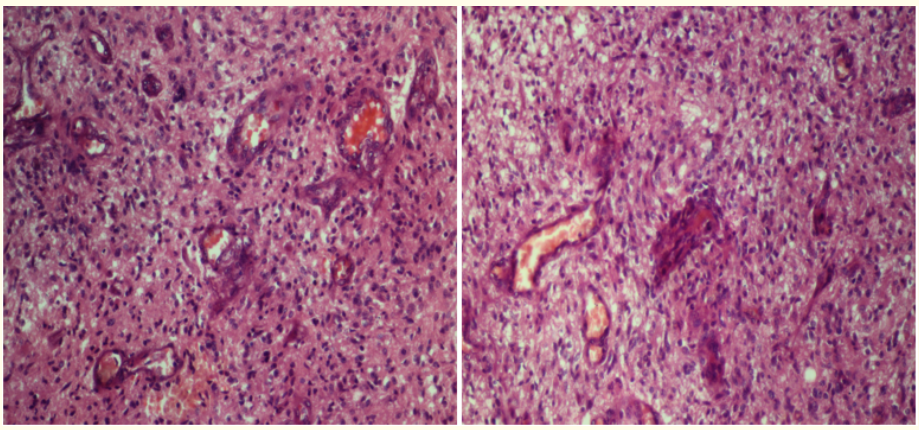

Figure 3: *Glioblastoma of patient M., who did not take a course of treatment with verapamil - hydrochloride. Tumor tissue is with moderate cell polymorphism, with severe endothelial hyperplasia and vascular proliferation in the zone of infiltrative tumor growth into the surrounding brain substance.*

Consequently, the effective inhibition of growth of glioblastoma obtained in the work was achieved using the mechanisms of blocking the NMDA- receptor and the calcium channel on the membranes of glioblastoma cells and peripheral blood cells of patients, which leads to a change in the microenvironment of malignant brain tumors and inhibition of their growth.

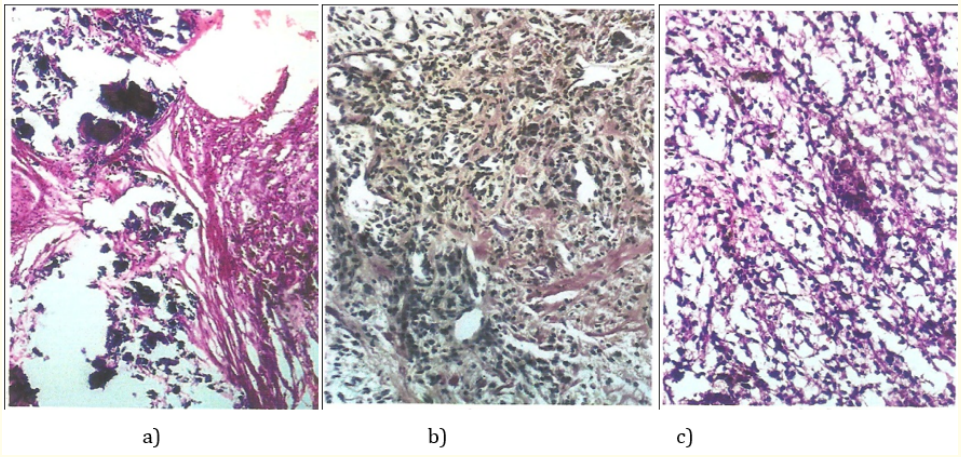

Figure 4: *Morphological studies of the glioblastoma tissue from patient B. a) areas of glioblastoma calcification and lysis. Staining by hematoxylin-eosin (x 200); b) areas of fibro-sclerotic transformation. Staining by pikrofuksin (x 200); c) areas of thin tissue of glioblastoma. Staining by hematoxylin-eosin (x 200). On histological specimens massive foci of calcium deposits with tissue lysis sites were found (a-b). In certain areas, glioblastoma tissue was subjected to fibro-sclerotic transformation along with areas of loosening of tumor tissue (c).*

Experimental results

However, the mechanisms, linking inflammation and stemness expression in cancer progression as well, as glioma malignancy, remain elusive [19,20]. The dependence of the lymphocytic blast transformation proliferation from TMP indices (the level of blood cells aggregation) is shown in figure 5. Dilutions of PHA from 1:10 to 1: 100 contribute to an increase in blood cell aggregation, and dilutions from a thousand to ten thousand lead to a decrease in blood cell aggregation. Accordingly, decreasing of blood cells transmembrane potential (SPR indices decrease) leads to increasing of lymphocytes blast transformation proliferation (LBTP) in the primary blood cell culture assay. With a decrease in the level of aggregation (SPR indices increase), the LBTP activity decrease and the number of the blasts cells also decrease.

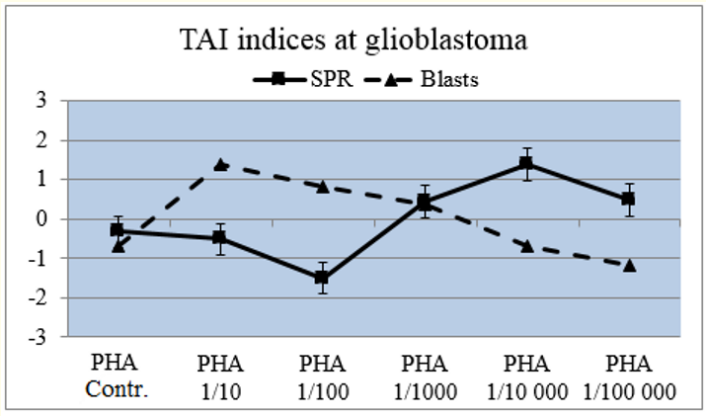

Figure 5: *Standardize SPR data on blood cells in comparison with number of blasts cells (in %) during lymphocyte cultivation by use of different PHA solutions at glioblastomas (in RBTL test).*

This pattern shows, that with the help of changes in TMP, it is possible to influence the proliferative activity and number of stem cells, including tumor stem cells. Thus, the effect of TAI on the malignant gliomas growth becomes understandable, since with a constantly low TMP, the number of stem cells accumulates, which leads to tumor progression and relapse in the postoperative period.

It should be noted, that with the addition of PHA in conventional dilution, the number of lymphoblasts is low, and with an additional dilution of 10 times the dilution, their number increases significantly. Perhaps, it is worth talking about relative immunosuppression in tumors?

All known methods of treatment of malignant tumors are aimed at cytoreduction and suppression of the tumor growth. Inflammation, contributing to an increase in cell mass with glioblastoma growth, is a protective-compensatory reaction, that cannot be completely suppressed. To TAI inhibition in patients it is necessary to act not on the third stage of inflammation with the mechanisms of proliferation, but on the first stage, on alteration, and to reduce the activity of calcium blockers and ionotropic NMDA - receptors. The latter activate inflammation and in the mechanisms of growth and destruction of brain cells during the tumor process play an important role, which can be indirectly determined by indicators of the level of blood cells aggregation [14,18]. This is achieved by reducing the appearance of necrotic tissues by suppressing the activity of NMDA-dependent calcium channels by verapamil. Thereby, conditions are created in the tumor microenvironment to hinder the reproduction of glioblastoma cells. The use of verapamil in traditional doses does not provide an antitumor effect, which was noted in animal experiments [10].

The effectiveness of such an approach to the treatment of glioblastomas can be compared with the data of other authors, in which, with early diagnosis of primary glioblastomas and new medical tactics, the life expectancy of patients averaged only 15.3 months [17].

The question of the adverse effect of calcium antagonists on the occurrence of cancer has been discussed. However, a critical analysis of data on the treatment of hypertension with these drugs allowed WHO experts to conclude that such information was unreasonable [22,23]. In addition, it was shown that with the introduction of certain carcinogens (7,12-dimethylbenzantracene) into animals, verapamil suppressed the development of tumors in rats [24]. Other researchers have shown the absence of tumor-stimulating activity of verapamil during prolonged exposure in rats [25]. As you know, the drug is used widely throughout the world for the treatment of cardiac arrhythmias, without showing pronounced adverse reactions. It was shown that verapamil did not increase the risk of complications in patients after myocardial infarction [22].

Verapamil improve the effectiveness of chemotherapy in patients [26]. The paper presents data on the treatment of 3 patients with glioblastomas only by verapamil, after irradiation courses, but without chemotherapy, which certainly indicates the presence of antitumor activity in this drug.

Traditional methods of glioblastoma treatment should be combined with techniques of low concentrations of verapamil - hydrochloride in the late postoperative period, since the activation of TAI after chemotherapy and radiotherapy contributes to the growth of tumor residues in the brain. The results obtained can be used to decide the question of the individual tactics of patient treating. The successful use of verapamil in the treatment of malignant tumors is determined by taking into account the mechanism of development of tumor-associated inflammation, which contributes to the occurrence of relapse of glioblast and its correction by pathogenetic methods.

Conclusion

It is very important to understand mechanisms of glioma progression, promoting patients death. Lowering of transmembrane potential data over a long period of time lead to blasts cells appears in abundance and it migrating to gliomas necrotic centre. But blast cells genome contains substantial number of chromosomal aberrations, that can prevent from normal regeneration processes [21]. Verapamil application promotes to TMP level increase (blood cells aggregation decrease), lymphocyte proliferative activity decrease. Such pathogenic approach in clinical conditions can lead to substantial suppression of further glioma progression in remote postoperative period.

The paper presents for the first time the results of treatment of highly malignant glioblastomas with the low concentrations of the dependent calcium blocker verapamil - hydrochloride. The results indicate a high antitumor activity of the drug, both when used together with traditional methods of treatment or separately. The treatment method is pathogenetic in contrast to the empirical one, which is mainly used by modern chemotherapy. With low concentrations of verapamil - hydrochloride, NMDA - receptor activity is reduced and calcium channels are blocked. This, in turn, leads to changes in the transmembrane potential on the blood cells membranes, which contribute to the suppression of the tumor-associated inflammation and decrease of stem cells number. TAI determines the tumor microenvironment, which promotes tumor growth and recurrence of glioblastoma in the postoperative period, in particular. Suppression of TAI helps prevent recurrence and, therefore, prolongs the life expectancy of patients with glioblastoma. The presented method can be used after courses of traditional postoperative chemotherapy throughout the patient's life, since this method, unlike other anti-inflammatory drugs, is not toxic.

Our results can be useful for treatment not only of patients with brain malignant tumors, but for another kind of malignant tumors with TAI.

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