

## Some Aspects of the Systemic Mechanism of Brain Malignant Gliomas Progression and Methodological Approaches to its Correction

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### Abstract

The growth of malignant gliomas was considered at a systemic level, which allows us to understand the basic shifts in the protective-compensatory reactions of the body, leading to the progression of brain tumors. It was shown that activation of NMDA-receptors affects the transmembrane potential, the indices of which are mediated through the level of aggregation of blood cells of patients with malignant gliomas. An increase in the level of aggregation of blood cells corresponds to stage II of the *in vivo* inflammatory process, which is a protective-compensatory reaction of the body. Verapamil is an NMDA- receptor dependent blocker of slow calcium L- channels, because they localized in the structure of NMDA- receptors. Using a highly sensitive Plasmon biosensor, the level of aggregation of blood cells was studied before surgery and on the 7<sup>th</sup> day after surgery. The effect of low concentrations of verapamil-hydrochloride on the indicators of blood cell aggregation in *in vitro* experiments made it possible to determine the differences between non-tumor inflammation and tumor-associated inflammation in glioblastomas. Tumor-associated inflammation is aseptic microinflammation, which is rarely detected by traditional laboratory tests and requires the use of biosensor equipment. Further, the use of verapamil-hydrochloride in dilutions with distillate water 10,000 times allowed to reveal the mechanism of “non-healing wounds” in malignant tumors. The oxidation enzymes of the polyamines DAO and PAO, which carry out repair processes in the III stage of inflammation, at low concentrations of verapamil- hydrochloride were characterized by a sharp decrease in activity in glioblastomas compared with identical indices in spinal hernias. These results are one of the experimental confirmations of the incompleteness of stage III of inflammation in malignant gliomas of the brain. This leads to the transition of reparative processes during inflammation to the processes of regeneration by mesenchymal stem cells through the epithelial - mesenchymal transition (EMT) and the subsequent progression of the tumor. It was shown that verapamil - hydrochloride in low concentrations reduces the expression of Snail, one of the main EMT genes, by 50%. The existing relationship between the development of glioma progression and the activity of ionotropic receptors that affect the transmembrane potential, defines a new methodology for therapeutic measures in the postoperative period. It consists in the suppression of tumor-associated inflammation by inhibitors of ionotropic receptors and ion channel blockers, stabilization of cell membranes, and inhibition of EMT. These therapeutic effects should be carried out after completing courses of any types of traditional therapy for malignant tumors.

**Keywords:** Glioblastoma; Systemic Mechanism of Progression; Tumor-Associated Inflammation; Blood Cells Aggregation; Surface Plasmon Resonance; Low Concentrations of Verapamil

## **Introduction**

The methodological approach to research in many areas of human cognition, called on behalf of the philosopher William Ockham (1285 - 1349 years) "Occam's razor", says: "It is not necessary to multiply things without necessity" or "Diversity should not be assumed without necessity".

The Occam principle should be applied to the analysis of the mechanisms of tumor progression in brain gliomas, which, as is commonly believed, consist in changes in the initially normal cells of the body into randomly proliferating malignant cell clones, that migrate from the main tumor focus to neighboring healthy tissues, that give relapse after removal of the primary tumor focus.

Applying the principle of "Occam's razor", it can be shown that most of the indicated characteristics of malignant tumors should be classified as pathological regenerative processes, that mimic tumor progression in the presence of chronic inflammation.

Glioblastoma is the most malignant of all gliomas, leading to the death of patients on average after 0.5 - 2 years from the time of diagnosis. Brain gliomas in the process of growth progress from benign to malignant forms. For effective treatment, it is necessary to clearly understand the mechanisms of glioma progression in order to develop pathogenetic methods for correcting this pathological process.

Currently, most treatment methods in oncology are cytoreduction of malignant cells. The methodology for killing cells was developed at the time of the rapid development of microbiological studies, in which the goal was to destroy bacteria with antibiotics and other aseptic reagents, and the results were achieved by treatment with maximum doses. This methodology has been used in oncology practice. However, in contrast to the positive results for septic diseases, treatment with toxic drugs of cancer was not so successful. What is the reason? The fact is that in the first case, the goal is the destruction of microorganism's alien to the body, and in the second case, cells of the tissues of the human body. The destruction of body tissues is always accompanied by the activation of the inflammatory process, which seeks to make up for the removed cell mass, since inflammation is a protective-compensatory reaction. Tumor growth is characterized not only by cell proliferation, but also by necrosis of the tumor tissue, which also contributes to the onset of the inflammatory process [1-6]. The area of necrosis increases with the transition from the II degree of glioma malignancy to the IV degree of glioma malignancy (or glioblastoma).

Inflammation affects the appearance of tumors and accompanies the growth of malignant tumors [7-10]. It is believed, that tumor progression depends only on changes in the tumor cells themselves associated with genetic and protein transformations. However, there is an assumption that tumor progression also largely depends on the presence of tumor-associated inflammation (TAI), which occurs in response to the appearance of necrosis in the tumor tissue, and constantly stimulates the growth of this tissue. Inflammation can be taken as a malignant component in the tumor process, which must be suppressed in order to inhibit tumor growth [11].

As it known, the inflammatory process is involved in the processes of cellular and tissue repair. This is its restoration function, as a result of which damaged cells and body tissues continue to live and function. However, parenchymal organs restore their damaged structure due to protective influences from the stem cells of the body, which carry out the function of regenerating organ tissues considering the specifics of their structure. Between the mechanisms of repair due to inflammation and regeneration by stem cells, there are relationships that are essential in the development of malignant tumors, if these mechanisms are not separated in time among themselves. If they function simultaneously for a long time, while stem cells are exposed to alteration factors during inflammation, they become a source of malignant growth in the body. Investigations of the mechanisms of malignant transformation of stem cells into tumor stem cells are of great interest to researchers [12-15].

However, many researchers do not consider the influence of the regenerative process on the appearance of relapses and metastases of tumors, when stem cells migrate from the primary tumor focus to other organs, which may also contain damaged tissues. In the body,

there are mechanisms that impede the development of the inflammatory process. This is cell death by apoptosis, while the inflammatory process does not occur, and EMT [16-18], which also inhibits inflammation and activates the regenerative functions of stem cells. It was shown that a prolonged decrease in the transmembrane potential on blood cell membranes during inflammation changes the function of repair enzymes using polyamine oxidation enzymes as an example, contributing to their inactivation, resulting in an increase in the number of mesenchymal stem cells in the blood [19]. In this case, stem cells perform the function of regeneration in the presence of an inflammatory process that does not stop [7].

This conclusion leads to the development of a methodological approach at the system level, which consists in inhibiting the manifestations of tumor-associated inflammation with the growth of malignant tumors. As you know, there are three stages of inflammation. At the same time, at the last stage, a huge number of inflammatory factors are synthesized, the expression of a huge number of genes changes, among which it is difficult to single out a key factor in order to effectively use it to suppress inflammation. The molecular target should be sought in the development of the first stage of inflammation. Such a target can be ionotropic receptors and ion channels in the composition of these receptors, which affect the transmembrane potential, a decrease in which leads to the development of inflammation. One of the methodological approaches may be the blocking of calcium channels in the structure of NMDA- receptors (Figure 1) [20] and the result of blocking can be determined by the change in cell aggregation, which indirectly reflects the level of transmembrane potential.

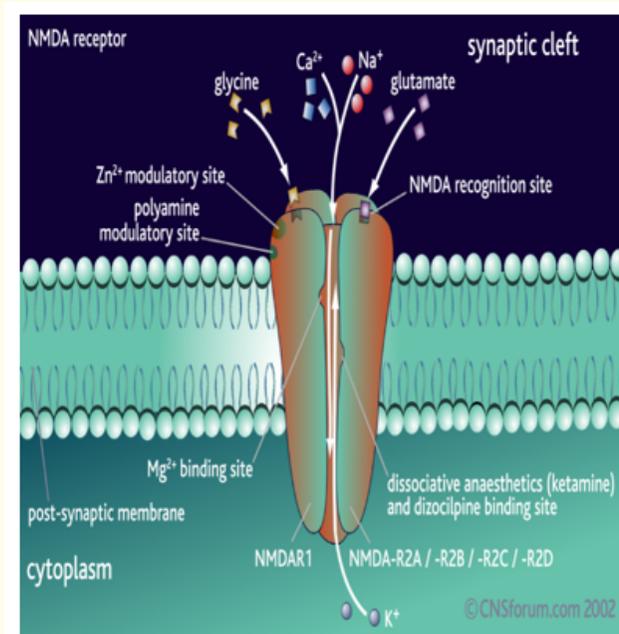


Figure 1: Structure of NMDA-receptor [20].

### Aim of the Study

Investigate the effect of low concentrations of verapamil-hydrochloride on peripheral blood cell aggregation to determine tumor - associated inflammation, reduce the activity of polyamine oxidation enzymes, and suppress Snail gene expression to reveal the systemic mechanism of progression of brain gliomas.

## Materials and Methods

**Method of blood cells aggregation determination:** The Plasmon biosensor was used to design based on the physical phenomenon of surface plasmon resonance [21, 22]. This device allows you to determine the nano-distance between blood cells placed on a glass plate coated with a layer of gold. A laser beam incident on the plate interacts with gold nano- particles, resulting in plasmon resonance, which is used to quantify the level of aggregation of blood cells [23]. In healthy individuals, blood cells are densely located on a gold coated plate, deflecting the laser beam more than by a standard unit of reference. In patients with malignant gliomas, blood cells on the plate are arranged in the form of “coin columns”, as a result of which empty spaces without blood cells remain on the plate. This arrangement of blood cells deflects the laser beam less than a standard unit.

Calcium channels were blocked using a selective verapamil blocker, which in different concentrations had different effects on the level of aggregation of blood cells *in vitro* [24].

To obtain the maximum signal, the cell mass of the peripheral blood of patients with glioblastomas was used [25]. Blood taken from peripheral vein of patients supplemented with heparin was centrifuged for 10 minutes at 1500 rpm. Verapamil- hydrochloride was added to the blood *in vitro* in a volume ratio of 1:10 (20 µl of verapamil - 200 µl of blood cells). Verapamil-hydrochloride (0.25% solution, “Farmak”) was dissolved in distillate, which was also added to the control blood sample in the same volumes.

**Method for determination of DAO and PAO enzymes activity:** The method for determining of the oxidation enzymes of the polyamines DAO and PAO was studied by the Gordon and Peter (1967) in the modification of S. P. Syatkin [26]. The Lowry method was investigated to determine the amount of protein required to calculate the activity of enzymes in nanokatal per mg of protein [27].

**Method for determination of gene Snail expression:** To determine the expression of the Snail gene, blood samples similar to those prepared to determine the level of blood cell aggregation obtained in 10 patients, after dilutions a thousand and ten thousand times of verapamil-hydrochloride were added a thousand and ten thousand times, were cultivated at + 37°C for 72 hours in DMEM nutrient medium with the addition of an antibiotic. Then the samples were centrifuged and the top layer containing leukocytes and lymphocytes was carefully collected. Total RNA was determined by the method of [28]. Then, the RNA was transformed into complementary DNA using the reverse transcriptase enzyme. After the PCR reaction, the synthesized amplicons were examined by 2% gel electrophoresis in TAE buffer with pH = 7.6.

Gene	Nucleotide consecution (5'-3')	Amplicon (nucleotide pairs)
<i>SNAIL</i>	F-CAGACCCACTCAGATGTCAA R-CATAGTTAGTCACACCTCGT	558
<i>GAPDH</i>	F-TGAAGGTCCGGAGTCAACGGATTTGGT R-CATGTGGGCCATGAGGTCCACCAC	260

**Table 1:** Primers composition for human gene expression investigation by RT-PCR method.

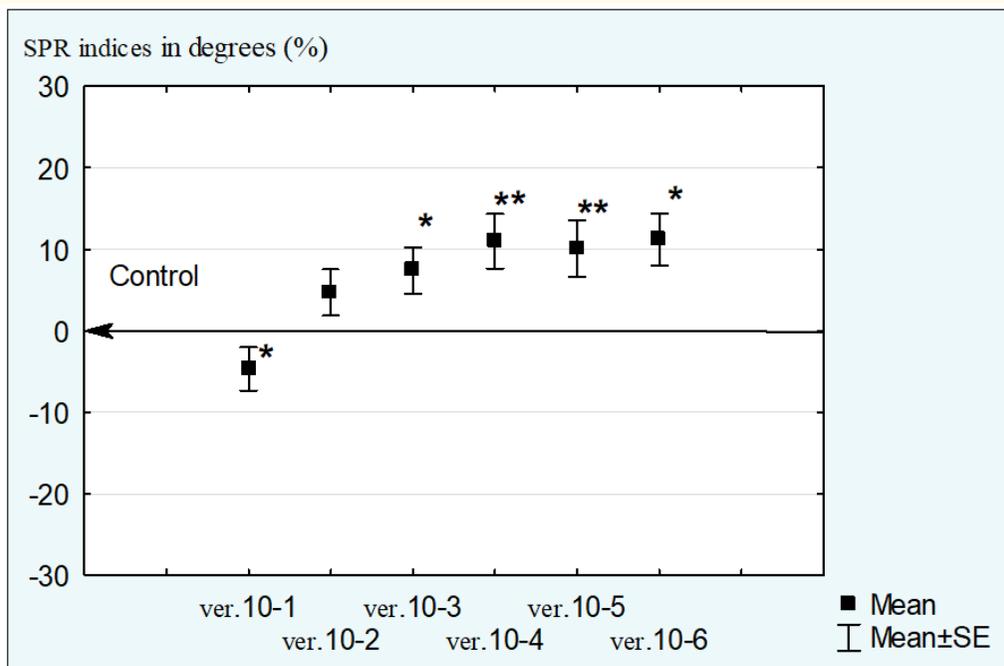
Statistical treatment of findings was realized by “Statistics-10v” package. Standardize of different indexes was realized by using of:  $X_n - X\bar{X}/\sigma$ , where  $X_n$  - individual meaning;  $X\bar{X}$  - average value;  $\sigma$  - standard deviation.

## Results and Discussion

### Determination of blood cells aggregation indices associated with changes in tumor-associated inflammation

Dilutions of verapamil - chloride increased the level of aggregation of blood cells by a factor of 10 and large dilutions (10,000 times or more) reduced this level in glioblastomas and other types of malignant tumors of the central nervous system.

Comparison of changes in blood cell aggregation with the addition of a pharmaceutical preparation of verapamil - hydrochloride in various concentrations at a dilution of 1:10 to 1: 100,000 for traumatic brain injury and malignant gliomas in patients allowed us to draw a significant conclusion that the presence of various dilutions of verapamil-hydrochloride can determine the presence of inflammation associated with and not associated with the tumor (Figure 2).

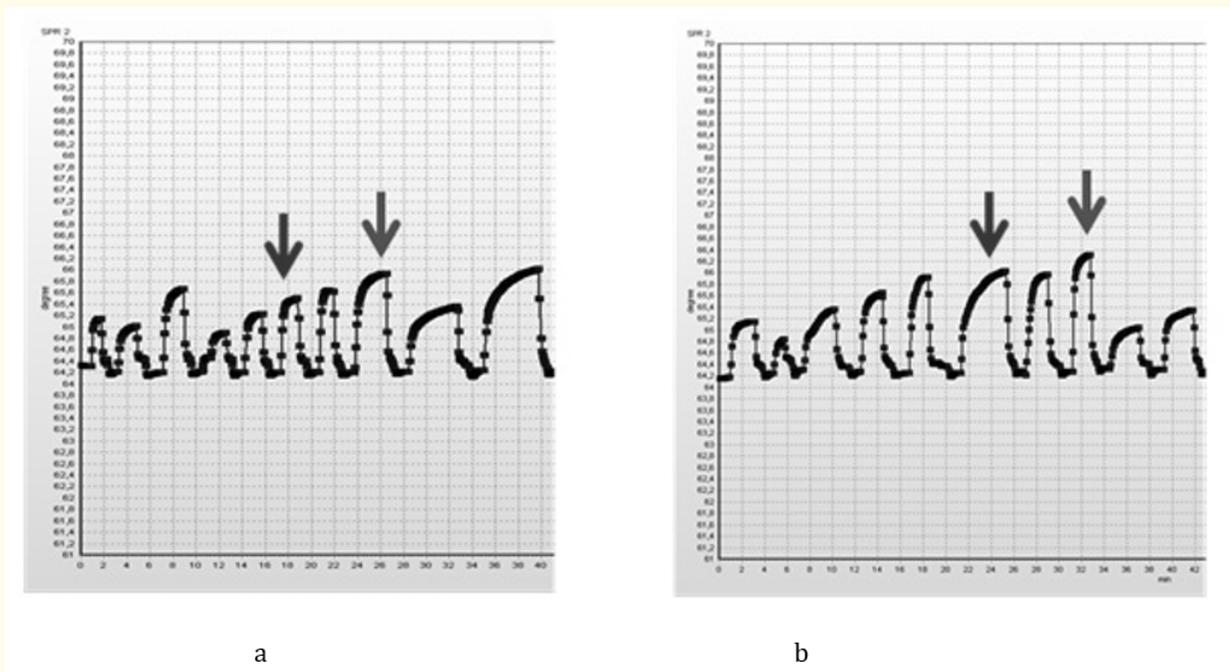


**Figure 2:** Effect of the different dilutions of verapamil-hydrochloride (from 10<sup>-1</sup> to 10<sup>-6</sup>) on the blood cells aggregation SPR indices of glioblastoma patients.

The two diagrams below (Figure 3a and 3b) show the data on the SPR indicators before surgery and on the 7<sup>th</sup> day after the operation to remove glioblastoma. Column 2 is the SPR of a blood sample without the addition of verapamil. After surgery, this indicator is significantly reduced, which indicates the development of the inflammatory process. It is important to note that without the addition of verapamil to the blood, the SPR values decrease if the level of aggregation of blood cells increases. With the addition of verapamil, PPR indicators, on the contrary, increase along with a decrease in the level of aggregation of blood cells.

To determine the type of inflammation that prevails by the SPR indices, it was determined that dilution of verapamil - hydrochloride 100 times on the 7<sup>th</sup> day after removal of the brain tumor helps to reduce the level of aggregation of blood cells (increase the SPR indicators - left arrow, 6th column), which indicates the presence in the patient's body of inflammation that is not associated with tumor growth. At the same time, a 10,000 dilution of verapamil - hydrochloride, which after the operation leads to a decrease in aggregation, may indicate the presence of tumor-associated inflammation in the body (arrow on the right). Such results can be explained by the time difference between the appearance of NMDA- receptor activator glutamate in synaptic clefts [30].

The dilution of verapamil- hydrochloride is 100 and 10,000 times comparable with the peculiarities of the release of glutamate in non-tumor and tumor diseases. Due to these features of activation of NMDA- receptors, it became possible to determine the aggregation of blood cells associated with tumor-associated inflammation.



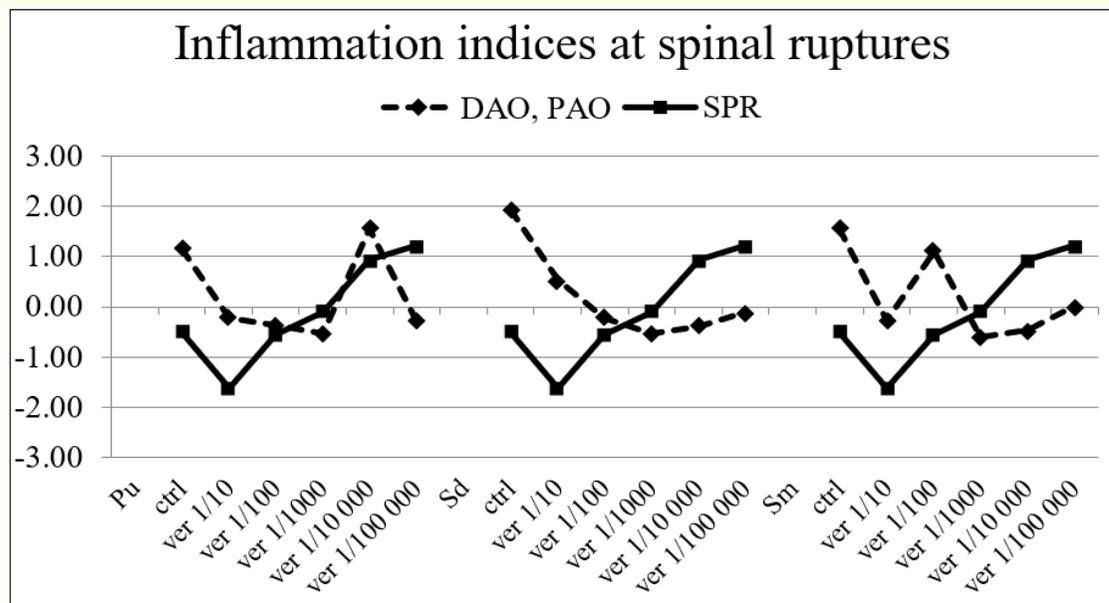
**Figure 3a and 3b:** Indices of SPR on blood cells in patient C. before (A) and on the 7<sup>th</sup> day. (B) after removal of glioblastoma. 1: column - SPR of blood plasma; 2<sup>nd</sup>: Blood; 3<sup>rd</sup>: Blood with double distillate; 4<sup>th</sup>: Blood with verapamil 1: 1; 5<sup>th</sup>: Blood with verapamil at a dilution of 1:10; 6<sup>th</sup>: Blood with verapamil 1: 100; 7<sup>th</sup>: Blood with verapamil 1: 1000; 8<sup>th</sup>: Blood with verapamil 1: 10.000; 9<sup>th</sup>: Blood with verapamil 1: 100.000; 10<sup>th</sup>: Blood with verapamil 1: 1 million.

These results lead to an understanding of the fact that when using any drugs to study the mechanisms of tumor growth, it is necessary to take into account indices of transmembrane potential. Tumor-associated inflammation develops against the background of a reduced transmembrane potential, while many enzymes and other potential dependent biologically active compounds can change their properties.

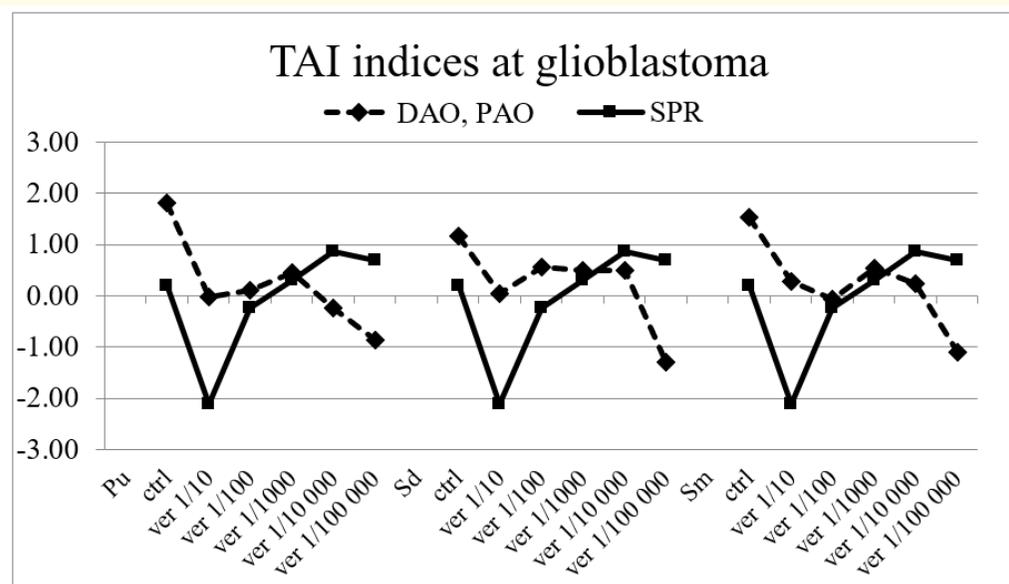
#### Determination of the activity of polyamine oxidation enzymes in connection with the study of the “non-healing wound” phenomenon

Determination of the activity of the enzymes diamino - oxidase (DAO) and polyamino - oxidase (PAO) involved in stage III of the inflammatory process may bring us closer to understanding the incompleteness of this stage of repair in malignant tumors. We carried out standardization of SPR indices with indices of DAO and PAO activity to study the laws of their dependence on the level of transmembrane potential on blood cells. Considering the importance of large dilutions of verapamil for detecting blood cell aggregation characterizing tumor-associated inflammation, it was possible to detect the characteristics of the activity of polyamine metabolism enzymes precisely by analyzing their changes in the framework of these dilutions (Figure 4 and 5).

A control group of patients with spinal hernias was selected as an example of non-tumor inflammation. SPR values for large dilutions of verapamil- hydrochloride (1: 10,000 and 1: 100,000) were more informative than 10 times dilution of verapamil. The activity of the enzymes DAO and PAO with these dilutions sharply decreases with glioblastomas compared with the identical indices with spinal



**Figure 4:** Standardize SPR indices on blood cells in comparison with DAO and PAO activity indices in supernatant during lymphocyte cultivation by use of different verapamil solutions at spinal ruptures (in RBTL test).



**Figure 5:** Standardize SPR indices on blood cells in comparison with DAO and PAO activity indices in supernatant during lymphocyte cultivation by use of different verapamil solutions in malignant gliomas (in RBTL test).

hernias. These results are one of the experimental confirmations of the incompleteness of stage III inflammation in malignant gliomas of the brain.

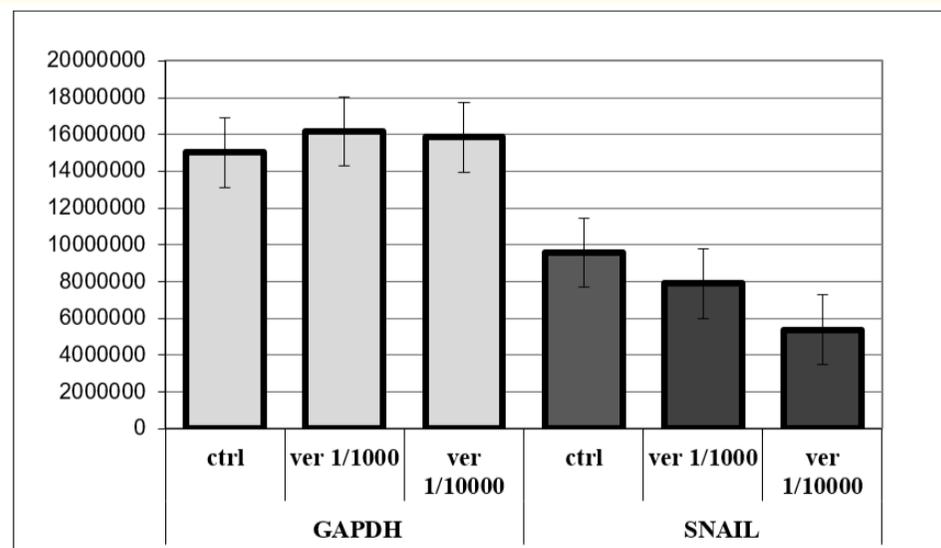
The differences in the activity of DAO and PAO enzymes between groups of patients with glioblastomas and spinal hernias are that in spinal hernias they acquire control values when exposed to low concentrations of verapamil- hydrochloride. In glioblastomas, DAO and PAO activity indicators remain low under the same conditions.

If the activity of enzymes is reduced for a long time, there is a change of repair processes to regeneration processes due to stem mesenchymal cells. Under normal conditions, these cells can differentiate into cells of damaged parenchymal organs, preserving their morphogenesis [11,30-33]. The change of repair processes to regeneration processes occurs under the strict control of the epithelial-mesenchymal transition (EMT) [16,34].

### The effect of low concentrations of verapamil -hydrochloride on Snail gene expression

It is known that mesenchymal cells migrating to the tumor site also have many chromosomal aberrations and genetic abnormalities [36,37], which will interfere with normal regeneration and promote the growth of the tumor node. Therefore, the search for drugs that reduce the activity of major genes in the implementation of EMT is relevant.

The effect of verapamil-hydrochloride in various dilutions was studied to suppress the expression of the Snail gene in lymphoblasts (Figure 6). Verapamil-hydrochloride at a dilution of 10,000 times has been shown to suppress expression of the Snail gene by almost 2 times compared with a dilution of 1000 times.



**Figure 6:** Densitogram of gene Snail expression indices under verapamil action in dilutions from 1000 to 10.000 times. Designation: 1, 2, 3: Gene GAPDH expression; ctrl: Gene Snail expression without verapamil; ver. 1/1000: Added of verapamil in 1000 times dilution; ver 1/10.000: Added of verapamil in 10.000 times dilution.

The research results show the dependence of the expression of the Snail EMT gene on the level of aggregation of blood cells, i.e. gene expression is voltage dependent. This opens new prospects for influencing the mechanism of EMT in order to reduce the accumulation of tumor stem cells and helps to normalize the tumor microenvironment.

### Conclusion

The result of the studies was a very important conclusion that the growth and progression of malignant brain tumors is influenced by tumor-associated inflammation, which contributes to the further growth of the tumor after surgery. A decrease in the level of transmembrane potential (TMP) is a sign of stage II of the inflammatory process, an indirect indicator of which is an increase in the level of aggregation of blood cells. Depending on the degree of reduction of the transmembrane potential, which is known to be mediated by the level of aggregation of blood cells, the inflammation will be of a non-tumor origin or tumor-associated inflammation.

The latter, due to a decrease in the activity of the oxidation enzymes of polyamines, diamino oxidase and polyamino oxidase, has an incomplete stage III of regeneration. This leads to an increase in the regenerative function of mesenchymal stem cells, which, due to the large number of genetic abnormalities, cannot provide normal regenerative function.

Currently, it is believed that one of the most characteristic properties of a tumor is genomic instability. There is evidence showing that with an increase in the degree of glioma malignancy, the number of chromosomal aberrations and genetic anomalies in the glioma nuclei increases [36]. However, some of these anomalies are used as targets for malignant tumors, and, as expected, without much success. When determining the number of chromosomal aberrations in peripheral blood lymphocytes with gliomas, it was concluded that an increase in the percentage of chromosomal aberrations in glioma progressions is associated with a decrease in the transmembrane potential, i.e. membrane integrity is a guarantee of the normal functioning of the cellular genome in blood cells and in glioma cells [37]. With the help of verapamil - hydrochloride, it is possible to reduce the level of aggregation of blood cells, which will lead to normalization of the activity of the polyamine synthesis enzymes DAO and PAO, a decrease in the activity of NMDA- receptors by blocking calcium channels, and a decrease in the expression of the Snail gene.

Thus, using verapamil - hydrochloride in large concentration dilutions, a new therapeutic target was determined. The treatment of patients with malignant tumors was used for glioblastomas, lung cancer, ovaries, and large intestines using verapamil-hydrochloride diluted 10,000 times. The results of treatment of patients with glioblastomas, who after surgery, radiation courses and chemotherapy were prescribed 10,000-fold dilution of verapamil, was determined by the patients' life expectancy. Compared with the average life expectancy of such patients in the postoperative period, which averages 9 months, it reached an average of 18-20 months, i.e. was 2 times higher. Some patients continue to live over 2.5 years. Patients with cancer of the lungs, ovaries and large intestine continue to live after surgery for 3 years without relapses and metastases.

If the main target in the pathogenesis of the disease becomes a therapeutic target, then its correction leads to effective treatment of patients. In this case, there is no need to conduct multi-target multistep cancer therapy.

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