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Optical *in vivo* and *ex vivo* imaging of glioma cells migration via the cerebral vessels: Prospective clinical application of the beta2-adrenoreceptors blockade for glioma treatment

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Malignant gliomas are highly invasive tumors that use the cerebral vessels for invasion due to high vascular fragility of the blood–brain barrier (BBB). On one hand, glioma is characterized by the BBB disruption, on the other hand, drug brain delivery via the BBB is a big challenge in glioma therapy. The limited information about vascular changes associated with glioma growth is a reason of slow progress in prevention of glioma development.

Here, we present *in vivo* and *ex vivo* study of the BBB disruption and glioma cells (GCs) migration in rats using fluorescence and confocal microscopy. We uncovered a local breach in the BBB in the main tumor mass but not within the border of normal and malignant cells, where the BBB was impermeable for high weight molecules. The migration of GCs were observed via the cerebral vessels with the intact BBB that was associated with macrophages infiltration.

The mechanisms underlying glioma progression remain unknown but there is an evidence that the sympathetic nervous system (SNS) via activation of vascular beta2-adrenoreceptors (B2-ADRs) can play an important role in tumor metastasis. Our results clearly show an increase in the expression of vascular B2-ADRs and production of the beta-arrestin-1 — co-factor of B2-ADRs signaling pathway in rats with glioma. Pharmacological blockade of B2-ADRs reduces the BBB disruption, macrophages infiltration, GCs migration and increases survival rate.

These data suggest that the blockade of B2-ADRs may be a novel adjuvant therapeutic strategy to reduce glioma progression and prevent metastasis.

Keywords: Glioma; macrophages; blood–brain barrier; beta-2-adrenoreceptors; beta-arrestin-1.

1. Introduction

Gliomas are lethal forms of brain tumors representing approximately 30% of all neoplasms.[1] The patients with malignant glioma can expect a median survival of only 15 months after diagnosis, less than 5% of patients live longer than five years due to 80% of aggressive glioma recurrence.[2,3] High rates of recurrence, poor treatment response, and dismal survival rates make malignant glioma the dreaded cancer.

Glioma spreads quickly and can colonize the entire brain, sending the tumor invasive cells far beyond the principal tumor mass.[4] Glioma formation is characterized by high density of microvessels that are highly defective with abnormal morphology and disruption of the blood–brain barrier (BBB) permeability.[4,5] Despite the numerous studies on glioma formation, mechanisms responsible for glioma progression and the cancer-related BBB injuries remain poorly understood.

In the past decade, it has become apparent that stress-related activation of sympathetic nervous system (SNS) plays an important role in tumor progression and regulation of cancer vascular micro-environments.[6–8] The clinical research suggests that glioma is often associated with high level of catecholamines and beta blockers improve outcomes of cancer patients.[9–13] The beta-adrenergic receptors transduce catecholamine signals to the G protein, which through a cascade of cellular chemical reactions generates highly specific signals. The beta2-adrenergic receptors (B2-ADRs) and beta-arrestins as co-factors in adrenergic signaling pathway are the most involved in the carcinogenic processes.[14,15]

Activation of the SNS promotes metastasis of tumors by stimulating macrophage infiltration, inflammation and angiogenesis.[6] Macrophages express B2-ADRs and respond to β-adrenergic signaling.[16] The high-level of B2-ADRs expression are associated with tumor-infiltrating macrophages that promote cancer progression via generation of a pro-inflammatory environment, acceleration of angiogenesis and chemoattraction of immune and tumor cells.[17,18]

However, the exact role of SNS and, particularly B2-ADRs, in mechanisms responsible for glioma progression remains unclear and requests further detailed studies. For better understanding of adrenergic mechanisms underlying glioma development, here we analyzed expression of B2-ADRs, production of beta-arrestin-1 (ARRB1) and effects of pharmacological modulation of B2-ADRs on the BBB permeability, macrophages infiltration and cancer cell migration in rats with fluorescent glioma.

2. Materials and Methods

Experiments were carried out on mongrel rats with a weight of 250 g. All procedures were performed in accordance with the "Guide for the Care and Use of

Laboratory Animals". The experimental protocol was approved by the Committee for the Care and Use of Laboratory Animals at Saratov State University (Protocol 7, 07.02.2018).

2.1. Preparation of fluorescent GCs

C6 glioma cells (GCs) were cultured in DMEM growth medium (Paneco, Russia) containing 2.5% embryonic calf serum (Biosera), 4 mm glutamine (Paneco, Russia), penicillin (50 IU/ml) and streptomycin (50 mg/ml) (Paneco, Russia). For removing GCs from the surface of the culture, plastic was used as the solution of Versene (Paneko, Russia) with an addition of 0.25% trypsin (Gibco). The C6 GCs were cultivated in a humid environment in the CO_2 incubator at $37°C$, with a content of 5% CO_2.

To obtain cell lines, stably expressing the protein TagRFP, C6 GCs were transfected with lentiviruses LVTTagRFP (Evrogen, cat. LP001) in case of multiplicity of infection of 10 viral particles per cell. The obtained cell lines were grown to the amount of not less than 10^6 cells and frozen in DMEM growth medium containing 45% embryonic calf serum and 10% DMSO. The expression of the corresponding fluorescent proteins in the obtained cell lines was confirmed by fluorescence microscopy performed in CCU "Simbiosis" IBPPM RAS.

2.2. Implantation of GCs

The GCs (5×10^5 cells per rat) were implanted into the caudate putamen area using stereotaxic apparatus (Narishige, Japan) at the following coordinates: Ap-1 mm; L-3.0 mm; V-4.5 mm, TBS-2.4 mm. The GCs were injected with a Hamilton microsyringe in a volume of 15 μl. After the implantation of GCs, the wound was closed and treated with a 2% solution of brilliant green. The animal was placed in a clean cage. The glioma growth was assessed by the magnetic resonance imaging of the brain on the Clinscan 7T tomograph ("Bruker", Germany) in T2-weighted images.

2.3. Preparation of fluorescent macrophages

Under inhalation anesthesia (2% isoflurane, 70% N_2O and 30% O_2), rats were injected into the abdomen with Eagle medium supplemented with 5% heparin. After massage for 5 min, the abdominal liquid was removed through an incision in the peritoneum, the incision was sutured and the animals recovered. The cell suspension was centrifuged for 1000 g for 10 min. The pellet was resuspended in Hanks solution, the procedure was repeated three times, and counted cells were taken. $1 \cdot 10^6$ peritoneal macrophages were incubated in DMEM + 10% fetal bovine serum with the addition of 20 kDa Conjugate TRITC-dextran, $37°C$, 5% CO_2 for 24 h. The fluorescent macrophages were further centrifuged for 15 min at 3000 g, re-suspended in physiological saline and were administered intravenously to the same animals to avoid immune response.

2.4. Electrophoretic, western blot and immunoassay analysis

Lysates of cerebral microvessels were obtained according to the protocol referred here.[19] The lysates were prepared in a licking buffer (1.5 mm KH_2PO_4, 8 mm Na_2HPO_4 (pH 7.3), 3 mm KCl, 137 mm NaCl and 1% Triton X-100) with a freshly prepared protease inhibitor mixture (Roche Applied Science). The sample was denatured in buffer with sodium dodecyl sulfate, boiling at $95–100°C$ for 5 min.

Electrophoresis was performed on the apparatus for vertical gel electrophoresis in the plates page ($14 \times 12 \times 0,2$ mm), using the system of Laemmli buffer solutions. The separating gel contained a gradient of acrylamide from 6% to 24%, concentrate-4%. Buffer of concentrating gel: 0.125 M TRIS pH 6.8, buffer of separating gel: 0.375 M TRIS pH 8.8. Electrode buffer: 0.960 M glycine, 0.125 M TRIS pH 8.3. All buffers contained 0.1% of SDS. Electrophoresis was performed in the stabilization of the current at 20 A on the plate.[20]

During immunoblotting, electric transfer of separated proteins to nitrocellulose filters ("Sigma", 0.22μm) was carried out. To determine the expression of the protein as a first antibody used to target rabbit primary antibody to B2-ADR (ABCAM, INC. USA, ab182136) at a dilution of 1:200 in PBS with 5% BSA with 0.2% Tween 20 at $4°C$ for the night. The second antibodies were goat anti-rabbit antibodies labeled with horseradish peroxidase (A0545Sigma, Merck, USA). The results were normalized according to actin expression (mouse anti-β-actin, 1:5000, Merck).

Beta-arrestin-1 level was measured in the brain and blood serum in rats by enzyme immunoassay (Beta-arrestin-1, ARRB1, ELISA) according to standard protocol.

2.5. Assessment of the BBB permeability

To evaluate the BBB permeability, we used two tests. The first test was a spectrofluorometric assay of Evans Blue dye (EB) extravasation. The EB (Sigma Chemical Co., St. Louis, USA) was injected in a single bolus dose (2 mg/25 g mouse, 1% solution in physiological 0.9% saline) into the right femoral vein. At the end of circulation time (30 min), mice were decapitated, their brains were rapidly collected and placed on ice. The EB extraction and visualization was performed according to Wang *et al.*[21]

The second test was confocal microscopy of TRITC-dextran 70 kDa (Sigma, St Louis, USA) extravasation.[22] In brief, TRITC-dextran was injected intravenously (4 mg/25 g mouse, 0.5% solution in 0.9% physiological saline) and circulated for 2 min. Afterwards, mice were decapitated and their brains rapidly removed and fixed in 4% paraformaldehyde for 24 h. These brains were cut into 50-μm thick slices on a vibratome (Leica VT 1000S Microsystem, Germany) and analyzed on a onfocal microscope (Leica TCS SP8, Leica, Germany). Approximately, 8–12 slices per animal from cortical and subcortical (excepting hypothalamus and choroid plexus where the BBB is leaky) regions were imaged.

For *in vivo* fluorescence imaging of the BBB permeability, we used FITC-dextran 70 kDa (Sigma, St Louis, USA) intravenous bolus injection (4 mg/25 g mouse, 0.5% solution in 0.9% physiological saline).

The expression of antigens on free-floating sections was evaluated using the standard method of simultaneous combined staining (abcam Protocol). Brain slices were blocked in 150 μl 10% BSA/0,2% Triton X-100/PBS for 2 h, then incubated overnight at 4°C with rabbit anti-rat GFAP (marker of astrocytes) antibody (1:500; Abcam, ab7260, Cambridge, USA), then continued the incubation for 2 h at room temperature. After several rinses in PBS, the slides were incubated for 3 h at room temperature with fluorescent-labeled secondary antibodies on 1% BSA/0,2% Triton X-100/PBS (1:200; Goat anti-Rb IgG-FITC Antibody, Merck,

USA, sc-2012 and Goat anti-Mouse IgM Antibody, Alexa Fluor 488, Merck, USA, Thermo Fisher, A-21042). The confocal microscopy was performed using an onfocal microscope Leica TCS SP8 (Leica, Germany). We used the program Gwyddion for analysis of photos obtained from confocal imaging.

2.6. The histological analysis

All mice were decapitated after the performed experiments. The samples were fixed in 10% buffered neutral formalin. The formalin fixed specimens were embedded in paraffin, sectioned (4 μm) and stained with haematoxylin and eosin. The histological sections were evaluated by light microscopy using the digital image analysis system Mikrovizor medical μVizo-103 (LOMO, Russia).

2.7. Fluorescence microscopy

Fluorescence microscope (Zeiss Imager A1, Germany) with filter sets 49002, 49004, 49019 (Chroma, USA) was used to visualize glioblastoma (excitation 555 nm and 650 nm) and fluorescein into blood vessel. The microscope was equipped with CMOS camera (acA1920-40uc, Basler AG, Germany) and 10 × 0.3 objective lens was used. Anaesthetized animal was positioned at the microscope stage using 3D printed homemade system.

2.8. Protocol 1: Fluorescence and confocal imaging of GCs migration and the BBB disruption

The fluorescence imaging of the BBB permeability and fluorescent glioma spearing were performed in rats from the control group ($n = 10$, 28 days after implantation of GCs). This procedure was carried out under inhalation anesthesia (2% isoflurane at 1L/min N$_2$O/O$_2$ – 70:30). For visualization of the cerebral vessels and the BBB permeability, we used intravenous bolus injection of FITC-dextran. Afterward, the brains of rats were collected for histological analysis of the brain tissues. For confocal imaging of the BBB leakage and glioma migration, we used additional 10 rats with glioma (28 days after implantation of cancer cells). The intravenous injection of TRITC-dextran was used for confocal analysis of the BBB disruption; FITC-dextran — for analysis of glioma migration.

2.9. *Protocol* 2: *Effects of pharmacological modulation of B2-ADRs on GCs migration, the BBB disruption and survival rate*

These experiments were performed using three groups of animals: first group included intact rats with glioma (the control group), which received physiological saline in the same volume as B2-ADRs antagonist/agonist; second and third groups of rats with glioma were treated with isoproterenol (agonist of B2-ADRs, 25 mg/kg/day, p.o., Sigma, St. Louis, MO, USA) and ICI-118551 (specific antagonist of B2-ADRs, 25 mg/kg/day, p.o., Sigma, St. Louis, MO, USA), respectively. The rats used drugs from the first day after GCs implantation.

Three indicated groups were divided on four sub-groups: 3, 7, 10 and 28 days after implantation of GCs for confocal and histological analysis of glioma metastasis. Each sub-group included 10 animals.

We evaluated the survival rate using additional 10 rats from each of the three main groups (control, ICI-118551 and isoproterenol) and allowed them to live till their death naturally.

The BBB permeability to EB albumin complex was evaluated by spectrofluorimetric assay in seven groups: (1) control (healthy rats), (2–5) intact rats in 3, 7, 10 and 28 days after implantation of cancer cells and (6,7) rats treated with isoproterenol (agonist of B2-ADRs, 25 mg/kg/day, p.o., Sigma, St. Louis, MO, USA) and ICI-118551 (specific antagonist of B2-ADRs, 25 mg/kg/day, p.o., Sigma, St. Louis, MO, USA), respectively. In 6 and 7 groups, the brains were taken for the analysis of the BBB permeability 28 days after cancer cells implantation. Each group included 10 animals.

The study of expression of B2-ADRs and level of ARRB1 in both the blood and the brain were performed in the following groups: (1) control (healthy rats); (2) untreated rats with glioma; (3) rats with glioma using ICI-118551; (4) rats with glioma using isoproterenol. We used rats 28 days after GCs implantation ($n = 8$ in each group).

2.10. *Statistical analysis*

The results were reported as mean $\pm$ standard error. The differences from the initial level in the same group were evaluated by the Wilcoxon test. Intergroup differences were evaluated using the Mann–Whitney test and ANOVA-2 (*post hoc* analysis with the Duncan's rank test). Significance levels were set at $p < 0.05$ for all analyses.

3. Results

3.1. *Fluorescence and confocal imaging of glioma migration and the BBB disruption*

In the first step of our work, we analyzed *in vivo* and *ex vivo* GCs migration from the main tumor mass and cerebrovascular changes associated with glioma growth using fluorescence and confocal microscopy, respectively.

Figure 1(a) demonstrates confocal imaging of fluorescent glioma (red color) and the cerebral vessels (green color) surrounding tumor mass. Histological analysis of the brain tissues showed cellular polymorphism, i.e., cells of different size and forms, that is typical sign of glioma (Fig. 1(b)). We observed cancer cells migration in 40% (4 of 10) rats in period between 14 and 28 days after cancer cell implantation. The glioma used the cerebral vessels for spreading from the main tumor mass to the normal brain tissues via the cerebral vessels (Figs. 1(c), 1(d) and 1(g)).

To study the BBB disruption in rats with glioma, we assessed FITC and TRITC-dextran 70 kDa extravasation and fluorescent microphages infiltration from the cerebral vessels into the brain parenchyma. We observed the high BBB permeability to FITC-dextran in the main tumor mass but not within the border of normal and malignant cells. Figure 1(e) shows significant FITC-dextran leakage (green color) in the main tumor mass (red color). However, no FITC-dextran leakage was observed in border between GCs and the normal brain tissues that suggest about the intact BBB (Fig. 1(f)). Figures 2(a)–2(c) demonstrate TRITC-dextran extravasation from the cerebral vessels into the brain parenchyma and its distribution between the astrocytes. These results show that astrocyte-mediated gliovascular coupling was lost in rats with glioma compared with the control group (Figs. 2(d)–2(f)). The increase in the BBB permeability in rats with glioma was associated with macrophages infiltration (Figs. 2(g) and 2(h)).

The survival rate of intact rats (the control group without drug treatment) was 28–35 days after GCs.
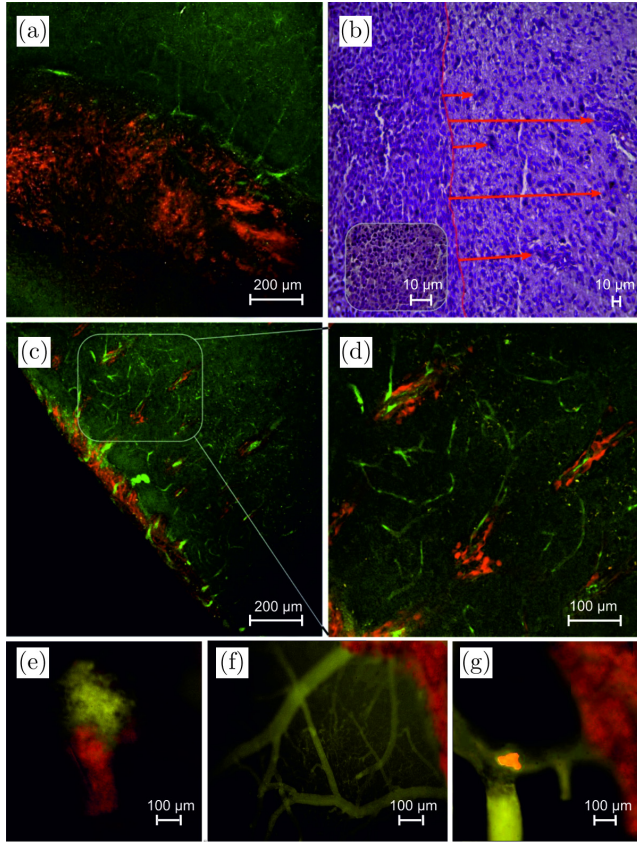
Fig. 1. The analysis of GCs migration and the BBB disruption: (a) confocal imaging of fluorescent glioma (red color) 28 days after GCs implantation; green color-the cerebral vessels filled FITC-dextran 70 kDa; (b) histological analysis of GCs migration (arrowed) from the main tumor mass in the normal brain tissues; figure at high magnification shows polymorphism of cancer cells; (c) GCs migration (circled) from initial tumor in the subcortical area; (d) the same area at high magnification from (c), shows migration of GCs along the cerebral vessels; (e) fluorescence microscopy of significant FITC-dextran (green color) extravasation in the main tumor mass (red color); (f) the intact BBB is impermeable for FITC-dextran that is inside of the cerebral vessels within the border between tumor mass and the normal brain tissues; (g) fluorescence microscopy of GCs (red color) migration via the cerebral vessel filled up by FITC-dextran. For confocal imaging, approximately 10–12 slices per animal ($n = 10$) were imaged.

3.2. Effects of pharmacological modulation of B2-ADRs on GCs migration, the BBB disruption and survival rate

During our first investigation phase, we analyzed the effects of pharmacological blockade and stimulation of B2-ADRs on survival rate and glioma spreading.
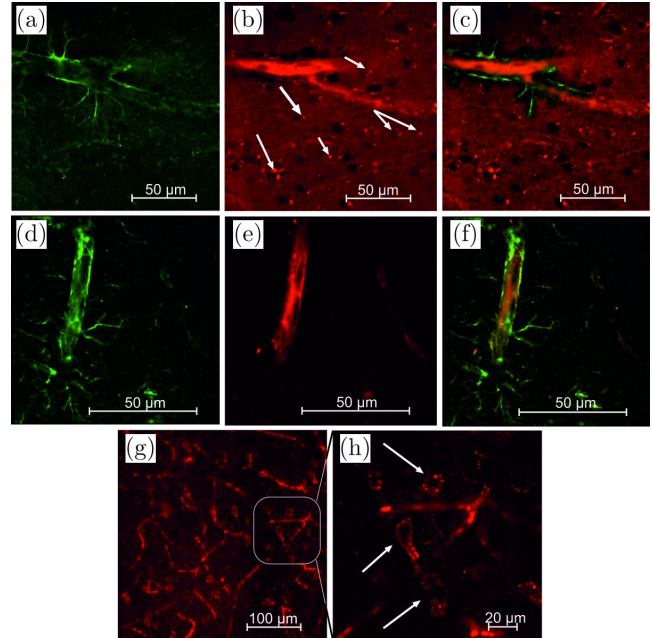

Fig. 2. The BBB disruption and macrophages infiltration in rats with glioma: (a)–(c) confocal imaging of TRITC-dextran extravasation (arrowed) that is observed around the cerebral vessel and astrocytes marked by GFAP (green color) that suggests the BBB leakage for high weight molecules and the loss of astrocyte-mediated gliovascular coupling; (d)–(f) the intact BBB is impermeable for TRITC-dextran that is inside of the cereberal vessel; (g) significant macrophages infiltration in the brain of rats with glioma, (h) the same area at higher magnification (arrowed) from (g). (a) and (d) GFAP; (b) and (e) TRITC-dextran; (c) and (f) merged a/b and d/e, respectively. 10 slices per animal ($n = 10$) were imaged.

All rats (10 of 10) treated with isoproterenol demonstrated metastasis seven days after implantation of GCs. Animals died between 18–22 days after cancer cell implantation, i.e., survival rate in this group was reduced 1.5-fold compared with the control group (see Sec. 3.1).

Blockade of B2-ADRs reduced GCs migration, in this group 20% of rats (2 of 10) demonstrated metastasis that was 2-fold lesser compared with the control (untreated) group. The survival of animals was increased to 43–47 days after the beginning of glioma development that was higher vs. the control group (see Sec. 3.1).

In the next set of experiments, we investigated changes in the BBB permeability to EB albumin complex in different stages of glioma formation without and with pharmacological modulation of B2-ADRs. Figure 3(a) shows gradual increase in EB leakage in 3–7–10–28 days after GCs implantation that suggests progression of the BBB breakdown
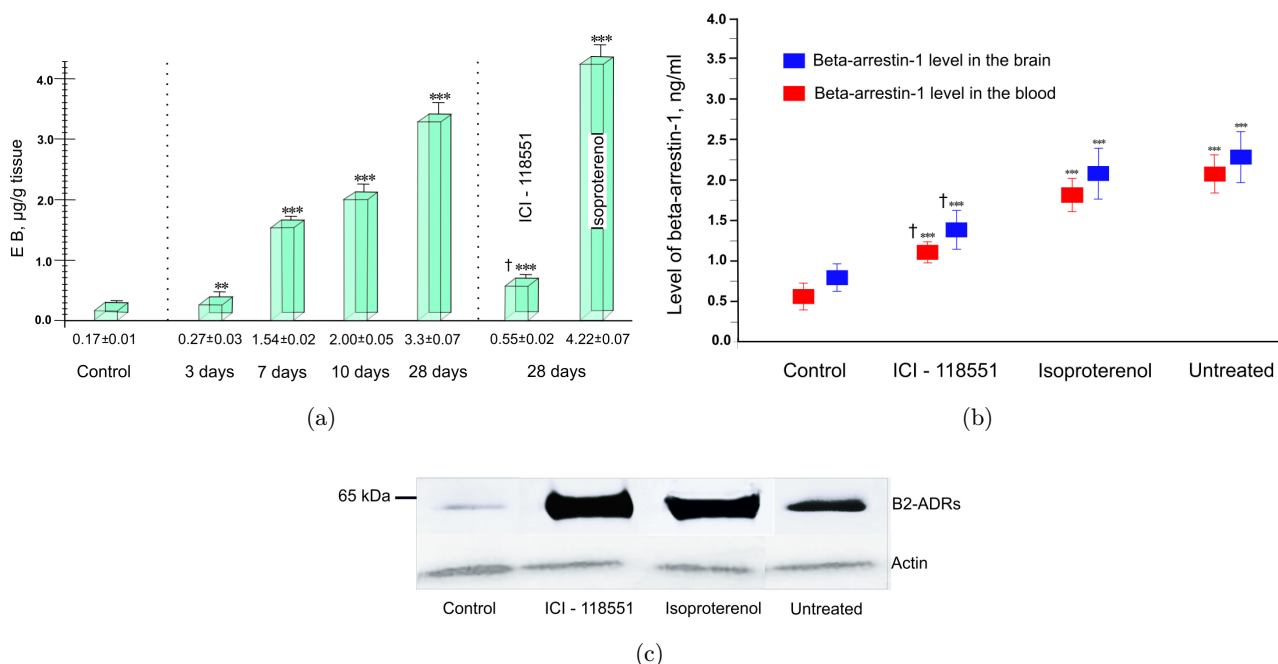
Fig. 3.   The expression of B2-ADRs, production of ARRB1 and the BBB permeability in rats with glioma: (a) The spectro-fluorimetric assay of EB leakage shows gradual increase in the BBB permeability to EB related to the degree of glioma progression. The pharmacological blockage of B2-ADRs by ICI-118551 reduced the BBB permeability to EB, while stimulation of B2-ADRs by isoproterenol caused the opposite effects. (b) The immunoassay analysis shows increase in production of ARRB1 in both blood and brain related to glioma progression. (c) The blotting analysis demonstrated increase in expression of vascular B2-ADRs in rats with glioma (28 days after GCs implantation). Here, ** $-$ $p < 0.01$; *** $-$ $p < 0.001$ vs. the control group; † $-$ $p < 0.05$ vs. untreated group.

with glioma growth in untreated group (without pharmacological modulation of B2-ADRs).

The expression of B2-ADRs and production of ARRB1 – co-factor of B2-ADRs signaling pathways were significantly increased in rats with glioma (28 days after GCs implantation) vs. the control group (Figs. 3(b) and 3(c)).

It is important to note that rats treated with ICH-118551 demonstrated significant reduction of the BBB breakdown compared with the control group, while the rats treated with isoproterenol showed the opposite effects. Indeed, the EB leakage was 2.2-fold ($p < 0.001$) lesser and 1.7-fold higher ($p < 0.001$) in groups using ICH-118551 and iso-proterenol vs. the control group, respectively (Fig. 3(a)). The ICH-118551 reduced hyperexpression of B2-ADRs and production of ARRB1 in rats with glioma. However, in this group expression of B2-ADRs and synthesis of ARRB1 remained to be higher vs. the control (healthy) group (Figs. 3(b) and 3(c)). The treatment with isoproterenol was not accompanied by significant changes in the expression of B2-ADRs and production of ARRB1 in rats with glioma (Figs. 3(b) and 3(c)).

4.   Discussion

Taken together, experimental data of this study demonstrate an important role of B2-ADRs in adrenergic mechanisms underlying glioma progression. The blockade of B2-ADRs by ICI-118551 reduced GC migration and increased survival rate of rats vs. the control group. The stimulation of B2-ADRs by isoproterenol caused the opposite effects to that associated with early migration of GCs and decrease in survival of animals compared with rats from the control group. The role of B2-ADRs in the pathogenesis of various types of cancer and the possible application of B2-ADRs-antagonists in antitumor therapy has been discussed in many reviews during the last decade.[6–8] Catecholamines and isoproterenol (B2-ADRs agonist) increase proliferation, migration and invasion of different cancer cells[23–26] and oncological patients often have high level catecholamines.[9] Anti-tumor effects of propranolol (non-selective blocker of B2-ADRs) had been shown for many cancer cell lines such as gastric, pancreatic, breast, leukemia, head and neck carcinoma.[27–31] The clinical epidemiological studies

suggest that therapy with beta-blockers improves outcomes.[10–13]

We found that glioma progression was associated with increase in expression of vascular B2-ADRs and level of ARRB1 in both blood and brain. The high expression of B2-ADRs also has been demonstrated in pediatric malignant brain tumors.[32] Hara *et al.* recently demonstrated that stress-induced catecholamines activate B2-ADRs-related DNA damage and production of ARRB1 as well as protein kinase, which phosphorylates HDM2, resulting in p53 degradation.[33] Wolter *et al.*, using human neuroblastoma cell lines, showed high expression of B2-ADRs in tumor and that inhibition of B2-ADRs by propranolol reduces viability of cancer cells via induction of apoptosis due to decrease in levels of the phosphorylated form of HDM2 and increase in levels of pro-apoptotic p53 family proteins.[34] In this work, authors also used specific beta-antagonists to demonstrate that B2-ADRs and not B1-ADRs blockade causes anti-tumor effects.

Taking into account all these facts, we assumed that activation of B2-ADRs-ARRB1-signaling pathways is one of the crucial mechanisms responsible for glioma progression and vascular abnormalities in tumor tissues.

Our results show that the degree of glioma progression was closely associated with the gradual BBB breakdown and that tumor migrated via the cerebral vessels. The experiments *in vitro* using rodent glioma cell lines also demonstrate that GCs travelling from the main mass of tumor through blood vessels and cancer cells migrate between endothelial cells and astrocyte endfeet.[35–37] This is associated with loss of astrocyte mediated gliovascular coupling and tight junctions thereby resulting in a focal breach in the BBB.[4,5,38] Even singly GCs are sufficient to locally open the BBB and disrupt gliovascular coupling.[4]

The blockade of B2-ADRs significantly reduced the BBB disruption that was associated with improving of survival rate. The mechanisms underlying this phenomenon remain unclear but there is evidence that glioma growth is accompanied by Ca^{2+}-dependent release of K^+ from endothelial cells and astrocytes via activation of G-protein-coupled receptors causing dilation of cerebral vessels.[4] The activation of B2-ADRs induces adrenergic vasorelaxation via stimulation of Ca^{2+}-activated potassium channels resulting in hyperpolarization of endothelial cells membrane.[39–41] We assume that the blockade of B2-ADRs protect the microenvironment of glioma due to prevention of destabilization of molecular mechanisms responsible for control of vascular tone through Ca^{2+}-dependent release of K^+. The fact that stimulation of B2-ADRs by isoproterenol aggravated the BBB disruption that was associated with early GCs migration confirms our hypothesis.

5. Conclusion

Collectively, our results clearly show that glioma progression is associated with the gradual BBB breakdown, increase in expression of vascular B2-ADRs and production of ARRB1-co-factor of B2-ADRs signaling pathway. Pharmacological blockade of B2-ADRs reduces the BBB disruption, B2-ADRs activation and increases survival rate. These data suggest that blockade of B2-ADRs-ARRB1 signaling pathways may improve the efficacy of traditional therapy in patients with glioma.

Conflict of Interest

The authors declare that there are no conflicts of interest related to this paper.

Acknowledgments

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