ЭКСПРЕССИЯ ГЕНОВ *PD-L1* И *PD-L2* В КЛЕТКАХ ГЛИОБЛАСТОМ ЧЕЛОВЕКА, РЕЗИСТЕНТНЫХ К ХИМИО- И ЛУЧЕВОЙ ТЕРАПИИ

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Резюме. Мембранные молекулы PD-L1 и PD-L2, лиганды рецептора PD1 Т-лимфоцитов, выполняют иммунорегуляторные функции. Их связывание с рецептором приводит к ингибированию пролиферации, снижению продукции цитокинов, цитотоксической реакции и апоптозу Т-лимфоцитов. Клетки многих опухолей, вне зависимости от гистогенеза, экспрессируют молекулы PD-L1, тем самым ограничивая развитие противоопухолевой иммунной реакции. Глиобластомы – высоко злокачественные рецидивирующие опухоли центральной нервной системы. Основным источником рецидивов глиобластом служат резистентные опухолевые клетки, имеющиеся исходно в гетерогенных по клеточному составу глиомах, а также формирующиеся в процессе терапии. Увеличение дозы цитостатиков или облучения при терапии рецидивов оказывается не эффективным. В отношении ряда опухолей, в том числе рака яичников и немелкоклеточного рака легкого, показано, что препараты, предотвращающие взаимодействие PD-L1/PD1, оказываются эффективными при лечении новообразований, резистентных к химио- и радиотерапии. Иммунотерапию, в частности с использованием препаратов, ингибирующих связывание молекул PD-L с рецептором, рассматривают в качестве способа преодоления резистентности глиобластом к терапии. Цель работы состояла в оценке уровня экспрессии генов *PD-L1* и *PD-L2* в резистентных клетках глиобластом линий A172, R1, T2 и T98G, возобновивших пролиферацию после действия максимальных для каждой линии сублетальных доз цитостатиков (фотемустина и темозоломида), а также фракционированного или одноразового гамма-облучения. Линия A172 относится к числу глиобластом, высокочувствительных к использованным воздействиям, T98G – высокорезистентная линия; линии R1 и T2 занимают в этом ряду промежуточное положение. В интактных клетках глиобластом A172, R1 и T2 уровень экспрессии генов

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PD-L1 и *PD-L2* был одинаково высоким, в клетках T98G он был достоверно меньшим. Воздействие цитостатиков или облучения на глиобластомы линий A172 и R1 существенно не изменяло экспрессии генов *PD-L1* и *PD-L2*, свойственной интактным клеткам. В клетках глиобластомы T2, и в особенности в клетках T98G, выявлено значительное увеличение уровня экспрессии этих генов, наиболее выраженное для гена *PD-L2*. Это усиление экспрессии может свидетельствовать об увеличенной злокачественности резистентных клеток T2 и T98G. Высокая экспрессия генов, отвечающих за продукцию PD-L1 и PD-L2, ограничивающих цитотоксическую реакцию организма против опухолевых клеток, является предпосылкой для использования лекарственных препаратов, мишенями которых являются эти белки, для элиминации резистентных к терапии клеток при глиобластомах.

Ключевые слова: глиобластомы, PD-L1, PD-L2, резистентные клетки, фотемустин, темозоломид, Гамма-нож, фракционированное облучение, A172, T98G, T2, R1

PD-L1 AND *PD-L2* GENE EXPRESSION IN HUMAN GLIOBLASTOMA CELLS RESISTANT TO CHEMO- AND RADIOTHERAPY

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Abstract. Membrane molecules PD-L1 and PD-L2, ligands of T lymphocytes PD1 receptor, perform immunoregulatory functions. Their binding to the receptor leads to inhibition of proliferation, reduction of cytokine production, cytotoxic response, and apoptosis of T lymphocytes. The cells of many tumors, regardless of their histogenesis, express PD-L1 molecules, thus limiting the development of an anti-tumor immune response. Glioblastomas are highly malignant recurrent tumors of the central nervous system. The main sources of glioblastoma recurrence are resistant tumor cells initially present in gliomas with heterogeneous cellular composition, as well as resistant cells that are formed during therapy. Increasing the dose of cytostatic drugs or radiation during relapse therapy is not effective in glioblastomas. It has been shown for a number of tumors, including ovarian cancer and non-small cell lung cancer, that drugs preventing PD-L1/PD1 interaction are effective in the treatment of neoplasms resistant to chemo- and radiotherapy. Immunotherapy using drugs that inhibit the binding of PD-L molecules to their receptor is considered as a way to overcome the resistance of glioblastomas to therapy. The aim of this work was to assess the level of *PD-L1* and *PD-L2* gene expression in resistant glioblastoma cells lines A172, R1, T2 and T98G, which resumed proliferation after exposure to the maximum for each line, sublethal doses of cytostatic drugs (fotemustine and temozolomide), fractionated or single gamma irradiation. A172 line belongs to glioblastomas that are highly sensitive to these influences, T98G is a highly resistant cell line, while R1 and T2 lines occupy an intermediate position. In intact glioblastoma A172, R1, and T2 cells the level of *PD-L1* and *PD-L2* gene expression was equally high, while in T98G cells it was significantly lower. Exposure of A172 and R1 glioblastoma lines to cytostatic drugs or irradiation did not significantly change the level of *PD-L1* and *PD-L2* genes expression typical for intact cells. In T2 glioblastoma cells, and especially in T98G cells, a significant increase in expression of these genes was found, most pronounced for *PD-L2* gene. This increase in expression may indicate an enhanced malignancy of resistant T2 and T98G cells. High expression of the genes responsible for the production of PD-L1 and PD-L2, which limit the cytotoxic response against tumor cells, is a prerequisite for the use of drugs targeted against PD-L1 and PD-L2 for the elimination of resistant cells in glioblastoma.

Keywords: glioblastoma, PD-L1, PD-L2, resistant cells, fotemustine, temozolomide, Gamma Knife, fractionated irradiation, A172, T98G, T2, R1

The work was performed according to Government Order "Study of resistant tumor cells on glioblastoma cultures in the simulation of stereotactic radiosurgery of recurrent glioblastoma" at the A. Granov Russian Research Center for Radiology and Surgical Technologies (St. Petersburg, Russia).

Introduction

Membrane molecules PD-L1 and PD-L2, ligands of T lymphocytes PD1 receptor, perform immunoregulatory functions. Binding of these molecules to the receptor leads to proliferation inhibition, reduction of cytokine production, cytotoxic response, and apoptosis of T lymphocytes. Many tumor cells, regardless of histogenesis, express PD-L1 molecules, thus restricting the development of an anti-tumor immune response. In addition to PD1, PD-L1 molecules bind to costimulatory molecules CD28, CD80, and CTLA-4. PD-L2 expression in tumors is detected much less frequently than PD-L1 expression, and PD-L2 molecules are only able to bind to PD1. Preventing PD-L1 and PD-L2 from interacting with their receptors can abolish T cell unresponsiveness to tumor antigens.

Glioblastomas are highly malignant tumors of the central nervous system. Despite the ongoing aggressive treatment, including surgical removal of the tumor and chemoradiotherapy, glioblastomas recur, the prognosis remains unfavorable, and the life expectancy of patients is measured in months. The main source of glioblastoma recurrence are resistant tumor cells initially present in gliomas that are heterogeneous in cell composition, as well as tumor cells whose resistance is formed during therapy. Increasing the doses of radiation or cytostatic drugs can't help to overcome the resistance of such glioma cells. Alternative ways of elimination of tumor cells resistant to chemoradiotherapy are being developed. For a long time, it was believed that the central nervous system was an immunologically privileged system. However, the discovery of the antigen-presenting function of microglia, possible ways of immune cell penetration through the blood-brain barrier, and increased vessel permeability in tumors changed the primary understanding. The issue of glioblastoma immunotherapy possibilities came up for discussion [11].

Most glioblastomas, especially of the mesenchymal subtype, are known to express PD-L1 [2, 7, 10]. Little information is available about the presence of PD-L2 in glioblastomas. However, a high expression level of this biomarker has been shown to correlate with an unfavorable prognosis [9]. High expression of PD-L1 and PD-L2 on tumor cells is a prerequisite for the use of drugs directed against PD1 ligands [1]. There are single indications that temozolomide-resistant glioblastoma cells have a high level of PD-L1 expression [8]. At the same time, there are no data about the level of expression of these target molecules in resistant cells of different glioblastoma lines that resume proliferation after cytostatic treatment, fractionated or single dose gamma irradiation.

The aim of the present work was to assess the expression levels of *PD-L1* and *PD-L2* genes in human glioblastoma cells resistant to chemotherapy and radiation therapy.

Materials and methods

Glioblastoma A172, R1, T2 and T98G cell lines with different properties, including various responses to irradiation and chemotherapy, were selected as research objects [3, 4]. Cells were cultured in plastic plates or ventilated vials at +37 °C, 6% CO_2, and 100% humidity, in α-MEM growth medium containing 5% fetal calf serum. Trypsin-EDTA solution was used for cell detachment.

Glioblastoma cells were treated with 0.1-5 mM temozolomide (Temodal pharmaceutical) for 24 h in growth medium, washed twice and cultured for 56 days under normal conditions. Every 3-4 days, half of the growth medium was replaced.

Glioblastoma cells were also treated with fotemustine (Mustophoran pharmaceutical) at drug concentrations of 0.5-750 μg/mL. Cells were incubated for 1 h in serum-free α-MEM medium containing fotemustine, washed three times, and cultured for two months more in standard growth medium.

Fractionated irradiation was performed on an Elekta Precise Treatment System™ Linear Accelerator (6 MeV, 460 cGy/min dose rate). Cells were irradiated with a single dose of 2 or 3 Gy/day or twice with 30 Gy, until a total dose of 36 or 51 Gy was reached. After irradiation, cells were cultured for two months.

Single stereotactic irradiation of glioblastomas with doses of 6-16 Gy was performed on a Leksell Gamma Knife® with 201-focused ^{60}Co radiation source at a dose rate of 3.236 Gy/min, 1.17 MeV energy, using a specially designed device for fixation and positioning of cell cultures during precision irradiation (patent for invention No. 2778859). The cells were then cultured for one month under normal conditions and their number was counted weekly.

Gene activity was studied by real-time polymerase chain reaction as described previously [4]. *PD-L1* gene was amplified using direct primers: TGGCATTTGCTGAACGCATTT and reverse primers: TGCAGCCAGGTCGTAATTGTTTT. The nucleotide sequences used for *PD-L2* amplification were ACCGTGAAAGAGCCCCACTTTG (forward) and GCGACCCCATAGATGATTATGC (reverse). The level of gene expression in intact cells was represented as the difference (ΔCt) between the threshold cycle of the studied gene and the compa-

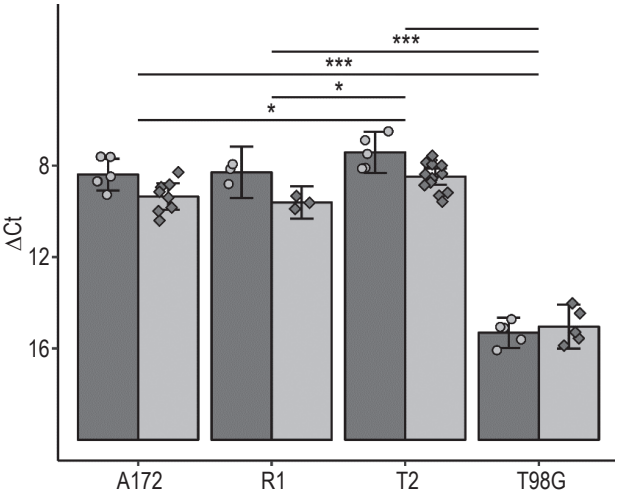

Figure 1. *PD-L1* and *PD-L2* gene expression in intact glioblastoma cells

Note. Horizontally – glioblastoma lines. Vertically – ΔCt, difference between threshold cycle of the studied gene and *GAPDH* housekeeping gene. Dark columns represent *PD-L1*, light columns represent *PD-L2*. Dots indicate individual values; bars denote the 95% confidence intervals. *, differences are significant at $p < 0.05$; ***, differences are significant at $p < 0.001$.

rison gene *GAPDH*. ΔΔCt value, the difference in the gene expression level in resistant and intact cells, served as an indicator of the difference in gene expression in the cells before and after the exposures. R language (version 4.1.2) was used for data processing and visualization. Statistical analysis was performed by creating linear regression models.

Results and discussion

PD-L1 and *PD-L2* gene expression was detected in all four tumor lines, which confirms the previous data about the activity of these genes in glioblastomas [9, 10]. In A172, R1 and T2 glioblastomas the level of *PD-L1* and *PD-L2* gene expression was equally high. In T98G, despite being considered the most aggressive and resistant to chemotherapy or radiation cell line, the level of *PD-L1* and *PD-L2* gene expression was significantly lower than in the other three lines (Figure 1) [5].

To date, there is no consensus which cells are considered resistant, and that causes certain difficulties when comparing data from different publications, especially in cases when the method of obtaining resistant cells is not specified [6].

In this work, the resistant A172, R1, T2, and T98G glioblastoma cells obtained in our laboratory earlier were examined. These cells were descendants of single cells that recovered their proliferative capacity after exposure to the highest sublethal doses of chemotherapeutic drugs or irradiation. The doses of exposures used to produce resistant cells (Table 1)

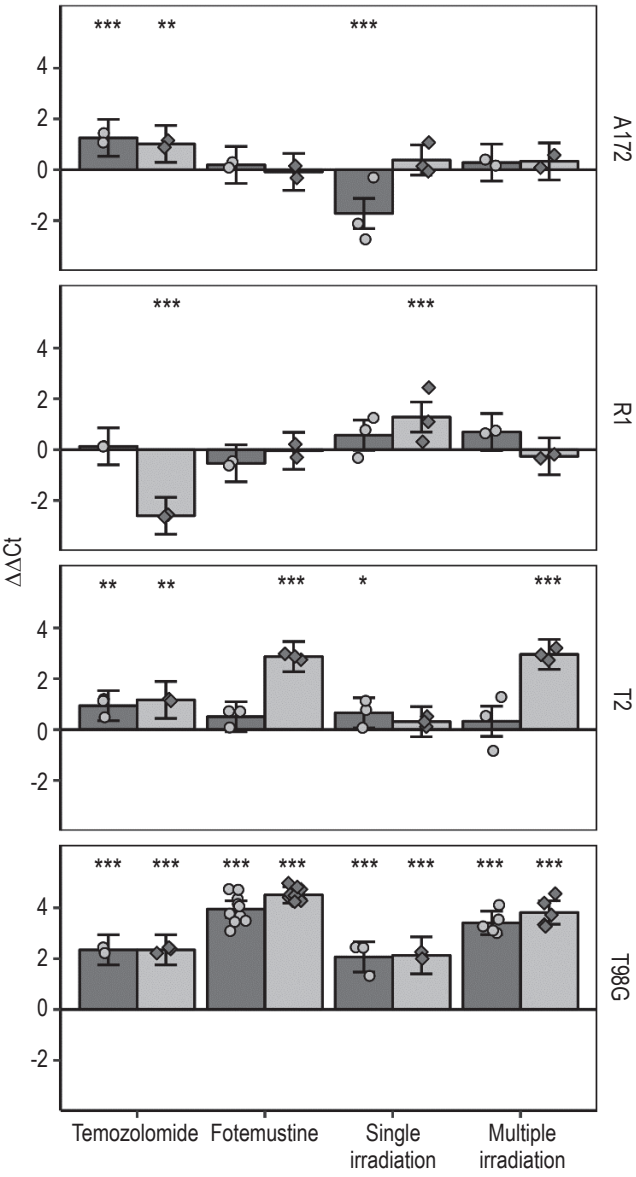

Figure 2. Changes in *PD-L1* and *PD-L2* gene expression in A172, R1, T2, and T98G glioblastoma cells resistant to chemotherapy and irradiation.

Note. Horizontally – effect on the cells, vertically – ΔΔCt, the difference in the gene expression level in resistant and intact cells. Horizontal lines correspond to the level of gene expression in intact cells. Dots indicate individual values; bars denote the 95% confidence intervals. *, differences are significant at $p < 0.05$; **, differences are significant at $p < 0.01$; ***, differences are significant at $p < 0.001$.

varied significantly, and were determined by the individual sensitivity of each glioblastoma cell line.

In resistant populations of A172 and R1 glioblastoma lines, which are among the more sensitive to the action of cytostatic drugs and irradiation, changes in *PD-L1* and *PD-L2* gene activity proved to be minimal. Resistant A172 cells that underwent single Gamma Knife irradiation had decreased *PD-L1* gene activity compared to intact cells, and proliferating cells after temozolomide action had slightly increased

TABLE 1. DOSES OF EXPOSURE TO CHEMOTHERAPY OR RADIATION AT WHICH RESISTANT CELL POPULATIONS WERE ISOLATED

Exposure	Cell lines			
	A172	R1	T2	T98G
Temozolomide, mM	0,1	1	1	5
Fotemustine, μg/mL	100	100	300	300
Fractionated irradiation, Gy	50 (2)*	36 (2)	51 (3)	60 (30)
Single irradiation with Gamma Knife, Gy	9	11	12	14

Note. *, total dose of fractionated irradiation; single dose of irradiation in brackets.

activity of the same gene. Some changes in *PD-L2* gene expression were detected in resistant R1 cells. *PD-L2* gene activity decreased in R1 cells exposed to temozolomide and slightly increased after a single irradiation. In T2 and T98G lines, which are considered to be more resistant to chemo- and radiotherapy and differ from A172 and R1 lines in their ability to form "dormant" polyploid cells, the resistant proliferating cells showed increased expression of the studied genes [5]. In T2 glioblastoma the increase in activity affected predominantly *PD-L2* gene, while in T98G line there was a significant increase in the expression of both genes (Figure 2).

Conclusion

The results indicate that glioblastoma cells resistant to alkylating antitumor drugs and irradiation actively express *PD-L1* and *PD-L2* genes. High levels of PD-L1 and PD-L2 molecules are unfavorable prognostic factors. At the same time, this indicator allows to consider immunotherapy with drugs that block PD-L1 and PD-L2 binding to their receptor as a possible means of elimination of therapy-resistant tumor cells [7, 8]. Several antitumor drugs with therapeutic effect based on inhibition of PD-L1 ligand binding to the receptors have been registered. These include Atezolizumab, Durvalumab, and Avelumab. All of these monoclonal antibody-based drugs block the interaction between PD-L1 and its receptors (PD1 and B7), but do not affect the interaction between PD1 and PD-L2.

For several other tumors, such as urothelial carcinoma, ovarian cancer, and non-small cell lung cancer, drugs that inhibit PD-L1/PD1 interaction have been shown to be effective in the treatment of neoplasms resistant to chemotherapy and radiotherapy. High expression of the genes responsible for the production of PD-L1 and PD-L2, which limit the cytotoxic response against tumor cells, is a prerequisite for the use of drugs targeted against PD-L1 and PD-L2 for the elimination of resistant cells in glioblastoma.

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