

## TGF- $\beta$ 1 EXPRESSION BY GLIOMA C6 CELLS *IN VITRO*

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The *aim* of the work was to study the impact of fetal rat brain cell supernatant (FRBCS) on the expression of transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) and p53 in C6 cells of rat glioma *in vitro*. *Materials and Methods*: FRBCS was obtained from suspensions of fetal rat brain cells on the 14<sup>th</sup> (E14) day of gestation. C6 glioma cells were cultured for 48 h in the presence of FRBCS or FRBCS + anti-TGF- $\beta$ 1 monoclonal antibody. Immunocytochemical staining for TGF- $\beta$ 1 and p53 was performed. *Results*: The proportion of TGF- $\beta$ 1-immunopositive tumor cells in C6 glioma cultures was statistically significantly higher than in the control cell cultures of normal fetal rat brain. FRBCS reduced the proportion of TGF- $\beta$ 1-immunopositive tumor cells and increased the proportion of p53-immunopositive cells in C6 glioma cultures. In cells cultured with FRBCS + anti-TGF- $\beta$ 1 monoclonal antibody, the above effects of FRBCS were abrogated. *Conclusion*: The obtained results suggest that TGF- $\beta$ 1 seems to be responsible for decrease in TGF- $\beta$ 1 expression and increase in p53 expression in C6 glioma cells treated with FRBCS.

*Key Words*: transforming growth factor  $\beta$ 1, p53, fetal rat brain cells supernatant, glioma C6, cell cultures, immunocytochemistry.

According to the National Cancer Registry, the incidence of malignant brain tumors in Ukraine in 2014 was 5.2 per 100,000 of population, and mortality — 3.9 per 100,000 [1]. Among primary tumors of central nervous system, glial tumors predominate. The malignant glioblastomas are difficult to treat because of their invasiveness and high recurrence rate. In neurooncology, criteria for individualization of patient treatment are actively developed by identifying a unique set of molecular changes in cancer cells providing for personalized or targeted therapy. As targets for personalized therapy of brain tumors, the markers of proliferation, neuroepithelial stem cell markers, regulators of cell proliferation, differentiation, survival and/or apoptosis are considered [2]. The use of neural stem and progenitor cells (NSC/NPC) is one of the approaches for the treatment of gliomas [3–6]. The similarities between NSC and brain cancer stem cells (CSC) are being studied [7–13]. It is believed that NSC/NPC may be used to induce long-term antitumor response by stimulating the immune system and for delivery of various biological agents to tumor [14, 15]. In the setting of experimental glioblastomas, it is shown that the NPC can migrate to the tumor inducing tumor cell death [15]. The use of NPC prolongs the survival of animals or almost completely inhibits glioma growth [16], but the mechanism of NPC antitumor effects remains unclear.

The cultures of tumor cells are widely used for developing new approaches to the treatment of brain cancer [17]. In previous studies, we demonstrated

cytotoxic and antimetabolic effects of fetal rat brain cell supernatant (FRBCS) on 101.8 and C6 glioma cells *in vitro* [18, 19] with reduction of the number of Ki-67-immunopositive cells [20] and CD133-positive cells in C6 glioma cell culture [21]. These properties seem to be mediated by soluble factors such as TGF- $\beta$  produced by NSC/NPC [18, 22, 23].

TGF- $\beta$  is known as an important mediator of the malignant phenotype of human brain gliomas [24] while TGF- $\beta$  signaling is involved in the regulation of proliferation, differentiation and survival/or apoptosis [25]. The antiproliferative effect of TGF- $\beta$ 1 on epithelial cells in the early stages and promoting effect in the later stages of tumor growth was shown [26]. It is believed that TGF- $\beta$ 1 signaling is a potential target for antitumor therapy, while the expression of TGF- $\beta$  increases significantly in gliomas with high degree of malignancy [25, 27, 28]. Inhibitors of TGF- $\beta$ 1 signaling reduce viability and invasive properties of gliomas modeled in animals [25].

The study of the possible pro-apoptotic effects of FRBCS and the expression of well-known tumor suppressor protein p53 is no less important. P53 functions as a transcription factor that regulates genes involved in the cellular response to stress; activation of p53 leads to the induction of DNA repair, cell cycle arrest and apoptosis, whereas loss of p53 responses due to mutations promotes uncontrolled cell proliferation [29].

The aim of the work was to study the impact of FRBCS on the expression of TGF- $\beta$ 1 and p53 in glioma cells *in vitro*.

### MATERIALS AND METHODS

**Cell lines.** Rat glioma cells C6 were obtained from the Bank of Cell Lines from Human and Animal Tissues, R.E. Kavetsky Institute of Experimental

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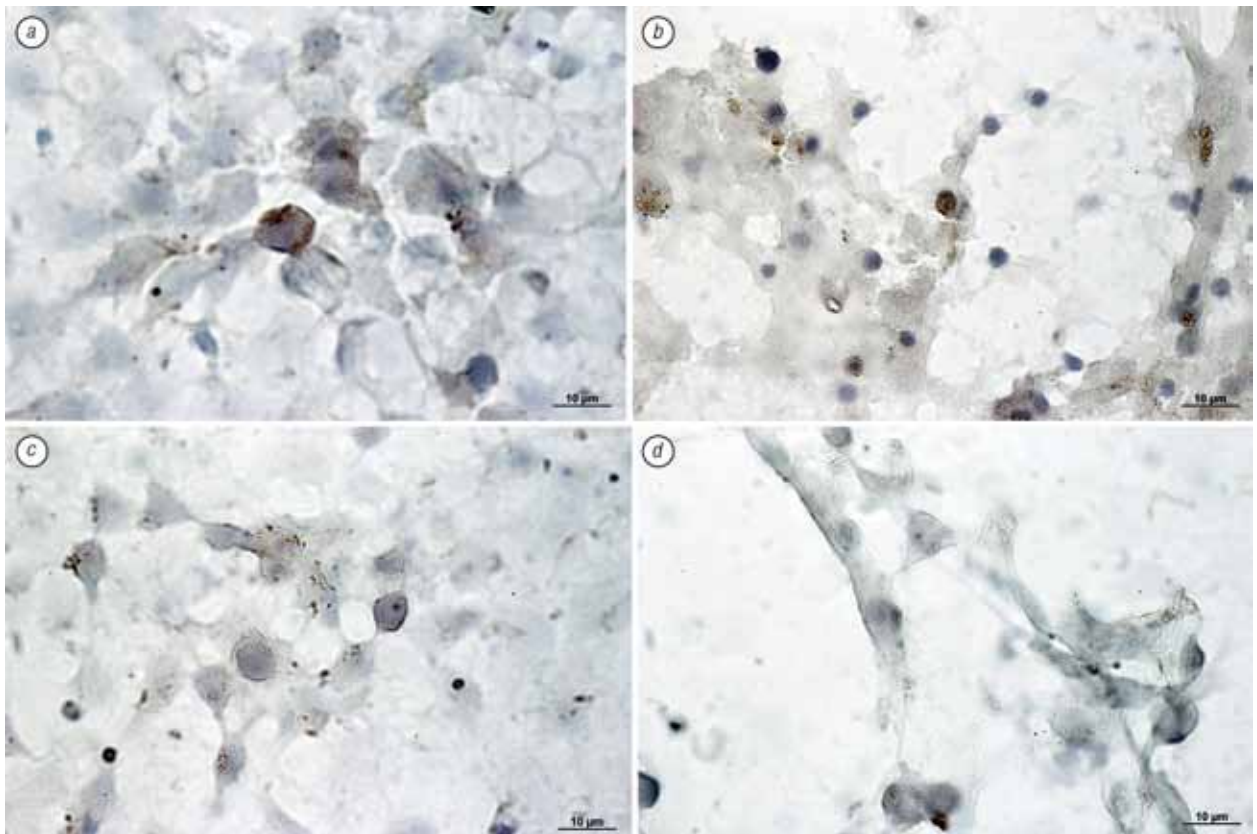
*Abbreviations used*: CSC – cancer stem cells; FRBCS – fetal rat brain cell supernatant; NPC – neural progenitor cells; NSC – neural stem cells; TGF- $\beta$ 1 – transforming growth factor  $\beta$ 1.

Pathology, Oncology and Radiobiology, the National Academy of Sciences, Kyiv). The cells of fetal rat brain (E14) were obtained under the protocol [21]. The viability of cells was determined in a standard test with 0.2% trypan blue (Merck, Germany) [21].  $1 \cdot 10^6$  cells were applied onto the adhesive coverslips coated with polyethylenimine (Sigma, USA), which were placed in Petri dishes and cultured in DMEM medium (2 ml, Sigma, USA), supplemented with 1% fetal calf serum (Sigma, USA), glucose (400 mg%) and insulin (0.2 U/ml). Cells were cultured in a CO<sub>2</sub>-incubator (37 °C, 95% humidity and 5% CO<sub>2</sub>) and observed in inverted microscope (Eclips TS 100, Japan) with microphotographic registration.

**FRBCS.** Fetal rat brain cells removed on the 14<sup>th</sup> (E14) day of gestation were cultured as previously described [18] and supernatant (0.10 mg/ml) was collected. Earlier we showed that 49–50% of E14 cells are nestin-immunopositive [30] and 37–40% — CD133-immunopositive [21], i.e. these cells are positive for NSC markers.

**Monoclonal antibodies.** Monoclonal antibody to TGF- $\beta$ 1 (anti-transforming growth factor- $\beta$ 1, clone 9016.2; Sigma, USA) and rabbit antibody to p53 (anti-TP53 antibody; Sigma-Aldrich, USA) were used. For FRBCS neutralization, FRBCS (0.10 mg/ml) was mixed with monoclonal antibody to TGF- $\beta$ 1 (0.10  $\mu$ g/ml) and incubated for 20–30 min prior to addition to the experimental cultures.

**Immunocytochemical staining for TGF- $\beta$ 1 and p53.** Cells fixed on coverslips were rehydrated, incubated in 0.1% solution of Triton X-100 (Sigma, USA) at room temperature for 30 min, and then washed three times for 5 min in 0.01 M phosphate buffer (pH 6.0). To block endogenous peroxidase, the coverslips were incubated in the dark for 10 min with 3% H<sub>2</sub>O<sub>2</sub> solution and then rinsed for 5 min in phosphate buffer. To block nonspecific background staining, coverslips were incubated 5 min with 1% solution of bovine serum albumin (Sigma, USA). Mouse monoclonal antibody to TGF- $\beta$ 1 or rabbit antibody to p53 were applied at a dilution of 1:100 for 60 min at room temperature. After triple washing in buffer, the secondary antibody (goat antimouse/antirabbit IgG peroxidase conjugated (Dako, Denmark) at a dilution of 1:200) were applied for 30 min at room temperature followed by treatment with diaminobenzidine solution (Dako, Denmark) for 2 min and the development of specific coloration was controlled under the microscope. After washing with distilled water, the cells were stained with hematoxylin and the specimens were embedded in balsam. Parallel studies were performed with positive and negative controls. The stained specimens were examined under Axiomager A2 microscope (Carl Zeiss Microscopy GmbH, Germany) with a broadband filter equipped with camera AxioCam MRc5 (Carl Zeiss Microscopy GmbH, Germany). TGF- $\beta$ 1- and p53-immunopositive and negative cells were counted



**Fig. 1.** Change in the TGF- $\beta$ 1 expression in C6 glioma cells treated with FRBCS. Immunocytochemical staining for TGF- $\beta$ 1 and counterstaining with hematoxylin,  $\times 2000$ , immersion: a — C6 cells, control; b — culture of fetal rat brain cells (E14), control; c — C6 cells incubated with FRBCS (E14), 0.10 mg/ml, 48 h; d — C6 cells incubated with a mixture of FRBCS (E14, 0.10 mg/ml) and antibody to TGF- $\beta$ 1 (0.10 mg/ml), 48 h

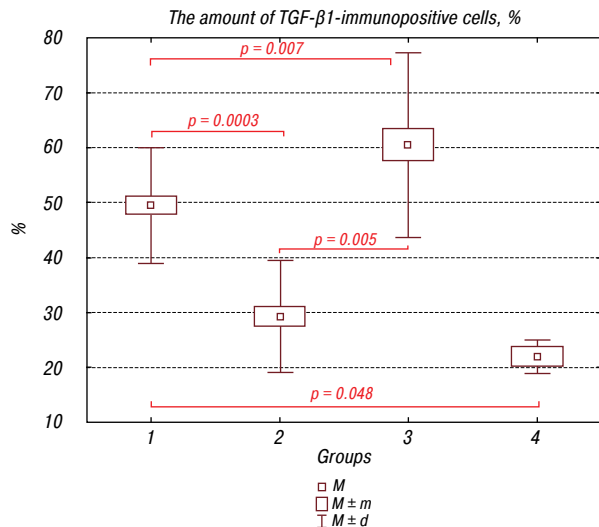
in 10 representative fields of view with standard measurement scale (object-micrometer). Digital images analysis was performed using the software “Zen Lite 2012” (Germany).

**Statistical analysis.** Statistica 8.0, software StatSoft, Inc. (2007) was used for nonparametric Mann — Whitney *U*-test for comparison of independent groups. Normality of data distribution was determined Shapiro — Wilkie test. A statistically significant difference was considered when  $p < 0.05$ , statistically highly significant — when  $p < 0.01$ .

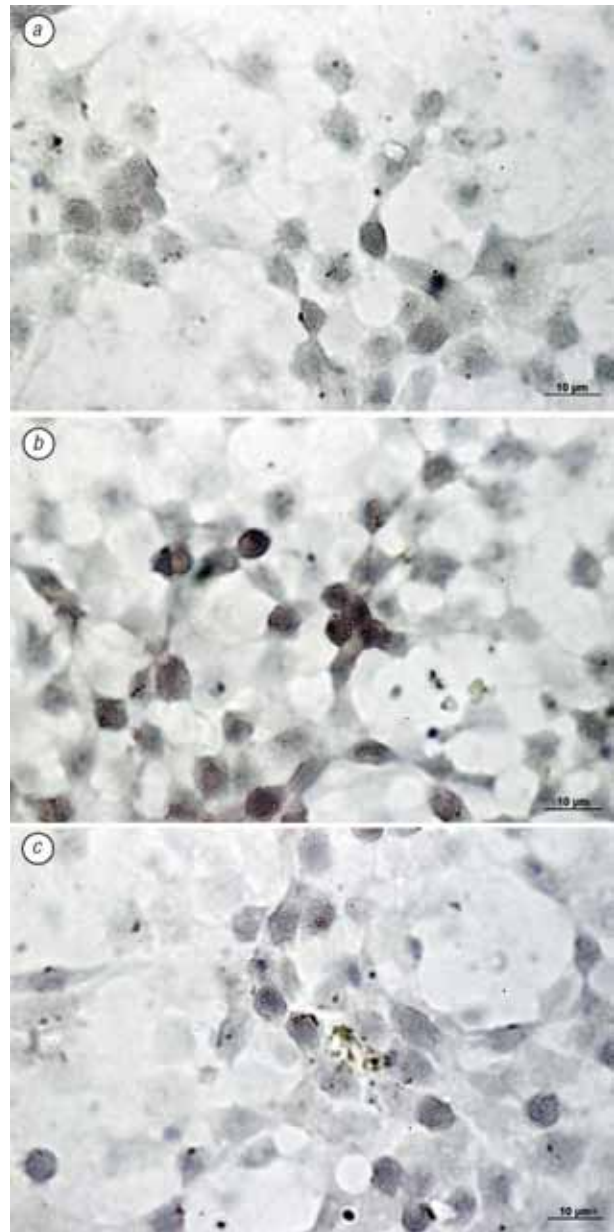
## RESULTS AND DISCUSSION

Immunostaining for TGF- $\beta$ 1 revealed  $48.81 \pm 7.91\%$  of TGF- $\beta$ 1-immunopositive cells in control glioma C6 specimens (Fig. 1, a; Fig. 2) that exceeds significantly the percentage of TGF- $\beta$ 1-immunopositive cells in, control cell cultures of normal fetal rat brain (E14) ( $22.04 \pm 2.33\%$ , Mann — Whitney *U*-test,  $p = 0.048$ ; Fig. 1, b; Fig. 2).

After 48 h of incubation of C6 glioma cultures with FRBCS in a concentration of 0.10 mg/ml, the fraction of TGF- $\beta$ 1-immunopositive tumor cells statistically significantly decreased to  $29.18 \pm 7.24\%$  (Mann — Whitney *U*-test,  $p = 0.0003$  compared to control; Fig. 1, c; Fig. 2). The incubation of C6 cells with a mixture of FRBCS and monoclonal antibodies to TGF- $\beta$ 1 abrogated the decrease of TGF- $\beta$ 1-immunopositive fraction in cultures treated with FRBCS as a single agent ( $p = 0.05$ , Mann — Whitney *U*-test; Fig. 1, d; Fig. 2). Earlier we reported that FRBCS contains TGF- $\beta$ 1 in an amount of 12.0 pg/ml [18]. Therefore, the decrease of TGF- $\beta$ 1-immunopositive cell count in glioma C6 culture after incubation with FRBCS may be presumably explained by the effects of TGF- $\beta$ 1 as the component of FRBCS.



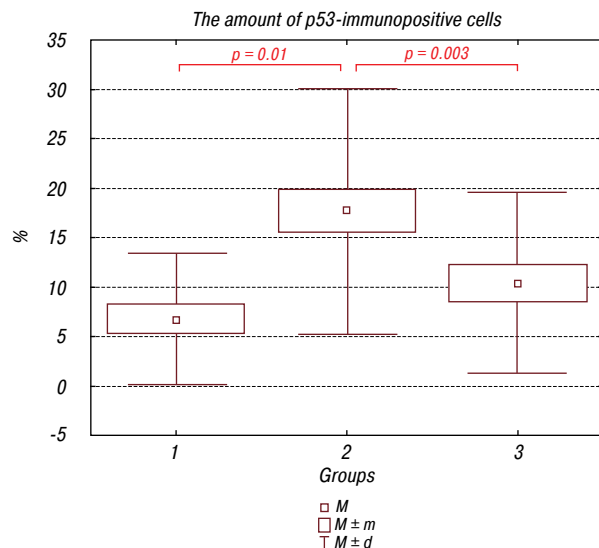
**Fig. 2.** Quantitative indicators of changes in the expression of TGF- $\beta$ 1 in C6 glioma cells treated with FRBCS, 0.10 mg/ml, 48 h: 1 — glioma C6 cells, control; 2 — glioma C6 cells incubated with FRBCS (E14), 0.10 mg/ml, 48 h; 3 — glioma C6 cells incubated with a mixture of FRBCS (E14, 0.10 mg/ml) and antibody to TGF- $\beta$ 1 (0.10 mg/ml), 48 h; 4 — culture of fetal rat brain cells (E14), control. M — mean value; m — standard error of the mean; d — standard deviation from the mean



**Fig. 3.** Change of the p53 expression in C6 glioma cells treated with FRBCS. Immunocytochemical staining for p53 and counterstaining with hematoxylin,  $\times 2000$ , immersion: a — C6 cells, control; b — C6 cells incubated with FRBCS (E14), 0.10 mg/ml, 48 h; c — C6 cells incubated with a mixture of FRBCS (E14, 0.10 mg/ml) and antibody to TGF- $\beta$ 1 (0.10 mg/ml), 48 h

We also examined the expression of p53 in C6 glioma cells treated with FRBCS. In control C6 cells the fraction of p53-immunopositive cells was  $8.94 \pm 6.05\%$  (Fig. 3, a; Fig. 4). After 48 h of incubation with FRBCS in a concentration of 0.10 mg/ml the fraction of p53-immunopositive C6 cells statistically significantly increased to  $18.47 \pm 10.62\%$  (Mann — Whitney *U*-test,  $p = 0.01$  compared to control; Fig. 3, b; Fig. 4). Again, this effect of FRBCS was canceled when monoclonal antibodies to TGF- $\beta$ 1 were added ( $p = 0.003$  compared with cultures incubated only with FRBCS, Mann — Whitney *U*-test; Fig. 3, c; Fig. 4). That is, while adding to the C6 glioma cells a mixture of FRBCS and monoclonal antibody to TGF- $\beta$ 1 the neutralization

of FRBCS effect took place because of binding molecules of TGF- $\beta$ 1 with specific monoclonal antibodies. In our view, this indicates that the established effect (increase of p53-immunopositive cells in glioma C6 cultures) after incubation with FRBCS takes place due to the influence of TGF- $\beta$ 1, which is a component of FRBCS.



**Fig. 4.** Quantitative indicators of changes in the expression of p53 in C6 glioma cells treated with FRBCS, 0.10 mg/ml, 48 h): 1 — glioma C6 cells, control; 2 — glioma C6 cells incubated with FRBCS (E14), 0.10 mg/ml, 48 h; 3 — glioma C6 cells incubated with a mixture of FRBCS (E14, 0.10 mg/ml) and antibody to p53 (0.10 mg/ml), 48 h; M — mean value; m — standard error of the mean; d — standard deviation from the mean

So we have shown, firstly: two-fold increase of cells expressing TGF- $\beta$ 1, in C6 glioma cell cultures compared with cell cultures of normal fetal brain; secondly: reduction of TGF- $\beta$ 1-immunopositive cell number in C6 glioma cultures after FRBCS treatment. The obtained results are generally agreed with the known data about mutations of the components of TGF- $\beta$ 1-signaling pathways in cells of gliomas, as well as autocrine-paracrine mechanism of TGF- $\beta$ 1, effects [24, 31].

In this regard it should be noted that under physiological conditions TGF- $\beta$  plays an important role in the embryo- and morphogenesis and in maintenance of tissue homeostasis [31, 32]. In particular, according to immunohistochemical studies of Pelton et al. [31], few TGF- $\beta$ 1-immunoreactive cells, and a significant number of TGF- $\beta$ 2-, TGF- $\beta$ 3-immunoreactive cells were identified in embryonic tissue of the central nervous system of mouse in fetuses aged 12.5–18.5 days. The authors concluded that the isoforms of the growth factor TGF- $\beta$  during embryogenesis of mammals work by both paracrine and autocrine mechanism, regulate differentiation (by stimulation or inhibition, depending on the type of cells), stimulate the formation of extracellular matrix, act as chemoattractant for certain cells, and also induce mesoderm formation during early development [31]. Other researchers have shown that multipotent human, rat and mouse NPC are able

to produce all isoforms of TGF- $\beta$ , in particular, TGF- $\beta$ 1, TGF- $\beta$ 2, which may explain the immunosuppressive nature of these cells [22, 23].

In setting of pathology, TGF- $\beta$  has a dual role. On the one hand, TGF- $\beta$  is an important mediator of the malignant phenotype of human brain gliomas being involved in the regulation of proliferation, differentiation and survival/or apoptosis [25], modulation of invasiveness, angiogenesis, evasion of immune control and maintenance of CSC in brain [24, 33–35]. On the other hand, TGF- $\beta$  is a strong inhibitor of proliferation of epithelial cells, astrocytes, immune cells and is considered as tumor-suppressive factor [24, 36]. Other authors reported TGF- $\beta$ 1 antiproliferative effect of TGF- $\beta$ 1 in a number of epithelial cells in the early stages and promoter effect in the later stages of tumor growth [26, 32]. Tumor-suppressive function of TGF- $\beta$  includes inhibition of proliferation, induction of apoptosis, regulation of autophagy, but with the development of tumors the response of cells to TGF- $\beta$  shifts. As a result TGF- $\beta$  acts as a potential promoter of cell motility, invasion, metastasis and CSC maintenance in brain [37]. In carcinogenesis, TGF- $\beta$  SMAD-dependent signaling pathway correlates with antiproliferative and tumor-suppressive functions of TGF- $\beta$ , while SMAD-independent way is involved in pro-tumor functions of TGF- $\beta$  [26, 32]. This occurs as a result of mutations of canonical TGF- $\beta$ -signaling pathway elements in malignant gliomas, which allow avoiding the antiproliferative influence of TGF- $\beta$ , thereby contributing to its promoting activity [24]. TGF- $\beta$  modulates the response of glioblastoma cells by autocrine way, all three isoforms of TGF- $\beta$  are expressed being biologically active in glioblastoma cells [24]. The total result of this multi-directional (antiproliferative or stimulating) effect of the TGF- $\beta$  can vary between different specimens of tumors and even between different parts of the same tumor [24].

It is believed that TGF- $\beta$ -signaling is a potential target for the therapy of cancer as the expression of TGF- $\beta$  isoforms increases significantly in gliomas with high degree of malignancy [24, 25, 27, 28, 32, 38], helping tumor to avoid immune recognition via various mechanisms, including inhibition of CD8<sup>+</sup> cytotoxic lymphocytes and natural killer cells [39] and stimulation of the generation of T regulatory cells. The three levels of therapeutic strategy of TGF- $\beta$ -signaling inhibition are considered: the ligand itself, the ligand — receptor interactions and the intracellular signaling cascade [32]. Inhibitors of TGF- $\beta$ -signaling, namely, neutralizing monoclonal antibodies to TGF- $\beta$  used in combination with vaccine based on glioma-associated antigen peptides reduce viability and invasive properties of gliomas in animals [25] contributing to lengthening the average life span mice with GL261 glioma [27]. The knockdown of TGF- $\beta$  receptor type 2 reduced invasiveness of glioma CSC *in vivo* [40].

We have demonstrated that FRBCS reduced the proportion of TGF- $\beta$ 1-immunopositive C6 glioma

cells and increased the proportion of p53-immunopositive cells. Nevertheless, a mixture of FRBCS and anti-TGF- $\beta$ 1 monoclonal antibody neutralized the above biological effects of FRBCS in C6 glioma cells. Thus, our data suggest that TGF- $\beta$ 1 seems to be responsible for decrease in TGF- $\beta$ 1 expression and increase in p53 expression in C6 glioma cells treated with FRBCS.

The obtained results, in our opinion, may become the basis for further research for the purpose of theoretical substantiation of complex pathogenetic therapy of the patients with gliomas including the use of the products derived from fetal neurogenic cells.

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