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ANTICANCER EFFICACY OF ALLOGENEIC VACCINE MODIFIED WITH PROGENITOR NEURAL CELLS SUPERNATANT IN RATS WITH GLIOMA 101.8

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The *aim* of the work was to investigate the antitumor efficacy of allogeneic tumor vaccine (ATV) modified with rat progenitor neural cell supernatant (RPNS) in rats with glioma 101.8. *Materials and Methods*: The study was performed on 74 white random-bred rats. ATV was developed on the basis of glioma 101.8 cell suspension modified with RPNS (0.02 or 0.10 mg/ml). RPNS was prepared from suspension made from whole rat brain tissue on 14th (E14) day of gestation. Model of brain glioma 101.8 was reproduced by intracerebral injection of glioma 101.8 cell suspension. ATV was injected intraperitoneally in a volume of 0.2 ml per animal once on the 10th day after tumor transplantation. For survival analysis Kaplan — Mayer multiple assessments method was used. Cytotoxic activity of rat lymphocytes (effector cells) was evaluated in MTT-colorimetric test by determining the state of mitochondrial dehydrogenase enzymes in target cells (allogeneic glioma 101.8 cells). *Results*: Intraperitoneal administration of ATV modified with 0.10 mg/ml RPNS significantly increased mean survival time and median survival of glioma-bearing rats compared with unvaccinated group (MST (19.9 ± 2.4), 21.4 days; *versus* MST (14.6 ± 2.8); 14 days; $p = 0.0002$, Gehan's — Wilcoxon test). Intraperitoneal administration of ATV modified with 0.10 mg/ml RPNS resulted in increased cytotoxic activity of immune cells of rats with glioma *in vitro* compared with this index in unvaccinated group ($p = 0.026$, U-Mann — Whitney test). *Conclusion*: Antitumor effect of vaccination with RPNS-modified ATV is realized *via* increased cytotoxic activity of immune cells, what could be used for further optimization of whole tumor cell vaccine.

Key Words: **allogeneic tumor vaccine, rat progenitor neural cells supernatant, glioma 101.8, mean survival time, lymphocyte cytotoxic function.**

Despite substantial progress in the development of modern methods of tumor treatment, the therapy of cancer patients, particularly patients with brain gliomas remains of low effectiveness.

Gliomas are the most common primary brain tumors, which are characterized by infiltrative growth and resistance to therapy. Patients with glioma have a poor prognosis. For immune recognition and elimination of tumor cells realization of a series of steps is necessary that are implemented by innate and adaptive arms of immune system, but in gliomas, especially malignant ones, several mechanisms are utilized to avoid the control by the immune system. These mechanisms include: 1) reducing the number and function of immune cells, along with an increase in the number of T-regulatory $CD4^+CD25^+FoxP3^+$ cells producing immunoinhibitory mediators [1–4]; 2) weakening of the immune response due to the formation of immunosuppressive tumor environment through the secretion of prostaglandin E2, IL-10, TGF-β, gangliosides [5]; 3) loss of expression of the classical major histocompatibility complex molecules (MHC), along with the appearance of expression of nonclassical molecules (HLA-G, -E) with immunomodulating properties [6–10].

Development of new approaches that will overcome mechanisms by which tumor avoid the control of the immune system and tumor suppressive effects on immunocompetent cells, is an important task. Advances in molecular biology of gliomas are quickly translated into innovative clinical trials of new methods of therapy, based on in-depth genomic, epigenetic, transcriptional and proteomic characteristics of tumor and factors of brain microenvironment interaction with the immune system. Along with the use of radiation, cryotherapy, chemotherapy, photodynamic therapy, oncolytic viruses etc. among novel approaches there are considered: 1) the use of facilities that enhance antigen presentation (targeted agents, vaccines, agonists of Toll-like receptors, type I interferons); 2) therapies targeting the growth factor receptors and adjoint downstream signaling pathways expressed by tumor, angiogenesis, modulation of tumor stem cells (TSC), the regulation of the cell cycle; 3) the use of agents aimed at reversal T-cell dysfunction (inhibitors of the "immune checkpoints" — an antibody against an cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death receptor 1 (PD-1)); 4) the use of agents aimed at other immunoinhibitory mechanisms (inhibitors of indolamine-dioxygenase, regulatory T cells, myeloid suppressor cells) [11, 12].

Immunotherapy of malignant gliomas using the innate ability of the immune system to recognize and destroy malignant cells is considered a promising as provides highly specific and less toxic therapy compared with conventional chemotherapy [4, 11].

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Abbreviations used: ATV – allogeneic tumor vaccine; CI – cytotoxic index; CTLs – cytotoxic lymphocytes; HLA – human leucocyte antigens; MHC – major histocompatibility complex; MST – mean survival time; MTT – 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide; NK – natural killer; NPC – neural progenitor cells; NSC – neural stem cells; RPNS – rat progenitor neural cells supernatant; TSC – tumor stem cells.

Immunotherapy of tumors includes the following approaches: the use of dendritic cells, adoptive T-cell therapy, anti-tumor antibodies, tumor vaccines, blockade of immune checkpoints [13]. Immunotherapy of gliomas is based mainly on active immunization against tumor antigens, that is an introduction of immunogen to generate response in tumor-carrier in order to eliminate malignant cells [14]. In recent years, new approaches for cancer vaccine generation are developed, based on the use of whole tumor cells or specific antigens (peptide vaccines) which are evaluated in clinical trials [15, 16].

Immunotherapy based on whole tumor cells vaccines is considered to be perspective [17]. In experimental and clinical studies the effectiveness of tumor autovaccine [18] and xenogeneic analogs of endogenous molecules has been demonstrated; such immunization is capable to overcome immune tolerance to tumor antigens and leads to a significant inhibition of tumor development [19]. The basis for the new biotherapeutic approach — design of the effective oncofetal tumor vaccine — is the use of embryonic and fetal tissues [20, 21]. Cellular and molecular similarities between TSC of brain tumors and normal neural stem cells (NSC) justify the development of treatment strategies using NSC and neurogenic progenitor cells (NPC) in malignant gliomas [22–24].

The aim of the work was to investigate the antitumor efficacy of allogeneic tumor vaccine (ATV) modified by rat progenitor neural cells supernatant (RPNS) in rats with glioma 101.8.

MATERIALS AND METHODS

The study was performed on white random-bred male rats (n = 74, weight 120 ± 10 g bred in the vivarium of SI "A.P. Romodanov Institute of Neurosurgery of the NAMS of Ukraine" ("INH NAMS")). The use and care of experimental animals have been performed in compliance with law of Ukraine "On protection of animals from cruelty", "European Convention for the protection of vertebrate animals used for experimental and other scientific purposes", in accordance with the principles of bioethics and biosafety regulations and were approved by the Committee on Bioethics Control of "INH NAMS". The animals were kept under standard vivarium conditions, anesthesia and euthanasia was performed under ether narcosis.

Animals were grouped as following: 1) rats with glioma 101.8 (n = 38); 2) rats with glioma 101.8 treated by administration of ATV modified with RPNS at a concentration of 0.02 mg/ml (n = 12); 3) rats with glioma 101.8 treated by administration of ATV modified with RPNS at a concentration of 0.10 mg/ml (n = 12); 4) intact rats (control, n = 12).

The model of brain glioma was reproduced by intracerebral injection of 0.02 ml ($3.5 \cdot 10^5$) glioma 101.8 cell suspension (Russian Collection of Cell Cultures, Institute of Human Morphology of Russian Academy of Sciences, Moscow, Russian Federation) in the left hemisphere of the rat brain to a depth of 1.5–2.0 mm. Strain 101.8 is anaplastic glioma in which astrocytes, oligodendrocytes and ependyma simultaneously have undergone malignization, and its histobiological properties are close to the human malignant gliomas [25].

RPNS was prepared from a suspension of neurogenic rat brain cells on 14th (E14) day of gestation. Native rat brain tissue was released from envelopes in buffered saline, transferred to DMEM medium (Sigma, Germany) and suspended by repeated pipetting. Cells were precipitated by centrifugation for 5 min at 1500 rpm, washed in DMEM, and resuspended in fresh DMEM. The viability of cells in suspension was determined in a standard test with 0.2% trypan blue (Merck, Germany). The concentration of cells was adjusted to $6.0 \cdot 10^6$/ml, concanavalin A (0.10 mg/ml) was added to the resulting cell suspension, and the cells were incubated for 2 h in a CO_2-incubator at 37.0 °C, constant humidity of 95% and 5% CO_2. After incubation, the cells were precipitated by centrifugation for 5 min at 1500 rpm, washed in medium DMEM, resuspended in fresh DMEM medium and incubated at the same conditions for 24 h. After incubation, the cells were re-precipitated by centrifugation for 5 min at 1500 rpm, and the supernatant was collected. Protein concentration in supernatant was determined by the method of Lowry. RPNS was standardized to a concentration of 1.0 mg/ml, and stored in aliquots at −20.0 °C.

ATV was developed on the basis of glioma 101.8 cell suspension, modified with RPNS (0.02 or 0.10 mg/ml). RPNS (0.02 or 0.10 mg/ml) was added to a suspension of fresh-isolated tumor cells ($2.0 \cdot 10^6$), and then the suspension in the volume of 2 ml was incubated in biologically inert glass centrifuge tubes with periodic shaking for 24 h in a CO_2-incubator at a temperature of 37.0 °C, constant humidity of 95% and 5% CO_2. We have previously shown that incubation with RPNS has cytotoxic and antiproliferative effects on glioma 101.8 cells (cytotoxic index was 68–86%) [26]. After incubation, the cells were precipitated by centrifugation for 5 min at 1500 rpm, washed in DMEM and resuspended in fresh DMEM.

ATV was injected intraperitoneally in a volume of 0.2 ml ($2.8 \cdot 10^5$) per animal once on the 10th day after tumor transplantation.

As a source of immune cells (lymphocytes) spleens of experimental animals were used and examined at the peak of clinical manifestations of glioma 101.8 (17th day after tumor transplantation). The animals were anesthetized, the spleens were removed, splenocyte suspension was prepared by mechanical homogenization in RPMI medium and filtered through a nylon filter. Lymphocytes were isolated by centrifugation in fikoll-verografin gradient (ρ = 1.077) at 1500 rpm for 30 min, then washed twice in buffered saline pH 7.2–7.4. Cell viability was determined by the permeability of the plasma membrane for 0.2% trypan blue (Merck, Germany). The obtained cells were examined in MTT-colorimetric test by determining the state of mitochondrial dehydrogenase enzymes in target cells.

Study of lymphocyte cytotoxic activity in MTT-colorimetric test was conducted by the protocol [27]. Lymphocytes of animals from experimental groups were used as effector cells ($5 \cdot 10^7$/ml); glioma 101.8 cells were used as target cells ($1 \cdot 10^7$/ml). In previously studies using effector-target ratio of 20:1, 10:1, 5:1, it was found that the optimal ratio was 5:1, which was used in this study. The test was performed in the triplets. Cytotoxic activity of lymphocytes was represented by cytotoxic index (CI) expressed in percent:

$$CI = 100 - (OD_{e+t} - OD_e \cdot 100) / OD_t \%,$$

where OD_{e+t} — optical density values in wells with effector + target;

OD_e — optical density values in wells with effectors;

OD_t — optical density values in wells with targets.

Statistical analysis of data was performed using the statistical software package "Statistica 6.0" (Software StatSoft Inc., 2003). For survival analysis Kaplan — Mayer multiple assessments method with Gehan's — Wilcoxon criteria was used, which allows to assess animal survival, regardless of whether it is full or censored sampling. For statistical analysis of cytotoxic activity indexes ANOVA by Ranks Kruskal — Wallis test was used for comparison of multiple independent groups, and U-Mann — Whitney test for paired comparison of groups. Data are presented as M ± m (mean ± SE). $p < 0.05$ was considered as statistically significant difference, $p < 0.01$ as highly statistically significant difference.

RESULTS AND DISCUSSION

The effectiveness of the administration of RPNS-modified ATV in experimental animals with glioma 101.8 was determined using survival analysis. Application of Kaplan — Mayer multiple assessments method with Gehan's — Wilcoxon criteria has shown that survival rates of animals with glioma 101.8 and vaccinated animals were significantly different (criteria χ^2, $p = 0.0001$). The pairwise comparison of groups using Gehan's — Wilcoxon two-sampling criterion revealed the following differences.

Intraperitoneal administration of ATV modified with RPNS (0.02 mg/ml) in animals with glioma 101.8 slightly increased the mean survival time (MST) and median survival of tumor-bearing rats (MST (16.4 ± 1.9), 15.5 days compared with MST (14.6 ± 2.8); 14 days; $p = 0.147$) (Table 1, Figure). In contrast, these indices in rats with glioma 101.8 after administration of ATV, modified with RPNS (0.10 mg/ml), were significantly higher than these indices in unvaccinated rats (MST (19.9 ± 2.4); 21.4 days compared to MST (14.6 ± 2.8); 14 days; $p = 0.0002$), and indices of animals vaccinated with ATV, modified with RPNS (0.02 mg/ml) ($p = 0.026$). All animals with glioma 101.8 in untreated group died till 23th day, while the maximal life span of tumor-bearing animals vaccinated with ATV, modified with RPNS (0.10 mg/ml) was 25 days.

Comparison of the cytotoxic activity of lymphocytes of animals from experimental groups using ANOVA by Ranks Kruskal — Wallis test showed highly significant differences between them ($p = 0.004$). The pairwise comparison of groups using U-Mann — Whitney test revealed the following features.

Table 1. MST and median survival of experimental animals with glioma 101.8

№	Group	MST, days (M ± m)	50' percentile (median), days	25' percentile (lower quartile), days	75' percentile (upper quartile), days	p (Gehan's – Wilcoxon two-sampling criterion)
1	Rats with glioma 101.8 (n = 38)	14.6 ± 2.8	14.0	12.0	17.3	–
2	Rats with glioma 101.8 vaccinated with RPNS-modified ATV (0.02 mg/ml) (n = 12)	16.4 ± 1.9	15.5	14.0	17.7	$p_{2,1} = 0.147$
3	Rats with glioma 101.8 vaccinated with RPNS-modified ATV (0.10 mg/ml) (n = 12)	19.9 ± 2.4	21.4	17.8	22.9	$p_{3,1} = 0.0002$ $p_{3,2} = 0.026$

Note: the differences between groups are statistically significant ($p < 0.05$)

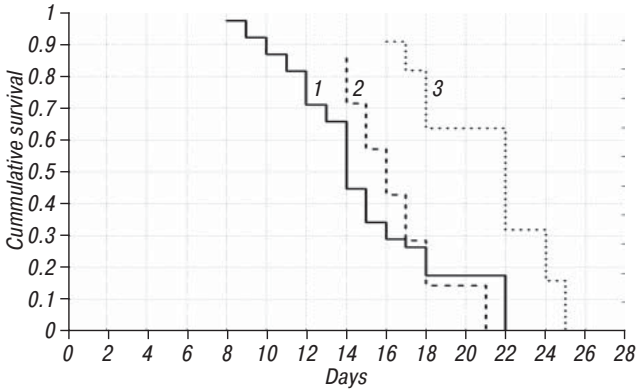

Figure. Survival curves of experimental animals with glioma 101.8 (1 — rats with glioma 101.8; 2 — rats with glioma 101.8 vaccinated with ATV modified with 0.02 mg/ml RPNS; 3 — rats with glioma 101.8 vaccinated with ATV modified with 0.10 mg/ml RPNS)

In intact rats the level of lymphocyte cytotoxic activity in MTT-test with glioma 101.8 cells (in allogeneic system) was at average 33.2 ± 1.1%.

The cytotoxicity index of lymphocytes from animals with glioma 101.8 reached at average 63.1 ± 11.1%, exceeding the same indicator of intact animals (U-Mann — Whitney test, $p = 0.003$, Table 2). After administration of ATV, modified with RPNS (0.02 mg/ml), in rats with glioma 101.8 on the 10th day of tumor transplantation, the cytotoxic activity of immune cells did not differ significantly from that index in unvaccinated tumor-bearing animals. However, after administration of ATV modified with RPNS (0.10 mg/ml), in rats with glioma 101.8 cytotoxic activity of lymphocytes was significantly increased by an average of 16% (U-Mann — Whitney test, $p = 0.026$) (see Table 2).

Thus, the i.p. administration of ATV modified with RPNS, led to an increase in the ability of immune cells to exert cytotoxic effects against allogeneic glioma cells *in vitro*. Administration of ATV, modified with RPNS at a concentration of 0.10 mg/ml, proved to be effective from the standpoint of life span extension.

Table 2. Cytotoxic activity of lymphocytes of experimental animals

№	Group	Number of animals, n	Cytotoxic index, % (M ± m)	p (U-Mann – Whitney criterion)
1	Intact rats (control) (n = 12)	6	33.2 ± 1.1	–
2	Rats with glioma 101.8 (n = 38)	6	63.1 ± 11.1	$p_{2,1} = 0.003$
3	Rats with glioma 101.8 vaccinated with ATV modified with 0.02 mg/ml RPNS (n = 12)	6	68.1 ± 4.9	$p_{3,1} = 0.029$ $p_{3,2} = 0.825$
4	Rats with glioma 101.8 vaccinated with ATV modified with 0.10 mg/ml RPNS (n = 12)	6	79.7 ± 7.5	$p_{4,1} = 0.009$ $p_{4,2} = 0.026$ $p_{4,3} = 0.114$

Note: the differences between groups are statistically significant ($p < 0.05$).

As it is shown above, in glioma-bearing animals cytotoxic function of immune cells is not reduced, conversely, CI of lymphocytes in the test with allogeneic glioma cells was higher than in intact (healthy) rats. But as far as tumor-bearing animals died till 23th day, it is evident that antitumor immunity in these animals is ineffective. This may be due to different mechanisms utilized by glioma allowing its escape from immune control. In gliomas, especially malignant, a partial or complete loss of expression of HLA antigens class I and/or II [6, 7] occurs, as well as down-regulation of transport molecules LMP2, TAP1 and β2-microglobulin [8]. On the other hand, glioblastoma cells and microglial cells/macrophages that infiltrate the tumor express nonclassical HLA-G and HLA-E molecules with immunomodulatory effects [9, 10]. It is shown that glioma tumor cells produce IL-10, which has immunosuppressive effects on the formation of local immunity by reducing the expression of class II MHC (DR) antigens on monocytes; inhibition of T-cell proliferation and allocytolytic activity of lymphocytes [3]. Reduced expression of class II MHC antigens is one of the reasons for the lack of antitumor activity of $CD4^+$ T-lymphocytes. For enhancing the efficiency of cellular immunotherapy of gliomas the tumor cells needs to become more sensitive to cytolysis by alloreactive cytotoxic lymphocytes (CTLs). For this purpose the proinflammatory cytokines IFN-γ, IL-1β, TNF-α are used, which can increase the expression of MHC antigens class I and II *in vitro* and *in vivo* in cells of gliosarcoma 9L, medulloblastoma, malignant glioma, glioma lines RG2, C6, 9L [28, 29]. Increased expression of MHC antigens I and II class was accompanied by increased animal survival and decreased tumor volume, elevated infiltration of the tumor by $CD4^+$ and $CD8^+$ T-cells, better recognition of allogeneic CTLs in cytotoxic tests.

One can assume that under the influence of RPNS, signaling cascades in glioma cells could be triggered leading to increased expression of MHC class I antigens and components of mechanism of the antigen processing, what promote glioma cells recognition by immunocompetent cells. In favor of such assumptions, the known data evidence that the rat NPC can express and produce a variety of different cytokines, in particular IL-1α, IL-1β, IL-6, IL-10, TGF-β1, TGF-β2, TNF-α [30, 31], LIF [32]. It is likely that IL-1β and TNF-α may be present in RPNS, and can increase the expression of MHC antigens I and II class by glioma cells.

It should be mentioned that we have used a modified MTT-test that allows to determine the total cytotoxic activity of effector immune cells — CTLs as well as natural killer (NK) cells. It can be assumed that the use of RPNS-modified ATV increases the efficiency of recognition of allogeneic tumor cells through a more effective presentation of antigens and, accordingly, leads to increased cytotoxic activity of immune cells. Since these immune cells (CTLs and NK-cells) play a key role in providing of antitumor immunity, we can assume that i.p. administration of RPNS-modified ATV induces a more effective immune response against glioma 101.8 cells, allowing to extend the life span of tumor-bearing animals.

Tumor-suppressive effect *in vivo* after i.p. administration of RPNS-modified ATV is obviously caused by the increase of efficiency of cytotoxic functions of immune cells. This observation could create the basis for further optimization of whole cell tumor vaccine. It is believed that the future of immunotherapy of gliomas is a combination of active vaccination and inhibition of immunological checkpoints [4]. It is possible that the method of modification of ATV using RPNS may be effective in the clinical development of individualized, specific for a particular patient targeted therapy based on individual molecular profile of glioma.

CONCLUSION

Intraperitoneal administration of RPNS-modified ATV in animals with glioma 101.8 increased MST and median survival of glioma 101.8 bearing rats presumably *via* elevation of cytotoxic activity of immune cells.

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