CHAPTER TWELVE

Pro-inflammatory modification of cancer cells microsurroundings increases the survival rates for rats with low differentiated malignant glioma of brain

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Abstract

Rationale: Glioblastoma multiforme (GBM) is one of the most aggressive human brain tumors. The prognosis is unfavorable with a median survival of 15 months. GBM aggressive nature is associated with a special phenotype of cancer cells that develops because of the transforming growth factor β (TGF-β). The study was aimed at providing experimental justification in vivo of a possibility to suppress TGF-β production in a tumor via pro-inflammatory modification of cancer cell microenvironment, using CD45+ mononuclear cells of the red bone marrow.

Materials and methods: The experiment used animals with transplanted C6 glioma. The animals were divided into 4 groups: (I) control ($N = 60$); (II) group of rats ($N = 30$) that received granulocyte colony-stimulating factor (G-CSF) to recruit CD45+ bone marrow mononuclear cells into their systemic circulation (G-CSF group); (III) group of rats ($N = 30$) that received pro-inflammatory therapy to trigger systemic inflammatory reaction by injecting bacterial lipopolysaccharides (LPS) and interferon-γ (IFNγ); (IV) rats ($N = 30$), stimulated with G-CSF, followed by pro-inflammatory therapy. Stereotaxic modeling of a brain tumor in experimental animals, as well as a combination of morphological, immunocytochemical analyses and immunosorbent assay were used.

Results: TGF-β1 production in the tumor tissue resulted being inversely proportional to the intensity of proliferation processes and directly proportional to the size of necrosis areas, peaking on the 28th day of the experiment. Stimulation of experimental animals with G-CSF recruits CD45+ mononuclear stem and progenitor cells into the systemic circulation of experimental animals with C6 glioma, accompanied by intensification of microglial proliferation in the tumor and infiltration of the tumor tissue with microglial cells. Pro-inflammatory therapy against G-CSF stimulation results in polarization of microglia/macrophages population together with intensified antigen presentation, lower production of TGF-β and IL10, increased synthesis of pro-inflammatory cytokines TNFα and IL1 in the tumor lesion and adjacent brain matter, remodeling of tumor matrix and higher survival rates for the experimental animals.

Conclusions: Pro-inflammatory inflammatory modification of cancer cell microenvironment suppresses TGFβ production in a tumor and increases survival rates of the rats with transplanted poorly differentiated malignant brain glioma.

1. Introduction

The analysts of the World Health Organization (McGuire, 2016; Plummer et al., 2016) report that cancer remains the one of the main reason of human mortality in the majority of world countries. Enormous efforts of the international medical community produced better results in treating

cancer of some types and locations (Cree et al., 2017). Nevertheless, glioblastoma multiforme (GBM), the most wide-spread invasive malignant primary brain tumor, holds a special place among oncological diseases (Omuro & DeAngelis, 2013). It predominantly develops in mature age and is slightly more common for men. Prognosis is unfavorable (Hottinger, Pacheco, & Stupp, 2016; Stupp et al., 2014), and the existing complex GBM treatment protocol ensures the median survival of 15 months with about a quarter of patients managing to live for 2 years since being diagnosed.

For a long time its treatment resistance has been associated with cancer stem cells (CSCs) that dominate the GBM cell hierarchy (Bradshaw et al., 2016) and possess unique signaling and morphogenic properties (Lathia, Mack, Mulkearns-Hubert, Valentim, & Rich, 2015). However, the attempts at targeted elimination of GBM cells that are immunopositive to the main CSCs markers were not very successful as a method of treatment (Gimple, Bhargava, Dixit, & Rich, 2019; Safari & Khoshnevisan, 2015).

Further studies demonstrated (Brown et al., 2017; Seidel, Garvalov, & Acker, 2015) the invasive growth—a key property of GBM—to be more associated not with one CSCs clone, but with the aggressive phenotype of GBM cells that develops due to complex intercellular interactions, accompanied by some cytokines, where the transforming growth factor β (TGF-β) plays a crucial part. By approximating the key properties of molecular phenotypes of GBM differentiated cells (Bryukhovetskiy et al., 2016; Bryukhovetskiy et al., 2016; Bryukhovetskiy, Bryukhovetskiy, Khotimchenko, & Mischenko, 2016; Milkina et al., 2018) and CSCs, this anti-inflammatory cytokine triggers appositional growth, remodels extracellular matrix and creates immunosuppressing microenvironment, making it possible for cancer cells to fulfill their invasive potential.

Radiation, chemotherapy and new targeted medication are unable to suppress TGF-β activity in the tumor lesion. A possible solution to this issue is total pro-inflammatory modification of the tumor lesion with CD45+ mononuclear stem cells of the bone marrow, the main part of them being hematopoietic stem cell (HSCs). When introduced into the circulation of animals with transplanted glioma, these cells migrate toward the neoplastic tissue, accumulate in the invasion and necrosis areas and transform microglial cells (Bryukhovetskiy, Bryukhovetskiy, et al., 2016; Bryukhovetskiy, Dyuizen, et al., 2016; Bryukhovetskiy, Manzhulo, et al., 2016). Theoretically speaking, triggering systemic inflammatory response provides classical pro-inflammatory activation of microglia and lowers TGF-β production in the neoplastic lesion.

The research aimed at providing experimental justification of a possibility to create pro-inflammatory modification of cancer cell microenvironment to suppress TGF-β production in a tumor, using CD45+ red bone marrow mononuclear cells, recruited into the systemic circulation in vivo.

The research objectives included the following steps:

(a) To study the patterns of TGF-β1 production in the brain tissue at different stages of oncological process in vivo;

(b) To demonstrate the possibility of recruiting autologous CD45+ mononuclear cells and HSCs into the blood vessel of animals with transplanted poorly differentiated glioma;

(c) To study the influence of systemic inflammatory response to TGF-β1 production in the tumor lesion after recruiting CD45+ mononuclear cells and HSCs into the blood flow of rats with transplanted poorly differentiated glioma;

(d) To conduct a comparative analysis of survival rates in these animal groups.

2. Materials and methods

2.1 Study design

The experiment used animals with transplanted brain glioma. The animals were divided into 4 groups: (I) control (N = 60); (II) group of rats (N = 30) that received granulocyte colony-stimulating factor (G-CSF) to recruit CD45+ bone marrow mononuclear cells into the systemic circulation (G-CSF group); (III) group of rats (N = 30) that received pro-inflammatory therapy to trigger systemic inflammatory reaction by injecting bacterial lipopolysaccharides (LPS) and interferon-γ (IFNγ) (pro-inflammation therapy (PT) group); (IV) rats (N = 30), stimulated with G-CSF, followed by pro-inflammatory therapy (G-CSF + PT group).

2.2 Experimental animals

The use of animals in this study was discussed and approved by the Ethical Committee of the FEFU School of Biomedicine (Minutes №1 and №2 of 05.03.2019). Animals were treated and housed in conditions in compliance with the GLP standards and the WMA Declaration of Helsinki on ethical principles of animal treatment in experiments. The research is conducted in accordance with the U.K. Animals (Scientific Procedures) Act and associated guidelines, the EU Directive 2010/63/EU for animal experiments, or

the National Institutes of Health guide for the care and use of laboratory animals. All animal studies should also comply with the ARRIVE guidelines and the AVMA euthanasia guidelines 2013.

The study used 150 male Wistar rats, weighing 150 ± 10 g, age—12–14 weeks. The rats were obtained from the breeding facility at the Research and Development Division of the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry (Pushchino, Moscow Region, Russia). The animals were kept in standard conditions of humidity, temperature (24 ± 2 °C), and light (12 h light/12 h dark). The animals were housed in large spacious cages, bedded with husk. They were fed with a standard rat pelleted diet and had free access to water throughout the experimental period.

2.3 Tumor cells

The study used cells of poorly differentiated C6 glioma line (cat. no ATCC® CCL-107™). The cells were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin and 5 μg/mL, in standard conditions (5% CO_2 and 37 °C), all chemicals were obtained from Gibco (Thermo Fisher Scientific, Inc., USA). The cells were cultured until 80% confluence and used in the experiment after the third passage since the moment of being obtained from the manufacturer. The cell line was tested for mycoplasma contamination with Universal Mycoplasma Detection Kit (ATCC® 30-1012K™).

2.4 Modeling glial brain tumor

The animals were anesthetized with intraperitoneal injection of 2 mg substance, containing Zoletil 100 (Virbac, France) + Rometar (Bioveta a.s. Czech) at a 1:4 ratio. A scalp incision was performed, the burr hole was inserted, using Ideal Micro Drill Surgical Drill (Harvard apparatus, US). Cancer cells (0.5×10 (Bryukhovetskiy & Shevchenko, 2016)) in 5 μL of sterile 0.14 M NaCl solution were injected with a Hamilton syringe (600 Series, Hamilton, US), injection speed 1 μL/min, into caudoputamen with SR-5R-HT stereotaxic instrument for rats (Narishige, Japan) based on the coordinates from the rat brain atlas (Paxinos, Watson, & Emson, 1980): Ap-1; L-3.0; V-4.5, TBS-2.4 mm. The tumor was verified in 10 days with magnetic resonance imaging on the Bruker's PharmaScan® (Massachusetts, US).

2.5 Recruitment of CD45+bone marrow mononuclear cells

Animals were subcutaneously injected with granulocyte colony-stimulating factor (G-CSF) (Filgrastim, cat. no. Y0001173 Sigma-Aldrich, Saint Louis, MO, US), 4 mg/day, for 7 days. Immobilization of CD45+ cells and HSCs was checked by testing experimental animals' blood with Navios™ flow cytometer (Beckman Coulter, US). Anti-rat CD45 antibodies (OX-1 conjugated with APC/Cy7, cat. no. 202216, Biolegend Inc., US) were used in the study. Cytofluorometry data were processed with Navios Software v.1.2 and Kaluza™ v.1.2 (Beckman Coulter, US).

2.6 Pro-inflammatory therapy

To trigger systemic inflammatory response bacterial lipopolysaccharide *E. coli* serotype O111:B4 (cat. no L2630 Sigma-Aldrich, Saint Louis, MO, US) (dissolved in 0.5 mL of sterile saline) was intraperitoneally injected for 7 days at a dose of 5 mg/kg body weight. Four hours later the animals were intraperitoneally injected with 9 μg of Recombinant Rat IFNγ (cat. no. I3275 Sigma-Aldrich, Saint Louis, MO, US), dissolved in 0.5 mL of sterile saline.

2.7 Morphological analysis

The rat brain tissue was obtained after decapitating the animals. The material was divided into two parts: one part was analyzed morphologically and immunohistochemically, another part was used to measure the amount of cytokines in the brain tissue, based on the method described below. The material for morphological and immunohistochemical analysis was fixed (4% PFA) for 12 h with subsequent PBS washing. For morphological analysis paraffin-embedded brain tissue sections (7 μm thick) were dewaxed and stained with hematoxylin and eosin according to the standard procedure. All used chemicals were produced by Sigma-Aldrich (Saint Louis, MO, US).

2.8 Immunohistochemical analysis

The analysis was based on the standard procedure. Primary antibodies to microglia/macrophage-specific protein IBA1-1 (ab107159), proliferating cells nuclear antigen (PCNA) (ab 29) and membrane protein CD 86 expressed on antigen-presenting cells (ab213044) and mannose receptor CD206 (ab64693) were used in compliance with the manufacturer's (Abcam, USA) manual. Secondary antibodies, labeled with Horseradish P Anti-Rabbit (PI-1000), Anti-Mouse (PI-2000), peroxidase PI-1000

(anti-rabbit); PI-2000 (anti-mouse), were used in compliance with the manufacturer's (Vector Laboratories) manual. NovaREDsubstratekit chromogen (Vector Laboratories; SK-4800) was used for immunoperoxidase reaction. The sections were thoroughly washed with 0.1 M PBS, dehydrated and covered with balsam, then studied with AxioScope A1 (Carl Zeiss, Germany).

2.9 Immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) of the brain tissue for main cytokines IL-1 (ab100768), IL-10 (ab100765), TNF-α (ab100747) and TGF-β1 (ab119558) was performed with Abcam kits (USA). The rat brain was extracted, one section was separated for morphological and immunohistochemical analyses, and the remaining material was immediately frozen in liquid nitrogen at −70°C. The glioma tissue with the surrounding brain matter (5 m from the tumor edge) was analyzed. Tissue homogenates were prepared with a buffer solution, containing 0.25 M sucrose, 0.01 M Tris-HCl, 0001 M EDTA, 0.01 HEPES. All chemicals were produced by Sigma-Aldrich (Saint Louis, MO, USA). Lysing buffer was added to the tissue at a ratio of 5 mL to 1 mg of tissue, the tissue was homogenated, centrifuged, and supernatant was collected. Protein content was measured with the Pierce BCA Protein Assay Kit (Thermo Scientific). Optical density was defined at a wavelength of 450 nm with BioRad xMark Microplate Spectrophotometer.

2.10 Statistical analysis of the results

The obtained data were processed with Graph Pad Prism 4.00 (GraphPad Software Inc., US). The results were used to calculate the arithmetic mean and its standard error. The results were presented as M ± SEM. Student's *t*-test was applied for paired samples. Differences between groups were considered significant with $P < 0.0.05$. Kaplan-Meier estimator was used for survival analysis.

3. Results

Patterns of TGF-β1 production in the brain tissue at different stages of cancer development.

Transplantation of glioma cells resulted in immediate creation of large tumors, rising above the convexital brain surface as a pale pink nodus,

contrasting with the brain tissue in color. Tumor development was accompanied by swelling and deformation of brain structures, disappearance of major brain convolutions, compression of cerebral ventricles and severe deformation of brain structures.

On the 14th day of the experiment the morphological study of the glioma tissue revealed accumulation of neoplastic cells, forming multiple blood vessels (Fig. 1A, B). Some areas of the tumor lesion had higher concentration of glioma cells around blood vessels, creating thick sphere-shaped or "rosette"-like conglomerates, limited by necrosis areas in the periphery (Fig. 1C). In the periphery of the tumor lesion glioma cells without clear borders invaded the brain matter with multiple solitary cancerous elements and secondary conglomerates.

The developed tumor was characterized by high proliferation speed. PCNA cells (Fig. 1D) were evenly spread in the center of glioma and adjacent brain matter where they were located in blood vessels and created secondary satellite lesions. Tumor development in the rat brain was accompanied by intense reaction of astrocytic glia (Fig. 1E, F). GFAP + cells with large body and short projections were located in the brain tissue, adjacent to the tumor lesion, and in some more distant areas, but were absent in the tumor tissue. In their turn, tightly knit groups of cell elements, immunoreactive to microglia/macrophage-specific protein IBA-1, were identified both in the center of the neoplastic lesion, and the periphery of the tumor, as well as in the brain tissue, blood vessels, surrounding the tumor.

On the 28th day of the experiment the morphology of glioma started to be dominated by extensive necrosis (Fig. 2A, B), containing empty blood vessels, surrounded by an assembly of several cell elements. Proliferation intensity had significantly dropped by that time, while PCNA + cells flocked toward the tumor edge in tight groups and were visible in the areas of invasion into the brain matter with dystrophic changes (Fig. 2D, E). Location of astrocytic glia elements did not show any significant differences, as compared with the 14th day of the experiment. GFAP + cells were located along the tumor lesion edge (Fig. 2F), had a stellate shape and multiple projections, closely interacting with each other.

The amount of microglia cells in the tumor tissue by the 28th day of the experiment had significantly dropped. Iba + cells were distributed in the center of the glial lesion as clusters or surrounded necrosis areas, creating pseudopalisade-like structures (Fig. 2G). Along the invasive edge of the tumor microglial cells had more dense accumulation, creating a band-like shape between the tumor tissue and brain matter (Fig. 2H), peppered with

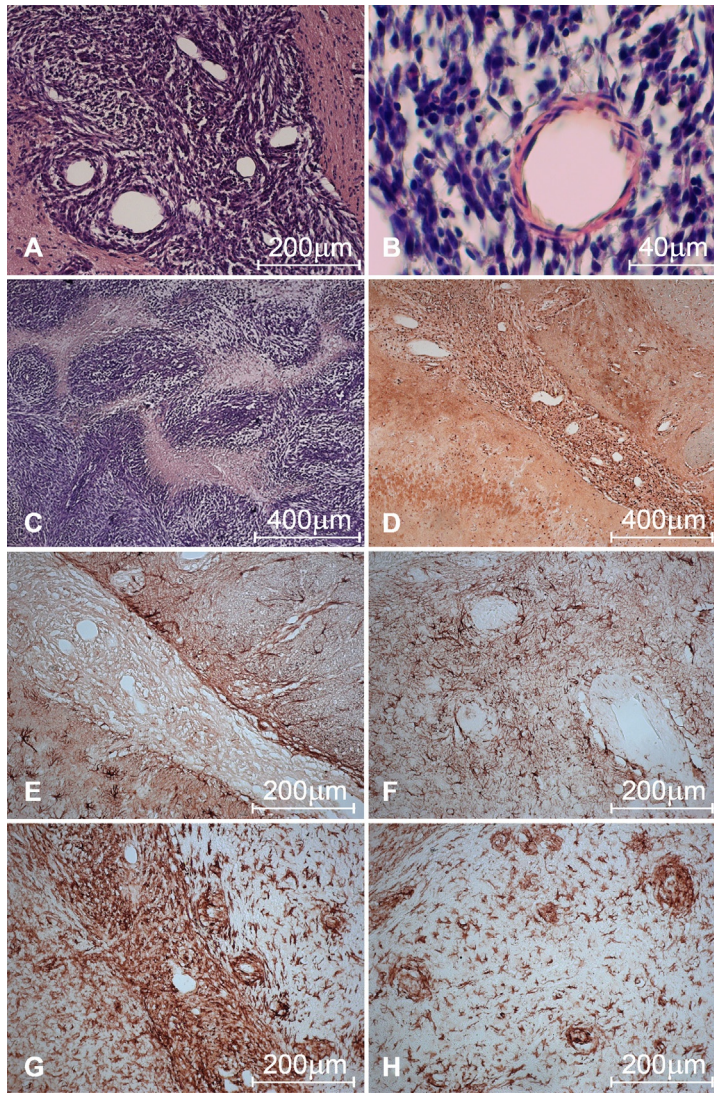

Fig. 1 Morphological and immunohistochemical description of the tumor lesion in the rat brain with transplanted C6 glioma, control group, 14th day of the experiment. (A) Tumor tissue in the rat brain contains multiple blood vessels. (B) Neoplastic blood vessel in the tumor. (C) Clusters of cancer cells around neoplastic blood vessels with creation of pseudo-rosettes, surrounded by necrosis areas. (D) Staining the tumor with antibodies against PCNA. (E and F) staining with antibodies against GFAP, center of tumor lesion; clusters of stellate cells with long projections along the periphery of the tumor lesion and close to (F) the tumor. (G and H) staining with antibodies against microglia/macrophage-specific protein Iba1, g—tumor, p—adjacent brain tissue.

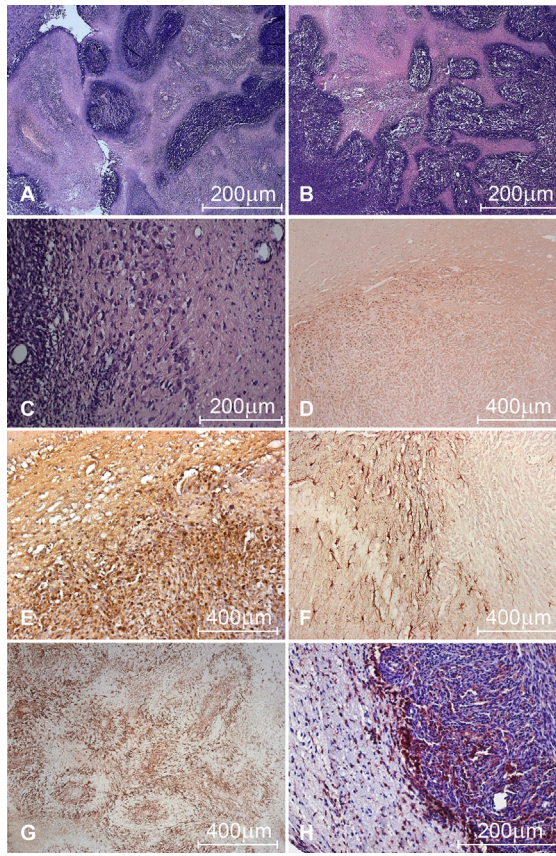

Fig. 2 Morphological and immunohistochemical description of the tumor lesion in the rat brain with transplanted C6 glioma, control group, 28th day of the experiment. (A and B) Tumor tissue in the rat brain contains large necrosis areas. (C) Edge of neoplastic lesion, tumor cells invasion into the brain matter. (D) Staining of the tumor lesion with antibodies against PCNA. (E) Staining of the tumor edge with antibodies against PCNA, more densely organized clusters of PCNA+ are visible in the areas of cancer cells invasion into the brain matter with dystrophic changes. (F) Staining of the tumor edge with antibodies against GFAP, clusters of stellate cells with long projections along the tumor periphery. (G) Staining of the tumor with antibodies against microglia/macrophage-specific protein Iba1, Iba+cell elements form clusters around the necrosis areas and pseudo-rosettes, creating pseudopalisade-like structures. (H) Staining of the tumor edge with antibodies against microglia/macrophage-specific protein Iba1, the specimen was additionally stained with hematoxylin and eosin, Iba1+ cell clusters were visible along the edge of the tumor lesion, creating a band-like shape.

cancer cells. Decrease in proliferation and the amount of microglial cells together with intensification of necrosis in the tumor lesion by the 28th day of the experiment were accompanied by the increase of TGF-β1 content in the brain tissue (Fig. 3). For that reason the 28th day was selected as the checkpoint to proceed with the experiment.

Recruitment of CD45 + autologous mononuclear cells of the bone marrow into the blood vessel of rats with transplanted C6 glioma.

Injecting animals, having C6 glioma, with G-CSF was accompanied by an increase in CD45 + cells amount in the overall number of lymphocytes, recruited into the blood vessel. Meanwhile, the amount of HSC-like cells among bone marrow mononuclear cells, immunopositive to CD45 membrane antigen, had increased more than 19-fold by the 7th day of the experiment (Fig. 4A–D).

Influence of the systemic inflammatory response on TGF-β1 production in the tumor lesion when immobilizing CD45 + mononuclear cells of bone marrow in the blood vessel of rats with C6 glioma.

On the 28th day of the experiment the animals of the groups II (G-CSF) and IV (G-CSF + PT) exhibited significant (Fig. 5A) increase of PCNA + cells in the tumor tissue, as compared with the control group and rats, that received only PT (group III). Unlike the control group (Fig. 2D, E), multiple PCNA + cells in the group IV rats (G-CSF + PT) were dispersed throughout the whole tumor tissue (Fig. 6A) and represented tightly knit groups in the area of neoplastic cells invasion into the brain matter (Fig. 6B).

Increased proliferation in the tumor tissue, discovered in the groups II (G-CSF) and IV (G-CSF + PT), was accompanied by the extension of glioma staining to microglia/macrophage-specific protein Iba-1 (Fig. 5B). Unlike the control group (Fig. 2G, H), rounded and ameboid-shaped cells were located both in the center of the tumor lesion, and close to the edges of tumor invasion (Fig. 6C, D), as well as visible in the adjacent brain matter. The rats that only received PT (group III) did not reveal any significant differences from the control group in their microglial response.

On the 28th day of the experiment a growing amount of Iba1 + elements of microglia in the tumor tissue of the groups II and IV was followed by polarization of microglia populations (Fig. 5C, D). Detection of solitary microglial cells, expressing CD86 in glioma tissue and lesion-adjacent brain matter of the control group rats, indicated a small amount of antigen, being present in the brain tissue (Fig. 7A). The amount of these cells showed a notable increase in the groups II and III (Fig. 5C), and reached its peak values

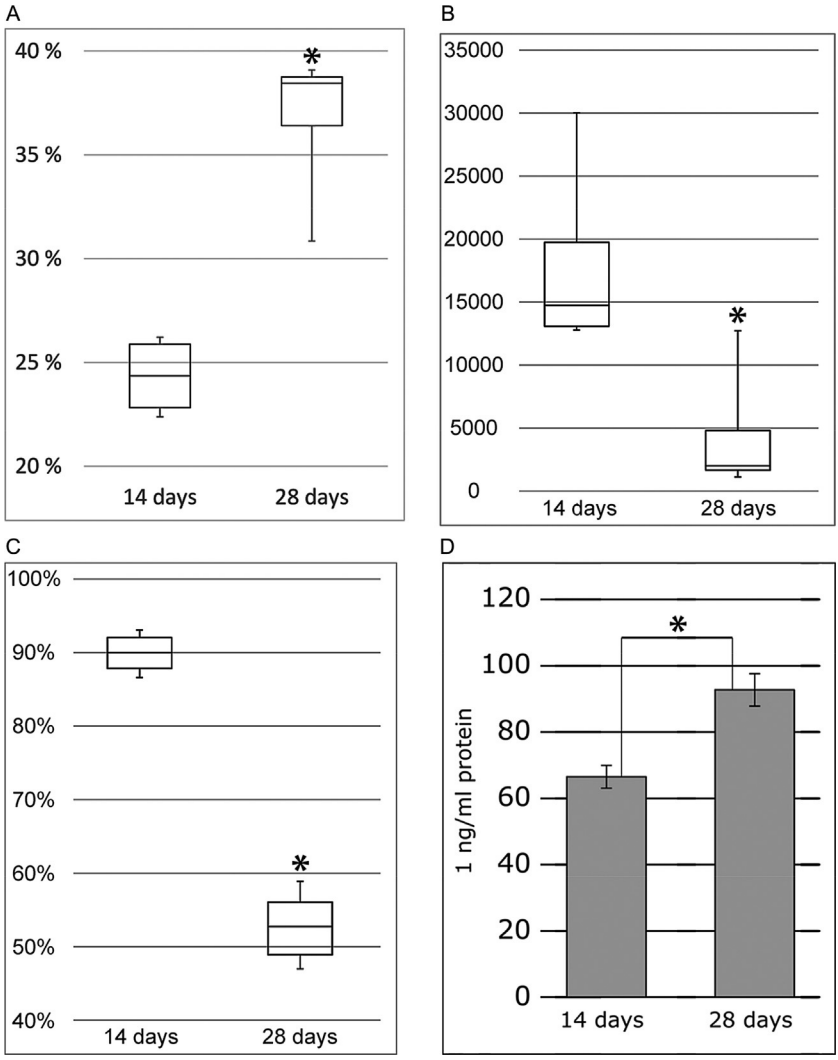

Fig. 3 The results of morphological, immunohistochemical analyses and immunosorbent assay of the tumor tissue in the control group rats on the 14th and 28th days of the experiment. (A) Necrosis area size in the tumor tissue. (B) Amount of PCNA+ elements in the tumor. (C) Ratio of the slide area with antibody staining against Iba1 to the overall area of the slide. (D) TGFβ1 amount (ng/mL) in the glioma tissue samples with adjacent brain matter. The results are presented as M ± SEM. *Differences between groups, as compared with the control group (group I), were considered significant with $P < 0.05$.

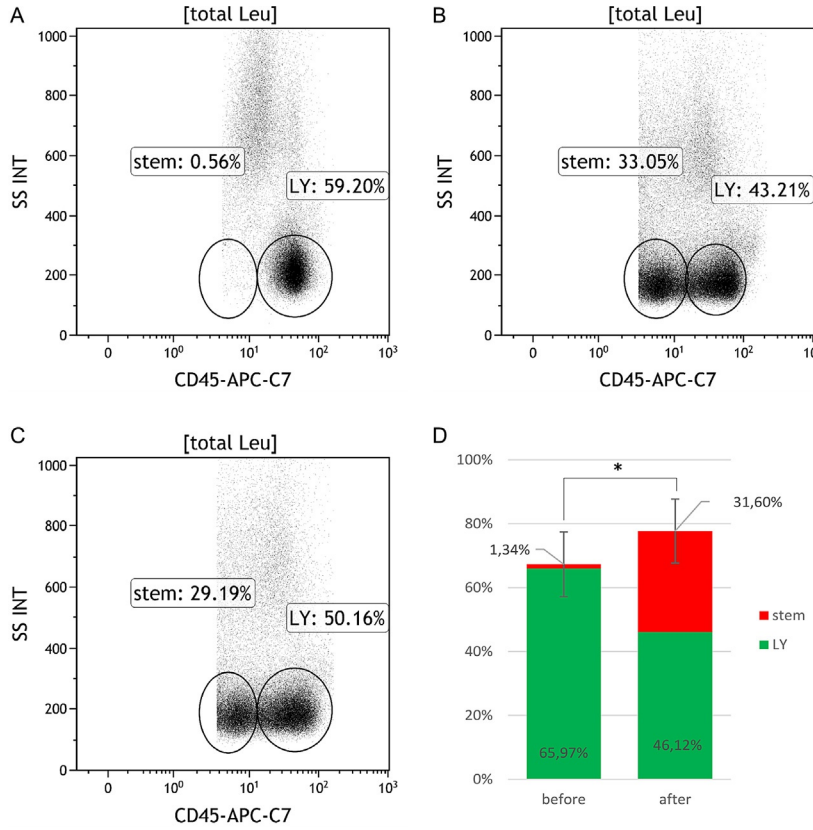

Fig. 4 The cytometry results for the blood samples of C6 glioma rats, stimulated with G-CSF. Amount of CD45+ mononuclear cells and HSC-like cells (A) before and (B and C) after stimulation of G-CSF. (D) CD45+ cells percentage in the overall amount of lymphocytes. The number of HSC-like cells in the overall amount of CD45+ mononuclear cells is highlighted with a red marker. The cytometry results for CD45+ cells population, side population of HSC-like cells was identified with the standard protocol (Telford, 2010). *Differences between groups, as compared with the control (before stimulation), were considered significant with $P < 0.05$.

in the group IV where multiple CD86+ cells of round, stellate or polygonal shape formed tightly knit clusters along the tumor edges and actively invaded neoplastic tissue (Fig. 7B).

In contrast, large quantities of cells, immunopositive to mannose receptor CD206, were discovered in the tumor and adjacent brain tissue (Fig. 7C, D). Their amount was a little higher in the group II of rats that received G-CSF, while the lowest amount was discovered in the groups III and IV (Fig. 5D).

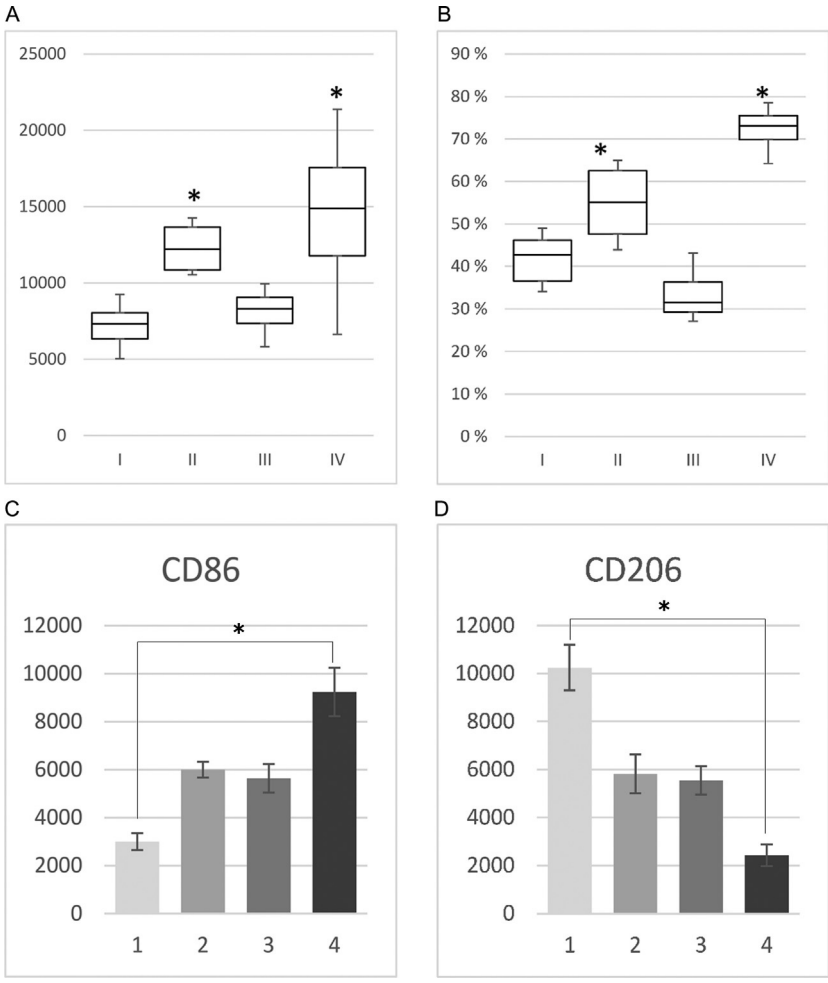

Fig. 5 The results of morphological, immunohistochemical analyses and immunosorbent assay of the tumor tissue in different groups of rats on the 28th day of the experiment. (A) The amount of PCNA+ cells in the tumor tissue. (B) Area of staining the tumor tissue with antibodies against Iba1. (C and D) The amount of cells, immunoreactive to CB86 (C) and CD206 antigens (D). The results are presented as M ± SEM. *Differences between groups, as compared with the control group (group I), were considered significant with $P < 0.05$.

Immunosorbent assay revealed significant differences between key pro- and anti-inflammatory cytokines in experimental groups of rats. By the 28th day of the experiment the control group rats had the highest level of anti-inflammatory cytokines TGF-β1 and IL-10 in the tumor and the adjacent

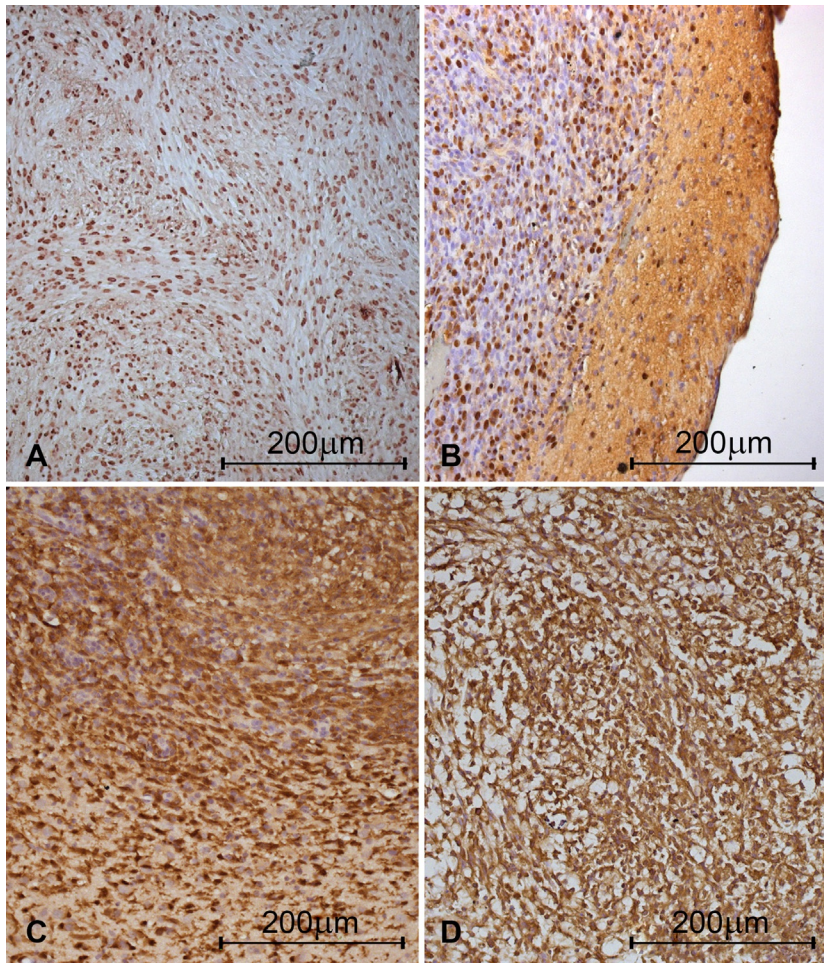

Fig. 6 Immunohistochemical description of the tumor lesion in the brain of group IV rats (G-CSF + PT), 28th day of the experiment. (A and B) Antibody staining against PCNA, (A)—center of the tumor lesion and (B)—the tumor edge, specimen was additionally stained with hematoxylin and eosin. Staining with antibodies against microglia/macrophage-specific protein Iba1, (A)—center tumor, (B)—tumor edge.

brain tissue (Fig. 8A, B). The groups II (G-CSF) and III (PT) exhibited a significant increase in the amount of these cytokines, while the group IV (G-CSF + PT) showed the smallest amount of them. These changes coincided with a surge in the amount of pro-inflammatory cytokines TNFα and IL-1 in this group of rats (Fig. 8C, D).

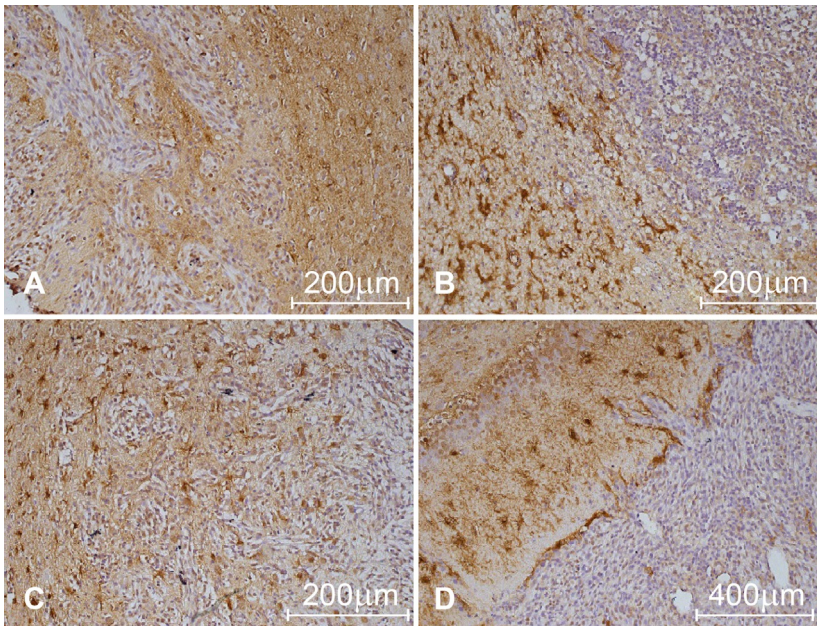

Fig. 7 Immunohistochemical description of the tumor lesion in the brain of the rat groups I (control) and IV (G-CSF + PT), stained with antibodies against CB86 and CB206 antigens. (A) The control group had solitary CD86+ cells in the tumor tissue and the adjacent brain matter. (B) Group IV exhibited CD86+ cell clusters in the brain matter, adjacent to the tumor. (C) Control group, multiple CD206+ cells in the brain tissue, adjacent to the tumor, among satellite glioma lesions; (D) group IV, solitary CD206+ elements in the tumor and adjacent brain tissue.

Comparative study of survival rates for animals with C6 glioma.

Animals of all experimental groups exhibited higher survival rates, as compared with the control group, but the highest rate was registered in the group IV (G-CSF + PT) (Fig. 9).

4. Discussion

As the experiment suggests, stereotaxic implantation of C6 glioma cells into the brain of immunocompetent Wistar rats allows to create a glial tumor with invasive growth, high proliferation speed of cancer cells, intensive angiogenesis and early development of necrosis areas, allowing us to classify this tumor as a highly aggressive malignant one, according to the Ste. Anne-Mayo System (SAMS), or Grade IV, based on the World Health Organization standards (Wesseling & Capper, 2018).

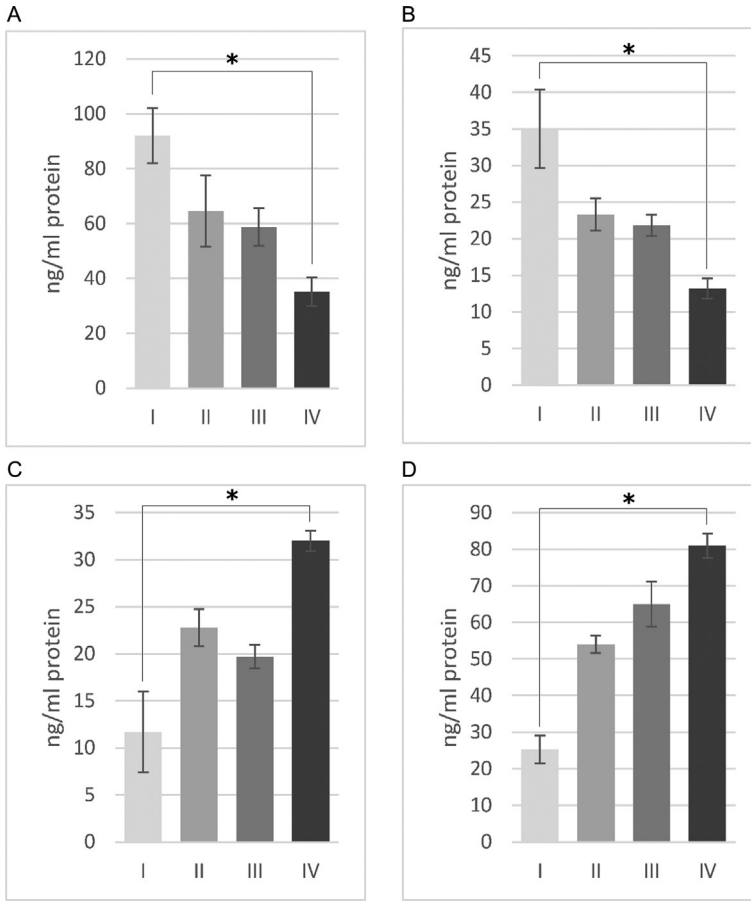

Fig. 8 The results of immunosorbent assay of the tumor tissue with adjacent brain matter on the content of the main anti-inflammatory and pro-inflammatory cytokines (A) TGF-β1, (B) interleukin 10, (C) TNFα, interleukin 1. The results are presented as M ± SEM. *Differences between groups, as compared with the control group (group I), were considered significant with $P < 0.05$. The results are presented as M ± SEM. Student's *t*-test was applied for paired samples.

It is crucial that this tumor repeats the main pathophysiological patterns of GBM. Despite intensive angiogenesis, there are progressing necrosis areas (Figs. 2A, B and 3A) and lower proliferation speed of cancer cells by the 28th day of the experiment, indirectly indicating the utmost importance of hypoxia as a main regulator of morphological changes in the tumor tissue.

We decided against staining the tumor with antibodies to the Hypoxia-Inducible Factor (HIF) in this experiment due to the existing extensive

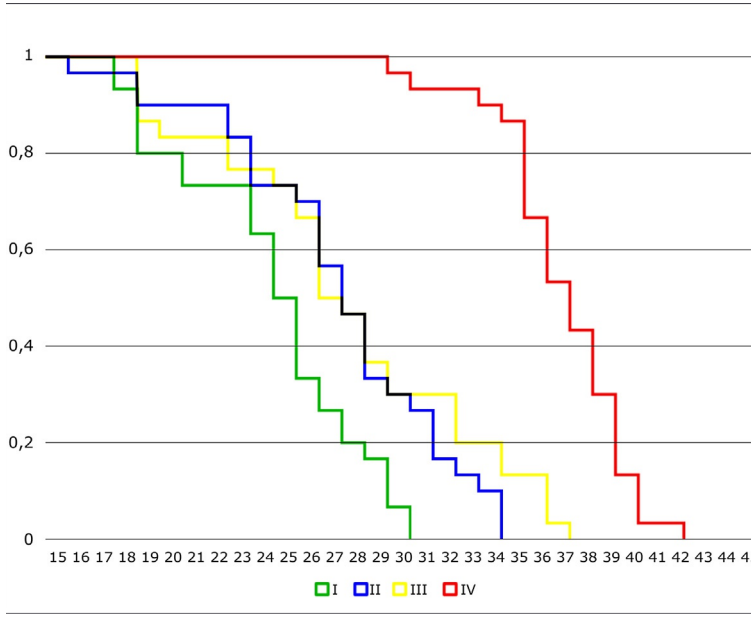

Fig. 9 Kaplan-Meier estimator was used for survival analysis. Green marker—group I (control), blue marker—group II (G-CSF), yellow marker—group III (PT), red marker—group IV (G-CSF + PT).

research on the matter and a direct connection between the amount of HIF in the glioma tissue and angiogenesis intensity (Clara et al., 2014). However, despite the intensity of angiogenesis, necrosis areas appear as early as the 14th day of the experiment, and they become more prominent in the overall morphological picture on the 28th day. The tumor still survives, and there is an active invasion of neoplastic cells and creation of satellite lesions visible along the border between the tumor and the brain matter (Fig. 2C).

It should be noted that proliferation continues in the areas, adjacent to the intact brain matter (Fig. 2D, E) where oxygenation level is higher than in the center of tumor lesion. In this case it is the high level of energy-consuming proliferation of multiple tumor cells that causes hypoxia, on the one hand, and induces TGF-β1 and VEGF synthesis in glioma cells, on the other hand. The first factor stimulates creation of intercellular matrix structures together with producing anti-inflammatory effect, while the second one triggers angiogenesis, directly related with matrix structures and, thus, provokes invasion, that is proven by the morphological analysis results.

Even these results of morphological analysis prove to be sufficient for a serious consideration of TGF-β1 role in oncologic process and

pathophysiological significance of its increased production in the invasive areas of the tumor and brain matter.

GBM cells are the main source of this cytokine in the tumor tissue (Massagué, 2008), with some contribution from activated microglial cells, producing TGF-β together with other anti-inflammatory cytokines. However, the number of microglial cells in the tumor tissue of the control group rats significantly drops by the 28th day of the experiment (Fig. 3C), and the rate of TGF-β production by fibroblasts is relatively low, as compared with neoplastic cells. There have been some indications that apart from GBM cells, bone marrow cells, recruited into the tumor, are directly involved in TGF-β synthesis (Hambardzumyan, Gutmann, & Kettenmann, 2016), but our data do not support this possibility.

There is a direct correlation between the TGFβ level in the blood serum and malignancy grade of a glioma (Rich, 2003). In normal conditions this cytokine induces apoptosis, controls proliferation and cell differentiation, Besides, our data suggest that TGF-β1 stimulation brings about a dramatic change in the molecular profile of GBM cells, represented by suppression of adhesive E-cadherin synthesis, increased production of migratory N-cadherin and components of actin-myosin cytoskeleton, and these changes are in full compliance with the primary function of TGF-β1 in organizing intercellular matrix and are reflected in the increase of mesenchymal phenotype proteins and matrix metalloproteinases synthesis (Bryukhovetskiy, Bryukhovetskiy, et al., 2016; Bryukhovetskiy, Dyuizen, et al., 2016; Bryukhovetskiy, Manzhulo, et al., 2016). In this respect, increased TGFβ production precedes angiogenic effects of VEGF (Krishnan et al., 2015) and potentiates them, hence, becoming a crucial factor of the tumor maturity and indicating the fact that the tumor contains cells, potentially capable of invading the surrounding tissues and organs, and, consequently, possessing all the properties of cancer cells.

Undoubtedly, invasive growth is a major criterion of tumor progression, and many authors believe this GBM characteristic to be associated mostly not just with any cancer cells, but CSCs in particular. A wide range of targeted drugs is aimed at damaging CSCs, however, the actual use of these drugs (Touat et al., 2015; Touat, Idbaih, Sanson, & Ligon, 2017) has not resulted in significant improvement of GBM survival rates, as large-scale clinical trials show. Meanwhile, the amount of CSCs in the tumor is always relatively small (Gimple et al., 2019; Lathia et al., 2015; Seidel et al., 2015), they are located in niches and fulfill their differentiation potential in creating neoplastic blood vessels, required for the tumor survival (Hambardzumyan

et al., 2016). Even though this research does not directly involve examining CSCs functions, the obtained data indicate the possibility of their importance for tumor progression to be overrated.

The results of our own research suggest that TGF-β stimulation approximates the molecular phenotype of differentiated GBM cells with CSCs, thus, closing the gap between the CSC amount and invasive properties of differentiated cancer cells and allowing the tumor to progress (Bryukhovetskiy & Shevchenko, 2016). So, the obtained results attest to the fact that suppressing TGF-β production in the tumor could be an important step in regulating GBM invasiveness by using antitumor potential of HSCs and mononuclear cells of the bone marrow.

CD45+ mononuclear cells of bone marrow have been widely used in oncology for over 50 years. They are involved in treatment protocols after high-dosage chemotherapy to decrease bone marrow depression, lower the risk of infection and hemorrhage complications and restoration of immune response. This type of cells, used in allogeneic transplantation, has its own curative effect due to donor immune cells reaction to the remaining tumor elements in a patient. Recruitment of these cells into the systemic blood flow and their subsequent immobilization always involves G-CSF, used in this research.

G-CSF (Pierce et al., 2017) stimulates proliferation and differentiation of neutrophil predecessors, accelerates their phagocytic and antibody-dependent cytotoxic activity against cancer cells, catalyzes natural killer cells, neutrophil adhesion, modulates expression of their receptors and their affinity. G-CSF is one of most powerful agents (Domingues, Nilsson, & Cao, 2017), recruiting stem and progenitor cells of bone marrow into the systemic circulation and inducing expression of CXCR4 receptor (Saba et al., 2015) that determines migration of these cells into the tumor lesion, as a response to increasing concentration gradiant of stromal cell-derived factor SDF1α (Bryukhovetskiy, Bryukhovetskiy, et al., 2016; Bryukhovetskiy, Dyuizen, et al., 2016; Bryukhovetskiy, Manzhulo, et al., 2016) and 80 other cytokines, using the corresponding receptor types to achieve this effect.

It is worth reminding about the design of this research. A significant part of cells, immobilized in the blood circulation with G-CSF, is represented by HSCs that are stained with antibodies against CD34 antigen. It is this phenomenon that conceptualized the creation of group II. Unfortunately, the majority of renowned producers do not supply antibodies against CD34+antigen to rats' HSCs, that somewhat complicates the matter. But as the experiment suggests, G-CSF stimulation provides not only rapid and

significant increase in the number of CD45+ cells in the overall amount of lymphocytes (Fig. 4), but it causes more than 19-fold increase in the amount of CD45+HSCs-like cells (Fig. 4A–D), being a part of the side population, defined as HSCs (Telford, 2010).

The earlier research of our team indicated that stem cells of higher mammals and humans were highly mobile toward glioma cells and carcinomas in vitro, and after intravenous injection of experimental animals with transplanted glial brain tumor in vivo, they migrate into the neoplastic lesion where they transform into microglial Iba+cells with high anti-tumor potential (Bryukhovetskiy et al., 2017).

Theoretically speaking, one of the CSCs suppression mechanisms is modulating monocyte-phagocyte system activity with CD45+ cell, recruited by G-CSF into the blood circulation. As demonstrated earlier (Bryukhovetskiy, Bryukhovetskiy, et al., 2016; Bryukhovetskiy, Dyuizen, et al., 2016; Bryukhovetskiy, Manzhulo, et al., 2016), injecting rats, having brain glioma, with haploidentical CD45+ rat cells rapidly increases the amount of cancer cells with microglia markers that are frequently associated with cancer progression. However, morphochemical modification of experimental glioma lesion as a response to CD45+ injection does not always reduce the life expectancy of rats with C6 glioma. The decisive factors here are the location and qualitative parameters of microglia.

The essential factor of bone marrow cells accumulation in the tumor is creation of neoplastic blood vessels as a direct response to intratumor hypoxia. In normal conditions, the amount of HSCs that are able to penetrate the intact blood-brain barrier, is relatively small. In vivo brain protection is provided by the resident microgliocytes, deriving from embryonic yolk sac; they maintain the population through proliferation, and bone marrow cells are not involved in this process.

Development of the blood vessels network allows GBM to recruit stem and progenitor cells of the bone marrow with their subsequent transformation into microglial cells. This fact suggests that the stimulating effect these cells have on tumor growth, noted by several researchers (Aderetti, Hira, Molenaar, & van Noorden, 2018; Wang et al., 2016), is partially preconditioned by hypoxia.

Hypoxia is likely to induce alternative activation of macrophages (M2 category) that increases their phagocytic activity, synthesis of interleukin-10, TGF-β and other anti-inflammatory cytokines (Nusblat, Carroll, & Roth, 2017), stimulating the remodeling of necrotized tissue that could be the reason for predominating amount of cells with M2 markers of

microglia in the microsurroundings of invasive GBM elements. Cells of this type create secretory loop, together with fibroblasts produce TGF-β and trigger generation of CSCs with epithelial-mesenchymal transition phenotype. Suppressing the activity of GBM cells with invasive phenotype is possible with classical activation of macrophages (M1 category), providing the grounds for groups II and IV creation.

The results, observed in the group III, were associated with the double stimulation of microglial cells that originate from monocyte-macrophages family. LPS (Café-Mendes et al., 2017; Fonceca et al., 2018) works through the innate immunity receptor TLR-4 and causes inflammasome development and secretion after processing preformed pro-inflammatory cytokines (IL-1, IL-6, TNFα) that is aptly demonstrated by the results, presented in the Fig. 8. Another major biological effect of IFNγ is stimulation of the cells from the monocyte/macrophage cell line, reflected in further increase in production of pro-inflammatory cytokines, assembly of proteasome complex and membrane expression of adhesive Iba-1 molecules and co-stimulating B7 (CD80/CD86) molecules, once again proved by the experiment (Figs. 6 and 7).

It had been reported (Colonna & Butovsky, 2017; Wolf, Boddeke, & Kettenmann, 2017) that brain microgliocytes, derived from embryonic yolk sac, maintain their population after via proliferation after closing of the blood-brain barrier; monocytes and HSCs are not involved into this process. That might explain the abundance of PCNA + cells, immunopositive to microglia markers, on the 14th day of the experiment. However, this resource depletes quickly, resulting in lower concentration of microgliocytes clusters on the 28th day of the experiment.

It should be taken into consideration that IFNγ activated macrophages regain their proliferation properties (Goldmann, Blank, & Prinz, 2016), and microglia is unlikely to be an exception to this rule, allowing to assume that there is another source of PCNA + cells in the periphery of the tumor lesion in the group IV on the 28th day of tumor growth, as well as explaining tumor shrinkage and increased number of Iba-1 cells of microglia along the periphery of the tumor and inside the lesion.

Another fundamental effect of IFNγ (Orihuela, McPherson, & Harry, 2016) is suppression of anti-inflammatory cytokines production that is also proven by the experiment through the example of TGF-β1 and IL-10 (Fig. 7). Combined effect of G-CSF, as well as LPS and IFNγ (group IV) provides maximum involvement of all mononucleocytes, stimulation of HSCs maturation toward myeloid line, multifaceted and thorough

stimulation of all (circulating and tissue) cells of monocyte/macrophage cell line, including microglial cells. It is the results of the group IV in all the tests that provide the most conclusive evidence and allow us to consider the possibility of experimental and clinical trials as a part of GBM therapy.

The results for the group IV, as well as the earlier data on the possibility of stimulating metalloproteinases activity in microglia cells (Bryukhovetskiy, Bryukhovetskiy, et al., 2016; Bryukhovetskiy, Dyuizen, et al., 2016; Bryukhovetskiy, Manzhulo, et al., 2016), allow us to create a complete picture with the data from immunohistochemical, morphological analysis and immunosorbent assay. Since metalloproteinases are crucial for matrix remodeling in case of inflammation, and increase in their production by the microglial cells could significantly change the matrix structure and decrease the neoangiogenesis speed in the tumor lesion that would result in lower growth rate and higher survival rate. This assumption is also proven by the increasing amount of Iba-1 cells of microglia in the lesion and around it and in no way contradicts the results of computer imaging and microscopic study.

Active angiogenesis facilitates disruption of the blood-brain barrier that allows to recruit stem and progenitor cells of the bone marrow into the tumor where they restore the population of microgliocytes. However, once these cells get into an immunosuppressive environment, created by TGFβ, these cells are directed by an alternative activation pathway which is proven by a significant increase in the amount of the cells, immunopositive to mannose receptor CD206—one of the main indicators of the alternative activation of macrophages (Hirosawa et al., 2018; Kaba et al., 2019; Scodeller et al., 2017; Suzuki et al., 2018)—in the tumor tissue of the rats in the group G-CSF. We believe analyzing VEGF dynamics in the tumor tissue and areas of circulation, using the suggested model, is also relevant and requires further studies.

It should be understood that recruiting CD45+ mononuclear cells of bone marrow into the blood circulation is a GBM treatment strategy of the utmost importance, since it increases the survival rates of the experimental animals. Still, the highest survival rates for rats with C6 glioma in the G-CSF+PT group indicate that local stimulation of inflammatory response is a leading mechanism of antitumor effect of CD45+ mononuclear cells of bone marrow.

Therefore, there are certain conclusions to be drawn from this study. TGF-β1 production in the tumor tissue is inversely proportionate to the intensity of proliferation processes. Stimulating experimental animals with

G-CSF recruits CD45 + mononuclear stem and progenitor cells into the systemic circulation of the experimental animals with C6 glioma, accompanied by the increase in microglial proliferation and tumor tissue infiltration with microglial cells. Pro-inflammatory therapy along with G-CSF stimulation intensifies antigen presentation, accompanied by the decrease in TGF-β production, remodeling of tumor matrix and higher survival rates of the experimental animals.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Informed consent was obtained from all individual participants included in the study, and all procedures performed in the studies, involving human participants, were in accordance with the ethical standards of the Far Eastern Federal University.

Authors' contributions

I.B. and A.B. wrote the manuscript, proposed the study idea, designed the study, offered support with the experiments, organized the scientific team, and cultured the cancer cells of glioma C6 for the experiment, operated on all experimental animals, provided scientific guidance and contributed to the immunohistochemical and enzyme-linked immunosorbent assay. S.Z. injected animals with G-CSF, carried out pro-inflammatory therapy and prepared all calculations, related to the mathematical analysis of the experiment results, analyzed the obtained data. A.B. and I.M. conducted morphological, immunohistochemical and immunosorbent assay. I.K. prepared a cytometric description of the mononuclear cells, recruited into the blood cells of experimental animals; Y.K., A.P., O.P. and H.S. took active part in the discussion of the experiment results and manuscript preparation.

All authors read and approved the manuscript and agree to be accountable for all aspects of the research to ensure that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Informed consent for publication was obtained from all individual participants, involved in the study.

Conflict of interest

The authors declare there are no conflicts of interests.

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