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TUMOR-ASSOCIATED INFLAMMATION MECHANISMS CORRECTION BY VERAPAMIL AT BRAIN GLIOMAS PROGRESSION

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ABSTRACT

Brain gliomas progression was investigated indirectly through tumor-associated inflammation (TAI) mechanisms.[1-5] Diamine oxidase and polyamine oxidase enzymes activity decrease is result in healing incomplete as "phenomenon persistent wound" at III stage of TAI. As a result of this phenomenon against a background of prolonged blood cells aggregation increasing is a significant blast cells accumulation. Increase of gene Snail expression level in blasts lead to epithelial - mesenchymal transition mechanism activation. Under dose of verapamil promote decreasing of blood cells aggregation, polyamines oxidation enzymes activity increasing, lymphocytes proliferate activity decreasing, gene Snail expression suppression in blast cells at TAI.

KEYWORDS: brain gliomas, tumor - associated inflammation, verapamil, gene Snail.

INTRODUCTION

Working up of malignant gliomas pathogenetic methods of treatment is of current importance. TAI plays a vital part at glioma progression mechanisms. Polyamine oxidation metabolism consist of diamine oxidase (EC 1.4.3.6.) and polyamine oxidase (EC 1.4.3.4.) main enzymes. DAO and PAO play a significant role in reparation mechanisms at TAI.[6-8] It is suppress cells proliferation realizing transduction to cells differentiation by polyamines oxidation. Decrease of DAO and PAO enzymes activity can influence on TAI completeness down to appearance of "persistent wound" syndrome at gliomas.[1]

As a result of such syndrome epithelial-mesenchymal transduction (EMT) take place in tumor microenvironment[9-12], because it essence of tissue inflammation reparation substitution by mesenchymal stem cells reparation.

E-cadherin gene expression suppression, vimentin gene expression, *Snail* transcriptional factors and other mesenchymal markers genes activity increase level take place in EMT realization.[13-14] As proof of direct TAI role in EMT mechanisms activation it is necessary to investigate Snail gene expression in low differentiate blood cells – lymphoblasts as progenitors of lymphocytes, that also take part in tissue reparation.[15-16]

TAI is detected not only in tumor environment, but on systemic level too by blood cells aggregation. Manifestation of early TAI stage may be corrected by blood cells aggregation level decreasing using NMDA-receptors blockers.[17-18] To investigate the TAI role at glioma progression and possibility for it correction by use of NMDA - dependent calcium blocker verapamil was the aim of the work.

MATERIALS AND METHODS

TAI features during glioma growth may be study in comparison with non tumor inflammation, for example at spinal ruptures. Distinction compare with different processes can lead to decoding of glioma progression mechanisms and methods of elaboration for it correction.

58 patients with gliomas of IV degree of malignancy (glioblastoma) and 45 patients with spinal ruptures in acute conditions were investigated. Patients were treated in the "A. P. Romodanov Neurosurgery Institute of NAMS of Ukraine". Venous blood was taken on an empty stomach with addition of heparin (0, 05 ml/5 ml of blood) before medical treatment.

Method of blood cells aggregation determination

New method for blood cells aggregation level was determined for TAI study at malignant gliomas by use of ultrasensitive instruments based on surface plasmon resonance phenomenon (SPR). 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. Transmembrane potential value and activity of glutamate

NMDA - receptors is of great importance in blood cells aggregation mechanisms. Highest possible SPR signal was taken on blood cells without plasma.[17-18] SPR unit is laser angle of deviation, that measured in relative numbers and converting in percents.

Method for determination of DAO and PAO enzymes activity

Determination of diamine and polyamine oxidative deaminization was realized by Gordon and Peter method (1967) in modification.[19] Enzymes activity was determined in nanokatal /mg of protein. Lowry method was used for determination of protein quantity.[20]

Method of lymphocyte proliferative activity determination

In 21 patients with malignant gliomas and 15 patients with spinal ruptures lymphocyte proliferative activity was investigated before medical treatment. NMDA-dependent calcium blocker verapamil was used in decreasing or increasing TMP level model on blood cells membrane mediate by blood cells aggregation level indices. Modification of lymphocytes blasttransformation reaction (LBTR) was realized *in vitro* by application of 0,25% verapamil solutions ("Pharmak"). 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 microliters (mcl) of blood cells without plasma, 60 mcl of different concentrations of verapamil, 60 mcl of phytohemagglutinin (PHA) ("Sigma", 5mg/5ml H_20) and 20 mcl of antibiotic was put into each 2-cm Petri dishes. Supernatant was used for determination of DAO and PAO activity.

Method for determination of gene Snail expression

Determination of gene Snail expression was realized in 5 patients with malignant gliomas and in 5 patients with CNS inflammations. Venous blood was taken on an empty stomach with addition of heparin before medical treatment. After centrifugation (1500 speed/min) during 10 minutes collected lymphocyte fraction from blood, that was cultivated for 72 hours as it was described early.

With aim of verapamil influence on embryonic genes expression investigation to each samples was added 60 mcl of preparation in dilution from 1:1000 to 1:10.000 times. Total RNA isolation from cultured leucocytes was realized by the method.[21]

RNA samples at first transformed into complementary DNA (cDNA) by use of reverse transcriptase. Then cDNA was use for PCR exponential amplification method. Synthesized amplicons was use in 2% agarous gel electrophoresis in TAE buffer (40 мM Tris acetate; 1мM EDTA; pH = 7,6).

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

Gene	Nucleotide consecution (5'–3')	Amplicon (nucleotide pairs)
SNAIL	F-CAGACCCACTCAGATGTCAA R-CATAGTTAGTCACACCTCGT	558
GAPDH	F-TGAAGGTCGGAGTCAACGGATTTGGT R-CATGTGGGCCATGAGGTCCACCAC	260

Statistical treatment of findings was realized by "Statistics–10v" package. Standardize of different indexes was realized by using of: $Xn - X\Sigma / \sigma$, where Xn – individual meaning; $X\Sigma$ – average value; σ – standard deviation.

RESULTS AND DISCUSSION

In fig. 1-3 application of verapamil dilutions (from 10 to 100.000 times) led to modifying of blood cells aggregation level *in vitro*. TAI stages simultaneously pass in organism, therefore TMP level is fluctuate at different pathology. Verapamil can stabilize such fluctuation.

Reparation processes reduction cause in glioma tissues was investigated by compare with standardize indexes of blood cells aggregation levels and DAO and PAO activity at spinal ruptures and malignant gliomas.

Use of verapamil dilutions from 10.000 to 100.000 times lead to blood cells aggregation level decrease, that is typical for III stage of not concerned with tumor growth inflammation (Fig. 1). The comparison of standardize SPR indices and enzyme activity to make it clear that decrease of aggregation level lead to increase of DAO activity (putrescine as substratum) and PAO activity (spermidine and spermine as substratums) is in average value at spinal ruptures (not tumor-associated inflammation).

Application of verapamil dilutions to 100.000 times result in blood cells aggregation level decreasing, that is typical for III inflammation stage and was observed decreasing of DAO and PAO activity at malignant gliomas (Fig. 2).

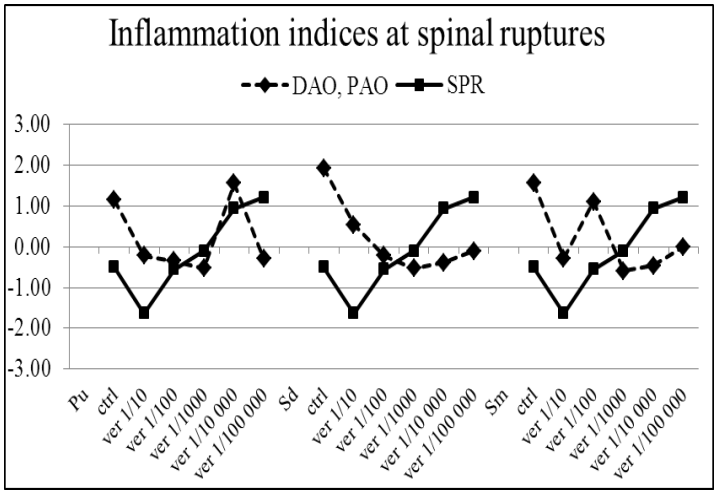

Figure: 1. Standardize SPR indices on blood cells in comparison with DAO and PAO activity indices in supernatant during lymphocyte cultivation by use of different verapamil solutions at spinal ruptures (in RBTL test).

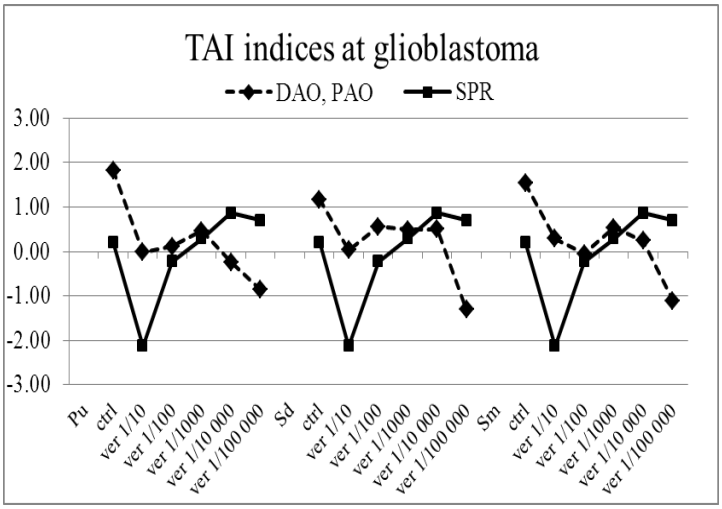

Figure: 2. Standardize SPR indices on blood cells in comparison with DAO and PAO activity indices in supernatant during lymphocyte cultivation by use of different verapamil solutions in malignant gliomas (in RBTL test).

Application of verapamil dilutions from 10 to 100 times lead to insignificant increasing of blasts cells against a background of aggregation level increasing in comparison with control indices without verapamil at spinal ruptures (Fig. 3).

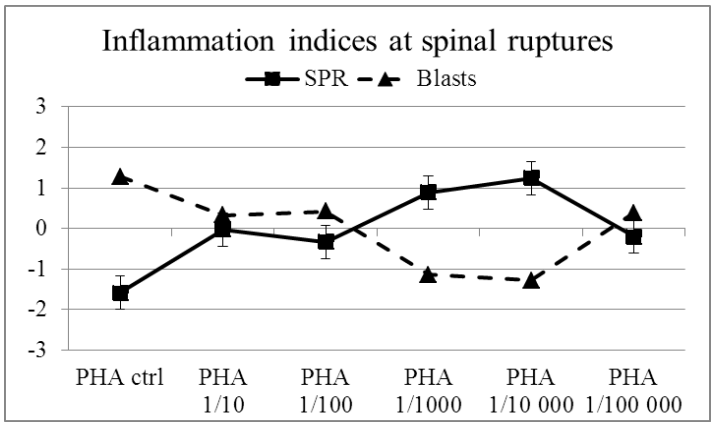

Figure: 3. Standardize SPR indices on blood cells in comparison with number of blasts cells (in %) during lymphocyte cultivation by use of different verapamil solutions at spinal ruptures (in RBTL test).
Designation: ctrl - blood samples without additional potency of PHA.

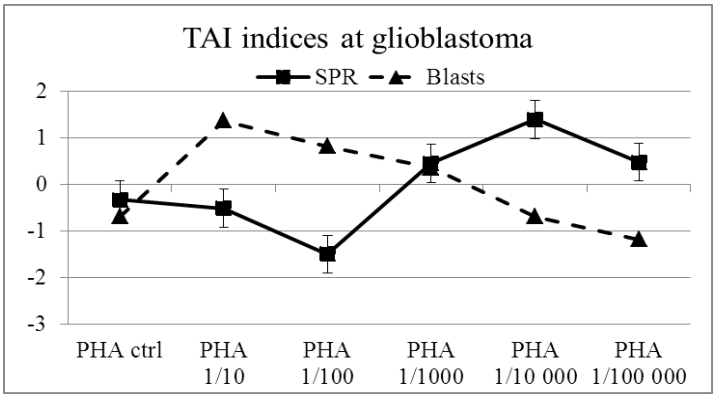

Figure: 4. Standardize SPR indices on blood cells in comparison with number of blasts cells (in %) during lymphocyte cultivation by use of different verapamil solutions at malignant gliomas (in RBTL test).

In control blood samples from patients with glioblastoma was marked decreasing of blast percent number against a background of the same increasing of aggregation level at spinal ruptures (Fig. 3-4).

At first sight, such result is a confirmation of immunosuppressive status at gliomas, when lymphocytes proliferative activity is decreasing in response on PHA impact.[22] But influence of verapamil to 10 times reveal, that immunosuppression is latent and depend from TMP level. In Fig. 4 shown, that increase of aggregation level lead to essential increase of lymphocyte proliferation activity under exposure of verapamil dilution in 10 times.

As soon as inflammation reparation processes is block up it can be activated reparation by stem mesenchimal cells. Such type of reparation is realized by epithelial-mesenchimal transition (EMT). It is very important, that EMT is realized normal embryogenesis and regeneration processes. Mesenchimal stem cells migrate from bone marrow to necrotic centre of different tissues and change into cells of tissues for reparation.[23-24] Migrating blast cells at malignant gliomas cannot normal recover tissues because it genome have great number of chromosomal aberrations.[25] It was first shown active expression of gene Snail in lymphoblast cells that is precursor of lymphocytes at gliomas of III and IV degrees of malignancy (Fig.5).

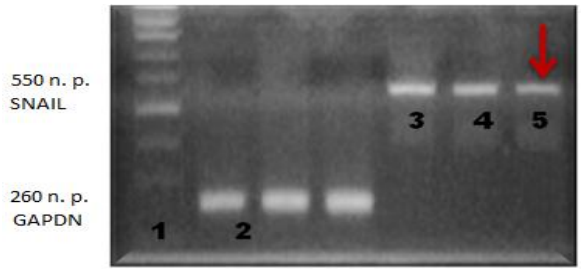

Figure 5. Gene Snail expression under verapamil condition from 1000 to 10.000 potency. Designation: 1 –collection of molecular markers GeneRuler with length from 250 to 10000 base pair; 2 - positive control with primers to gene GAPDH; 3- expression of gene Snail without verapamil; 4- expression of gene Snail under verapamil dilution to 1000 times impact; 5 - expression of gene Snail under verapamil dilution to 10.000 times impact.

Therefore, verapamil dilutions was successfully used for suppression of gene Snail expression in lymphoblasts *in vitro*. Thus, verapamil suppressed gene expression in physiological under doses. Gene Snail suppression effect under verapamil influence to 10.000 times dilutions was achieved to 50% (Fig. 6). Comparison this results with indices changes of aggregation levels under verapamil impact lead to conclusion, that gene Snail expression changes was transmembrane potential dependent.

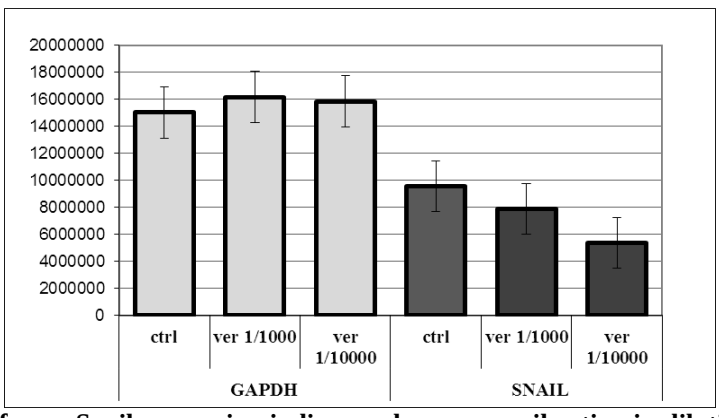

Figure 6. Densitogram of gene Snail expression indices under verapamil action in dilutions from 1000 to 10.000 times.

Designition: 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.

CONCLUSION

It is very important to understand mechanisms of glioma progression promoting patients death. Incomplete of III stage of TAI, when enzymes DAO and PAO lost its activity, offer one of basic reason of EMT activity. Lowering of transmembrane potential over a long period of time lead to blasts appears in abundance and it migrating to gliomas necrotic centre. But blast genome contains substantial number of chromosomal aberrations that can prevent from normal reparation processes and influence on gene Snail expression. Probably, lymphoblast with active expressing of gene Snail on against of background TMP decrease in cancer environment can influence on reprogramming stem cells processes from multipotent to pluripotent and even totipotent phenotypes. Subsequent proliferation of blast cells with large regenerative potential can help to expound of glioblastoma multiforme morphological phenomenon. Verapamil application promotes to TMP level increase (blood cells aggregation decrease), lymphocyte proliferative activity decrease, suppression of gene Snail expression in blast cells at TAI. Such pathogenetic approach in clinical conditions can lead to substantial suppression of further glioma progression in remote postoperative period.

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