ONCOLOGY

CYTOTOXIC EFFECT OF THE VV-GMCSF-LACT ONCOLYTIC VIRUS AGAINST 3D CULTURES OF HUMAN GLIOBLASTOMA CELLS U-87 MG

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ABSTRACT

Background. *One of the promising methods of treating tumors is virotherapy, which is based on direct lysis of cancer cells by a virus and a virus-mediated antitumor immune response of the body. For the recombinant vaccinia virus strain VV-GMCSF-Lact, producing human GMCSF and the oncotoxic protein lactaptin, cytotoxic and antitumor effects were shown in experiments in vitro and in vivo, respectively, when using adherent cultures of U-87 MG human glioblastoma cells. 3D cultures are a more relevant tumor model than adherent models, as they more fully reflect the realistic scenario of cancer development, as well as the response of the tumor to anticancer therapy.*

The aim of the study. *To evaluate the cytotoxic effect of the oncolytic virus VV-GMCSF-Lact against 3D cultures of human glioblastoma U-87 MG.*

Materials and methods. *The following methods were used in the work: cultivation of 3D cell cultures, cytofluorometry, microscopic analysis, virus titration, and statistical analysis.*

Results. *U-87 MG cells were transduced with a lentiviral vector carrying the GFP reporter gene. The cytotoxicity of the VV-GMCSF-Lact virus (IC50) against the studied cells was 0.024 PFU/cell. U-87 MG cells were cultured under conditions for the formation of 3D structures. Microscopic analysis showed the oncolytic effect of the virus on the cells of 3D cultures as early as 24 hours after the start of incubation. Flow cytometry showed an increase in the granularity of glioblastoma cells under the action of the virus, which indicates active replication of the virus in the cells. The virus titer was 0.44 PFU/cell.*

Conclusion. *The recombinant VV-GMCSF-Lact virus has a cytotoxic effect on 3D human glioblastoma U-87 MG cell cultures and actively replicates in them. In the future, to test the oncolytic effect of VV-GMCSF-Lact, it is planned to use not only 3D human glioblastoma cultures, but also cerebral organelles obtained in the process of cocultivation of glioblastoma cells and induced human pluripotent cells.*

Key words: *glioblastoma, neurospheres, oncolytic virus, VV-GMCSF-Lact*

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ЦИТОТОКСИЧЕСКОЕ ДЕЙСТВИЕ ОНКОЛИТИЧЕСКОГО ВИРУСА VV-GMCSF-LACT В ОТНОШЕНИИ 3D-КУЛЬТУР КЛЕТОК ГЛИОБЛАСТОМЫ ЧЕЛОВЕКА U-87 MG

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РЕЗЮМЕ

Обоснование. *Одним из перспективных методов лечения опухолей является виротерапия, в основе которой лежит прямой лизис вирусом опухолевых клеток и вирус-опосредованный противоопухолевый иммунный ответ организма. Для рекомбинантного штамма вируса осповакцины VV-GMCSF-Lact, продуцирующего GMCSF человека и онкотоксический белок лактаптин, показано цитотоксическое и противоопухолевое действие в экспериментах in vitro и in vivo соответственно при использовании адгезивных культур клеток U-87 MG глиобластомы человека. 3D-культуры являются более релевантной моделью опухоли в сравнении с адгезивными моделями, так как более полно отражают реалистичный сценарий развития опухолевого процесса, а также ответа опухоли на противоопухолевую терапию.*

Цель исследования. *Оценка цитотоксического действия онколитического вируса VV-GMCSF-Lact в отношении клеток 3D-культур глиобластомы человека U-87 MG.*

Методы. *В работе использовались следующие методы: культивирование 3D-культур клеток; цитофлуориметрия; микроскопический анализ; титрование вируса; статистическая обработка данных.*

Результаты. *Клетки U-87 MG были трансдуцированы лентивирусным вектором, несущим ген GFP. Цитотоксичность вируса VV-GMCSF-Lact (IC50) в отношении исследуемых клеток составила 0,024 БОЕ/клетку. Далее клетки U-87 MG культивировали в условиях формирования 3D-структур. С помощью микроскопического анализа показано онколитическое действие вируса на клетки 3D-культур уже спустя 24 часа после начала инкубации. Методом проточной цитофлуориметрии показано увеличение гранулярности клеток глиобластомы под действием вируса, что указывает на активную репликацию вируса в клетках. Титр вируса составил 0,44 БОЕ/клетку.*

Заключение. *Рекомбинантный вирус VV-GMCSF-Lact оказывает цитотоксическое действие на 3D-культуры клеток глиобластомы человека U-87 MG, активно реплицируется в них. В дальнейшем для тестирования онколитического действия VV-GMCSF-Lact планируется использовать не только 3D-культуры глиобластомы человека, но и церебральные органоиды, полученные в процессе сокультивирования клеток глиобластомы и индуцированных плюрипотентных клеток человека.*

Ключевые слова: *глиобластома, нейросферы, онколитический вирус, VV-GMCSF-Lact*

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INTRODUCTION

Human glioblastoma (GB) or astrocytoma of grade IV malignancy is one of the most common types of malignant primary brain tumours, being characterised by high invasiveness, heterogeneity and aggressiveness of the tumour process. With a conventional treatment regimen (the standard therapy for GB is the Stupp protocol) including surgical resection, radio- and chemotherapy with temozolomide, the survival rate of patients diagnosed with glioblastoma is only 26.5 %, with an overall survival rate of more than 10 years of 0.71 % [1]. Actually, the mean life expectancy after diagnosis is about 15 months, with a survival rate of usually no more than 3 months in the absence of therapy. The disease is more common in men than in women, and the average age of patients at the time of diagnosis is 64 years. As a result, the development of new therapeutic approaches for such difficult-to-treat cancers is urgently needed. One of the promising methods of tumour treatment is virotherapy, which is based on direct lysis of tumour cells by virus and virus-mediated anti-tumour host immune response. The anti-tumour efficacy of recombinant viruses can be enhanced by inserting genes of oncotoxic and immunomodulatory proteins into their genome.

The recombinant VV-GMCSF-Lact strain of smallpox vaccine virus producing human granulocyte-macrophage colony-stimulating factor (GMCSF) and the oncotoxic protein lactaptin was used in this study [2]. VV-GMCSF-Lact has previously been shown to have an anti-tumour effect against breast cancer and is currently in clinical trials (ClinicalTrials.gov: NCT05376527). Nevertheless, the activity spectrum of this virus is much broader: in particular, its cytotoxic and anti-tumour effects were revealed in *in vitro* and *in vivo* trials, respectively, using adherent cultures of human glioblastoma U-87 MG [3]. 3D cultures are known to be a more relevant tumour model as they allow a more complete reproduction of the tumour development scenario as well as the response to anti-tumour therapy.

THE AIM

Assessment of the oncolytic action of VV-GMCSF-Lact against 3D-cultured human glioblastoma U-87 MG cells. In order to better visualise the cytotoxic effect of the recombinant virus, in this study the human glioblastoma U-87 MG cells pre-transduced with lentivirus carrying a *GFP* reporter gene were used.

METHODS

Cell cultivation

U-87 cells transduced with lentivirus carrying *GFP* gene were cultured in complete growth medium DMEM/F12 supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 1X MEM essential amino acid solution and antibiotic solution (100 U/ml penicillin, 100 mg/ml streptomycin sulfate) at 37 °C in a CO_2 incubator. To obtain spheroids, AggreWell™800 microwell plates (STEMCELL Technologies, Canada) were treated with Anti-Adherence Rinsing Solution (STEMCELL Technologies, Canada) for 10 minutes and then washed with 1X PBS. Cells were dissociated using 0.25 % trypsin solution with EDTA and seeded at a rate of 3×10^6 cells per well in 2 ml of growth medium. Half the volume of the medium was changed the next day. On day 3, spheroids were washed out of micro-well plates and transferred to 10 cm dishes were cultured in a shaker incubator at 37 °C, 5 % CO_2 and constant agitation at 80 rpm, changing the medium every 3–4 days.

Assessment of cytotoxic activity of VV-GMCSF-Lact against U-87 MG cells

U-87 MG cells were seeded onto a 96-well plate at a concentration of 4×10^3 cells per well in 100 µL of phenol red-free Opti-MEM medium and incubated at 37 °C in a 5 % CO_2 atmosphere. After 24 h, VV-GMCSF-Lact was added to the cells. The multiplicity of virus infection ranged from 0.0012 to 10 PFU per cell. Cells were incubated with the virus for 72 h. Control cells were incubated under the same conditions without the addition of viral preparation. After incubation, 10 µL of Deep Blue Viability Kit reagent (BioLegend, USA) was added to the wells and incubated for 4 h at 37 °C. The optical density of the solution was measured at a wavelength of 570 nm (reference value – 620 nm) using an Apollo LB 912 spectrophotometer (Berthold Technologies, Germany). Cell viability was determined relative to the viability of control cells (100 %) ± SD following three independent experiments.

Assessment of cytotoxic activity of VV-GMCSF-Lact against 3D-cultured human glioblastoma U-87 MG cells

3D cultures of U-87 MG cells were placed in wells of a 96-well plate at 10 cells per well and cultured in 100 µL of complete DMEM/F12 culture medium. After 24 h, the medium was removed and replaced with 50 µl of virus suspension with a virus titre of 3.3×10^7 PFU/ml. 50 µl of 1 mM TRIS HCl (pH = 8.5) was added to control wells. The plate was centrifuged for 20 min at 500 rpm to sediment the virus. The cells were then incubated in a CO_2 incubator at 37 °C for 20 min, after which 50 µL of complete DMEM/F12 culture medium was added. The cytotoxic effects of the virus were analyzed at 24 and 72 hours and on day *7* after infection of cells.

Microscopy

3D cell culture samples after exposure to the virus were analysed *in vivo*, in transmitted light and in the FITC green channel using a Nicon Eclipse Ti microscope (Tokyo, Japan).

Cytofluorimetric analysis

Cell samples after virus exposure were dissociated enzymatically using Accumax reagent (Stemcell Technologies,

Canada) and analysed with a BD FACS Canto II flow cytometer (Becton Dickinson, USA) using BD Pharmigen FITC Annexin V Apoptosis Detection Kit I, according to the manufacturer's instructions with minor modifications: only one dye, propidium iodide (PI), was used, as Annexin V – FITC should be analysed in the green channel already occupied due to the transduction of U-87 MG cells. Cytofluorimetry results were analyzed in FlowJo™ v. 10 Software (Becton Dickinson, USA).

VV-GMCSF-Lact virus titration

Green monkey kidney 4647 cells were seeded in a 12-well plate containing 300,000 cells per well, in 2 ml of complete DMEM medium with 10 % fetal serum (FBS) and incubated at 37 °C in a 5 % CO_2 atmosphere until 90 % monolayer formation. 10-fold dilutions of the viral suspension in DMEM medium were prepared. Growth medium was removed from the wells of the plate and 100 µl of the appropriate dilution of virus was added to the centre of each well. The plate with cells was incubated at 37 °C in a CO_2 incubator for 1 hour. Then 2 ml of minimum essential medium (DMEM + 2 % FBS) was added to the wells and the plate was left at 37 °C in a CO_2 incubator for 48 hours. Afterwards, the minimum essential medium was removed, 1 ml of crystal violet solution was added to the wells with cells and left for 20 min at room temperature. Then the crystalline violet solution was removed and the plaques were counted.

Statistical analysis

Quantitative variables are presented as mean ± standard deviation (SD). Each experiment was repeated at least three times. Statistical analysis was performed using GraphPad 6.01 (GraphPad Software, USA). Two-factor analysis of variance was used to compare more than two data sets. Differences were considered statistically significant if $p < 0.05$.

RESULTS AND DISCUSSION

U-87 MG human glioblastoma cells were transduced with a lentiviral construct carrying a *GFP* reporter gene, as it is planned to use these cells for co-culture with human cerebral organoids in the future. The cytotoxicity of the virus against U-87 MG cells was determined by cell proliferative activity using resazurin as an indicator. The degree of reduction of resazurin and production of resorufin is proportional to the number of metabolically active cells. The IC50 (half maximal inhibitory concentration) measured in CompuSyn software was 0.024 PFU/cell, indicating a higher sensitivity of this cell culture to VV-GMCSF-Lact virus compared to the non-transduced lentivirus U-87 MG cell culture [4].

Formed 3D cultures of U-87 MG were incubated with VV-GMCSF-Lact, which had a titer of 3.3×10^7 PFU/ml. This amount of viral particles is comparable to that of intratumoural injection into the body of a laboratory animal in preclinical trials [3]. The 3D-cultured cells were further analyzed 24 and 72 h and 7 days after cell infection. It has been revealed that replication of some oncolytic viruses can increase as early as 24 h after infection of tumour cells, however, after 72 h it starts to decrease [5, 6].

Microscopic analysis of the samples was performed both in transmitted light and using a fluorescent filter for fluorescein isothiocyanate (FITC). In our study, oncolytic effect of VV-GMCSF-Lact was observed on a 3D culture of U-87 MG cells (Fig. 1).

The structure of 3D-cultures is destroyed and the amount of cellular debris increases with extending incubation time with virus ("3D-cultures with virus_24 hours", "3D-cultures with virus_72 hours", "3D-cultures with virus_7 days"), whereas in control wells 3D cultures ("Control 3D-cultures_24 hours", "Control 3D-cultures_72 hours", "Control 3D-cultures_72 hours", "Control 3D-cultures_7 days") remain unchanged, preserving clear outlines and smooth edges. The intensity of cell death reactions was assessed by the interaction of cells with propidium iodide (PI), a marker of necrosis. Unfortunately, no statistically significant difference was observed between groups in the number of stained cells in the PE channel, possibly due to the presence of necrotic core in 3D cultures. As tumour spheroid reaches a diameter of more than 500 µm, it is known that the spheroid usually exhibits a three-layer concentric structure including an outer layer of proliferating cells, a middle layer of quiescent cells and a central necrotic zone, with each region at different stages of the cell cycle [7]. The complexity of this multilayered structure may be caused by a lack of oxygen and nutrients, which is not observed in 2D tumour cell cultures. Drugs, soluble metabolites, as well as oxygen concentration and pH are known to exist as a gradient within the tumour: peripheral cells closer to blood vessels have greater access to soluble components and oxygen, which decreases as it diffuses through the extracellular matrix to the tumour core. The concentration gradients of growth factors, nutrients, and metabolites create intratumoural heterogeneity and affect signalling in the microenvironment, including cell function, proliferation, morphogenesis, and chemotaxis [8]. A concentration gradient, from a pharmacokinetic point of view, limits the penetration of drugs into the tumour and the attainment of a dosage sufficient to exert therapeutic effects on all cancer cells.

Using cytofluorimetric analysis, a statistically significant increase in side scatter intensity, i.e. cell granularity of 3D-cultured cells at time points 24 and 72 h (Fig. 2) was observed, which indirectly attributes to active replication of the virus in the cells. For instance, it was previously revealed that cell populations with higher SSC (side scatter) intensity had more inclusions, more organelles and virosomes (viral factories) [9, 10].

To assess the replication efficiency of VV-GMCSF-Lact in 3D-cultured human glioblastoma cells, the virus titre was determined on day 7 after cell infection [11]. The virus was revealed to replicate efficiently in spheroid cells; the titer was 0.44 PFU/cell, which is ≈18-fold higher than the IC50 for the corresponding adherent cultures, further confirming the literature data about the resistance of 3D cultures compared with adherent cells when testing the cytotoxic-

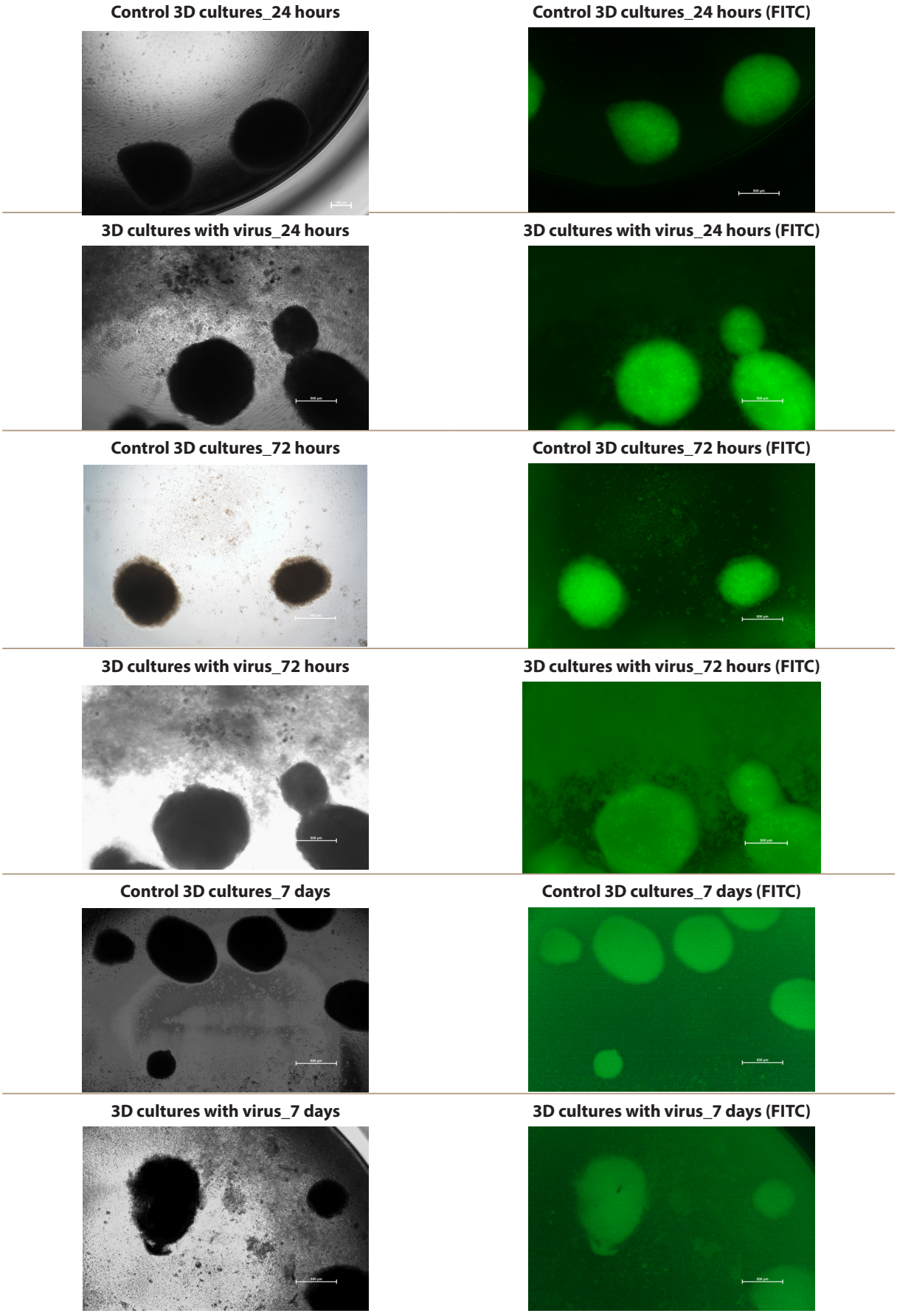

FIG. 1.

Microscopic image of 3D-cultured cells treated with oncolytic virus VV-GMCSF-Lact and untreated control 3D-cultured cells; microscope magnification × 40

ity of anti-tumour drugs [12]. It is likely that virus replication occurs in the outer cell layer of the spheroid, but further experiments are needed to pinpoint the exact localization of this process.

Consequently, cells cultured in larger three-dimensional aggregates mimic the *in vivo* state by being in different proliferative states based on nutrient access that is limited by a concentration gradient. Whilst 2D cultures are still predominantly used for drug development due to their simplicity and compatibility with screening platforms, 3D culture systems have numerous advantages over 2D cell culture. Specifically, 3D cell culture models more accurately reflect the pathophysiological microenvironment that allows tumour cells to aggregate, proliferate and exhibit phenotypes as they do within the body [13]. Complex cellular interactions between other cells and the three-dimensional matrix are crucial for preserving the structure, function and motility of tumour cells. Since cell migration occurs in three dimensions, the matrix provides a topology that mimics the three-dimensional architecture of tissue, allowing cells to attach and interact with the environment.

CONCLUSION

3D cultures are a more relevant model for testing anti-tumour drugs compared to adherent models. Additionally, the move to 3D preclinical models has become more attractive as improvements in tissue engi-

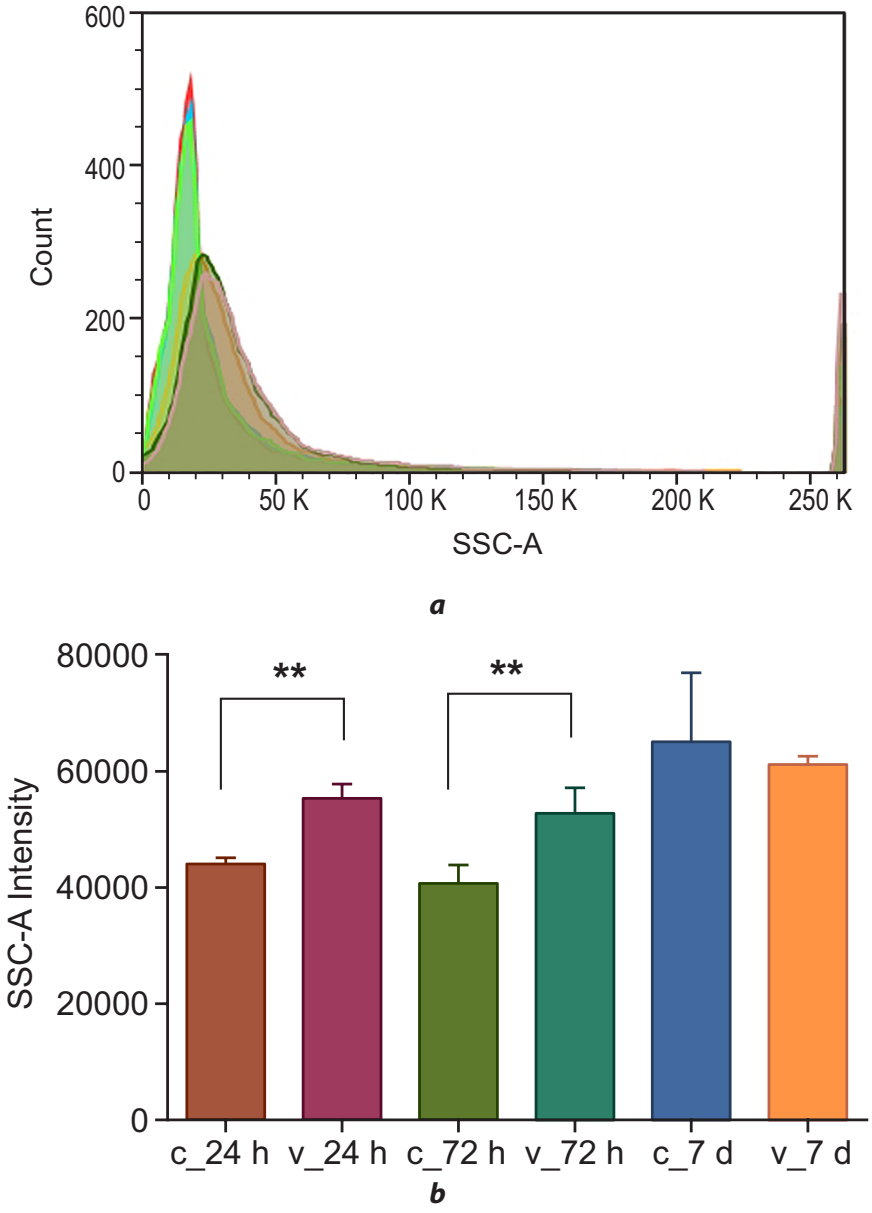

FIG. 2.

a *– distribution of 3D-cultured cells by side scatter intensity; flow cytometry;* ***b*** *– average side scatter intensity of 3D-cultured cells treated with virus (v_24 h, v_72 h, v_7 d) compared to control group cells (c_24 h, c_72 h, c_7 d)*

neering technologies have made 3D cell culture more adaptable and adjustable to microenvironmental factors so as to better reflect the functional pathology of tumours *in vivo*. The use of 3D cultures allows, among other things, to assess the ability of oncolytic drugs to penetrate the tumour and affect its internal structures. Meanwhile, the formation of a necrotic tumour core complicates the analysis of the oncolytic action of the virus by cytometry. Therefore, several analytical methods should be applied to assess the anti-tumour efficacy of therapeutic agents.

Subsequently, it is planned to use not only 3D cultures of human glioblastoma, but also chimeric cerebral organoids obtained by co-culturing tumour cells and human cerebral organoids to test the cytotoxic effect of VV-GMCSF-Lact [14]. Such a cell model allows to recreate three-dimensional cytoarchitectonics of some parts of the brain, which opens unique opportunities to study the interaction between tumour cells and brain cells, as well as the effect of oncolytic drug on healthy nervous tissue and glial tumour microenvironment [15].

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Conflict of interest

The authors of this article declare no conflicts of interest.

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