

HUMAN ENTEROVIRUSES EXHIBIT SELECTIVE ONCOLYTIC ACTIVITY IN THE MODEL OF HUMAN GLIOBLASTOMA MULTIFORME XENOGRAPTS IN IMMUNODEFICIENT MICE

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Stem cells that penetrated deeply into the brain tissue are the main reason behind the relapses of glioblastoma multiforme after surgery. Finding new approaches to counter such relapses, including those that make use of oncolytic viruses, is a pressing issue. This study aimed to determine the sensitivity of cells of human glioblastoma multiforme to non-pathogenic enteroviruses, *in vitro* and *in vivo* (mice xenografts model). Glioblastoma tumor cells were exposed to type 1 poliovirus (Sabin vaccine strain), Coxsackie virus A7 (strain LEV8), Coxsackie virus A9 (strain LEV9) and Coxsackie virus B5 (strain LEV14). The virus reproduction intensity and cytolitic activity were assessed through infection of monolayered glioblastoma cell cultures. The ability of glioblastoma cell cultures (enriched with tumor stem cells) to build subcutaneous tumors in immunodeficient mice after those cultures were exposed to viruses signaled the effectiveness of glioblastoma stem cells destruction. The study revealed that Coxsackie virus A7 and type 1 poliovirus possess the most pronounced oncolytic and replicative properties when tested on glioblastoma cells infected with viruses *in vitro* and on subcutaneous tumor xenografts in immunodeficient mice (*in vivo*). Type 1 poliovirus and Coxsackie virus A7 virus prevented development of tumors when glioblastoma neurospheric cell cultures were preincubated with viruses before subcutaneous implantation. Coxsackie virus B5 only managed to reduce the number of tumors developed, and Coxsackie virus A9 did not affect the tumor development at all. Thus, a number of non-pathogenic enteroviruses strains can destroy glioblastoma's stem cells, i.e. they show promise in the context of development of therapeutic agents for relapse-free treatment of glioblastomas.

Keywords: glioblastoma multiforme, oncolytic virus, non-pathogenic enteroviruses, personalized medicine, tumor relapse, experimental cancer therapy

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ЭНТЕРОВИРУСЫ ЧЕЛОВЕКА ПРОЯВЛЯЮТ ИЗБИРАТЕЛЬНУЮ ОНКОЛИТИЧЕСКУЮ АКТИВНОСТЬ НА МОДЕЛИ КСЕНОТРАНСПЛАНТАТОВ МУЛЬТИФОРМНОЙ ГЛИОБЛАСТОМЫ ЧЕЛОВЕКА В ИММУНОДЕФИЦИТНЫХ МЫШАХ

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Основным источником возникновения рецидивов мультиформной глиобластомы после хирургического вмешательства являются стволовые клетки, успевающие проникнуть глубоко в ткани мозга. В настоящее время актуален поиск новых подходов для борьбы с ними, в том числе с помощью онколитических вирусов. Целью работы было определение чувствительности к непатогенным энтеровирусам клеток мультиформной глиобластомы человека, поддерживаемых *in vitro* и в модели мышинных ксенотрансплантатов. Культуры опухолевых клеток глиобластом испытывали на чувствительность к полиовирусу 1 типа (штамм вакцины Сэбина), вирусу Коксаки А7 (штамм ЖЭВ8), Коксаки А9 (штамм ЖЭВ9) и Коксаки В5 (штамм ЖЭВ12). Количественную оценку репродукции вирусов и их цитолитическую активность проводили заражением монослойных культур клеток глиобластомы. Эффективность уничтожения стволовых клеток глиобластомы определяли по способности клеточных культур глиобластом, обогащенных опухолевыми стволовыми клетками, формировать подкожные опухоли у иммунодефицитных мышей после обработки вирусами. По результатам исследования наиболее выраженная онколитическая и репликационная активность выявлена у вируса Коксаки А7 и полиовируса 1 типа при тестировании в модели культур клеток глиобластом, инфицированных вирусами *in vitro*, а также *in vivo*, в модели подкожных опухолевых ксенотрансплантатов на иммунодефицитных мышах. Полиовирус 1 типа и вирус Коксаки А7 предотвращали образование опухолей после того как нейросферные культуры клеток глиобластом преинкубировали с вирусами перед подкожным введением. Вирус Коксаки В5 вызывал лишь частичное сокращение числа опухолей, а Коксаки А9 не влиял на опухолеобразование. Таким образом, ряд штаммов непатогенных энтеровирусов способен уничтожать стволовые клетки глиобластом и представляется перспективным при разработке терапевтических средств для безрецидивного лечения глиобластом.

Ключевые слова: мультиформная глиобластома, онколитический вирус, непатогенные энтеровирусы, персонализированная медицина, рецидив опухоли, экспериментальная терапия рака

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Brain tumors remain hard to treat, especially when they take form of a glioblastoma multiforme, which is the most malignant and practically incurable [1]. Such tumors grow and infiltrate functionally important areas of the brain, making surgery extremely difficult. Often, the risk of neurological disorders dictates minimal invasion, i.e. only partial removal of the tumor. However, even when it is removed completely, a relapse is imminent: glioblastoma stem cells that ignite the development of such tumors migrate deeply into the healthy areas of the brain and cannot be removed [2–4]. Chemotherapy and radiotherapy result in short-term remissions only; when they are over, the tumor resumes its growth [4]. Modern medicine is almost out of options when dealing with glioblastomas, which justifies the search for alternative approaches to targeted and effective destruction of malignant cells. Significant progress in understanding the mechanisms of malignant growth and specific properties of glioblastoma cells is a good basis for development of innovative therapies. The particularly promising one implies using non-pathogenic oncolytic viruses that recognize and destroy glioblastoma cells [5, 6].

Members of various virus families are used to design oncolytic viruses to treat human gliomas. Among these are herpes viruses, Newcastle Disease virus [7–11], adenoviruses [12–16], parvoviruses [17–19], reoviruses [20–23], enteroviruses [24–27] etc [1]. Numerous clinical trials have shown that oncolytic virus preparations are non-toxic [28–31]. In addition, unlike chemo- and targeted therapy, many oncolytic viruses can effectively kill tumor-initiating stem cells [16, 32–37], which is crucial for the complete recovery of patients. Today, there is a wide range of potentially therapeutic virus strains that can be used in further clinical trials. Properties of non-pathogenic strains of Coxsackie viruses (A7, A9 and B5 in particular) are being studied actively, and preliminary data suggest that they can potentially form therapeutic agents [38].

This study aimed at evaluating the response of glioblastoma tumor cells to oncolytic action of a number of non-pathogenic enteroviruses in *in vitro* and *in vivo* models in order to uncover and assess their therapeutic potentials for a treatment of glioblastomas.

MATERIALS AND METHODS

Obtaining primary cell cultures from glioblastoma tumors

Fragments of the freshly removed tumors were stored for up to 12 hours in ice-cold sterile culture medium. Pieces of tumors were mechanically separated from necrotic tissue, stroma, blood vessels, and then gently pushed through a nylon mesh (pore size — 50 μm). The tumor cell aggregate suspension was washed with PBS and incubated in 25 volumes of collagenase 4 solution (PanEco, Moscow) for 25 minutes at 30 °C. To obtain monolayer glioblastoma cells cultures, the suspension treated with collagenase was washed twice with DMEM (PanEco, Moscow) and placed in DMEM-F12 supplemented with 10% fetal bovine serum and 100 $\mu\text{g}/\text{ml}$ of penicillin and streptomycin at the density of 10^5 cells/ml. The incubation was at 37 °C in the atmosphere of 5% CO_2 ; the medium was replaced every 4 days up until the monolayers formed. 18–25 days after the incubation, monolayer cultures were placed to 10 cm culture dishes.

Growth of tumor xenografts in immunodeficient mice

We obtained the xenografts of subcutaneous tumors by injecting neurospheres, which were obtained by the alternative

method of plating cells from glioblastomas after treating tumor fragments with collagenase 4 (PanEco, Moscow). The plating conditions were as follows: density — 10^4 cells/ml; medium — DMEM-F12; supplements — 100 $\mu\text{g}/\text{ml}$ penicillin and streptomycin; growth factor — 20 ng/ml EGF and 10 ng/ml bFGF. The cultures stayed in a CO_2 incubator at 37 °C for 2 weeks; the medium was replaced every 4 days until visible neurospheres appeared. The neurospheres enriched with glioblastoma stem cells are highly tumorigenic when injected to immunodeficient mice. For implantation purposes, the neurospheres were washed twice with PBS, their numbers counted with a hemocytometer. 3–5-week-old athymic Balb/c mice received 200 neurospheres subcutaneously, injection location — shoulder. The tumor growth was monitored every 3 days; the total number of tumors was assessed on the day 21.

Strains of oncolytic viruses

We used non-pathogenic strains of human enteroviruses from the laboratory's collection: type 1 poliovirus (Sabin vaccine strain), Coxsackie virus A7 (strain LEV8), Coxsackie virus A9 (strain LEV9) and Coxsackie virus B5 (strain LEV14) [38, 39]. Enteroviruses were grown in Vero cells. Viral titres as TCID₅₀/ml were determined by infecting Vero cells with serial dilutions of the virus-containing liquid.

Testing sensitivity of glioblastomas cells to viruses

We used the tumor material from two patients with glioblastomas to determine the differential sensitivity of cells (after a minimal number of passages in the culture) to the panel of oncolytic enteroviruses. The cultures obtained from these patients were named GM-3564 and GM-3876. 96-well culture plates (SPL Lifesciences, Republic of Korea) were used to infect the cultures. Subconfluent one-day monolayers of GM-3564 and GM-3876 cell cultures, as well as Vero control cells, were incubated with 0.1 ml serial virus dilutions. We measured cell viability seven hours after infecting the cultures with serial tenfold dilutions of viral preparations (four non-pathogenic enteroviruses). After 72 hours, we evaluated the cytopathic activity using the CellTiter-Glo[®] Luminescent Cell Viability Assay kit (Promega, USA).

In vivo virus introduction

To measure the ability of viruses to prevent formation of tumors with neurospheres injected, we incubated neurospheres with viruses prior to subcutaneous administration (2×10^6 virus infectious units (i.u.), volume 0.1 ml, for 30 minutes at 37 °C).

RESULTS

We tested sensitivity of the monolayer glioblastoma cultures (obtained from tumors in two patients) to four strains of enteroviruses. GM-3564 cells were most susceptible to Coxsackie virus A7 and slightly less so to type I poliovirus (Fig. 1). GM-3876 culture showed quite the opposite: type I poliovirus was the strongest agent in its case and Coxsackie virus A7 produced a similar effect only when the dose was about ten times larger. Both cultures were relatively resistant to Coxsackie virus B5 and almost completely resistant to Coxsackie virus A9. The latter produced a slight toxic effect only at maximum doses. At the same time, all for strains were cytotoxic to the Vero cells (control) at approximately the same dosage. Thus, the two cultures of glioblastomas are apparently selective in their sensitivity to virus strains, and the lysing activity of those

strains depends on that sensitivity. The probable explanation is the differing ability to support replication of viruses the cultures exhibit.

In order to establish the ability of viral strains to prevent formation of subcutaneous tumors following introduction of xenografts into immunodeficient mice, we obtained cultures of neurospheres from tumor material of glioblastoma patients. The neurospheres received tumor-forming stem cells, which made their tumorigenicity significantly greater than that of monolayer glioblastoma cell cultures. Obtaining neurosphere cultures requires medium with specific growth factors, EGF and bFGF. In addition, the cells should be plated to a non-adhering plastic surface preventing attachment of cells. In such conditions, glioblastoma cells form spheroid aggregates, or neurospheres, which are enriched in tumor-forming stem cells. Since counting the number of individual cells in spheroids is difficult, in order to standardize the procedure of subcutaneous injections aiming tumor development we counted the number of neurospheres. In our preliminary study, we found that injecting 200 neurospheres leads to the formation of tumors within 1–2.5 weeks. The control group of five mice received 200 neurospheres in a volume of 0.5 ml subcutaneously in the shoulder region. We used the same number of mice to test the effect produced by viruses on tumor formation. Thus, each group of mice could develop up to 10 tumors. To test the ability of viruses to prevent formation of tumors from the injected neurospheres, the latter

were incubated with viruses (see Materials and Methods). We used five mice for each type of virus; they were injected with a suspension containing virus-treated neurospheres (two injection points). The resulting tumors were counted 21 days after the injection. Assessing the tumors, we did not take into account their size (Fig. 2). 9 tumors (out of 10) developed in the control group injected with GM-5564 neurospheres. Treatment with type 1 poliovirus completely suppressed the formation of the tumors; Coxsackie virus A7 showed a less prominent result: one tumor did develop, but at a later date. Treatment with the Coxsackie virus B5 lead to the formation of three tumors, while Coxsackie virus A9 showed almost no anti-tumor effect (8 tumors). Groups of mice that received the GM-3876 neurospheres showed principally similar results: 8 tumors in the control group (out of 10 possible), no tumors after treatment with poliovirus and Coxsackie virus A7, 4 tumors in the group treated with the Coxsackie virus B5, 8 tumors in the Coxsackie virus A9 group.

DISCUSSION

Neurosphere cultures are the most adequate model for analyzing the therapeutic potential of anti-glioblastoma agents [40]. This is due to the fact that neurospheres carry tumor stem cells, which are the main cause of relapses: they are very resistant to most therapeutic agents [41]. In this study,

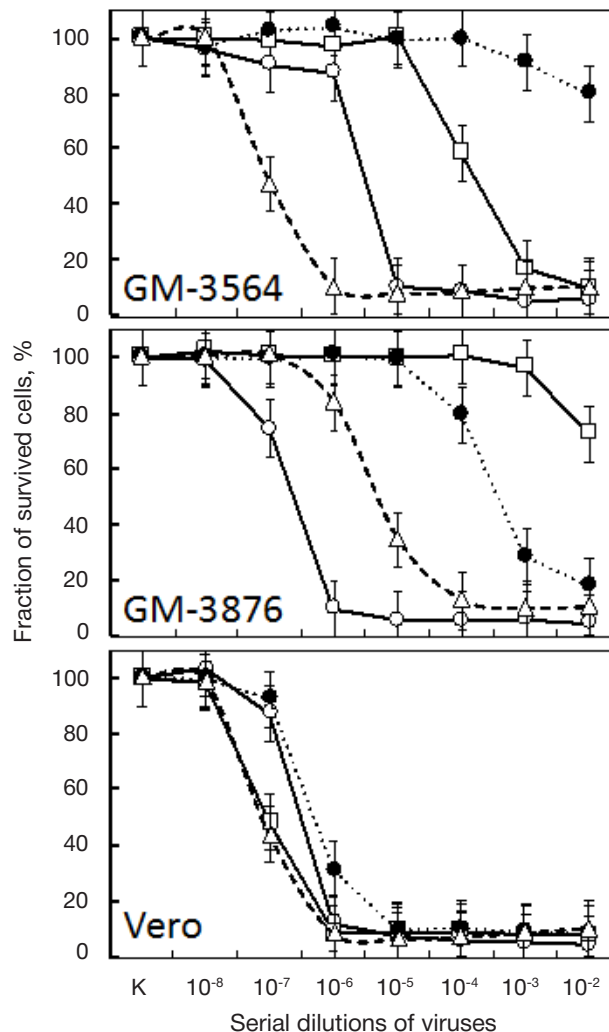


Fig. 1. Sensitivity of early-passage monolayer cell cultures obtained from two glioblastoma patients to four strains oncolytic enteroviruses: —○— Type 1 Poliovirus; - -Δ- - Coxsackie virus A7; —□— Coxsackie virus A9;●.... Coxsackie virus B5. Horizontal scale: 10^{-8} – 10^{-2} — ten-fold serial dilutions of viral stocks used for the infections. Vero — control cell line that is sensitive to all four types of viruses used

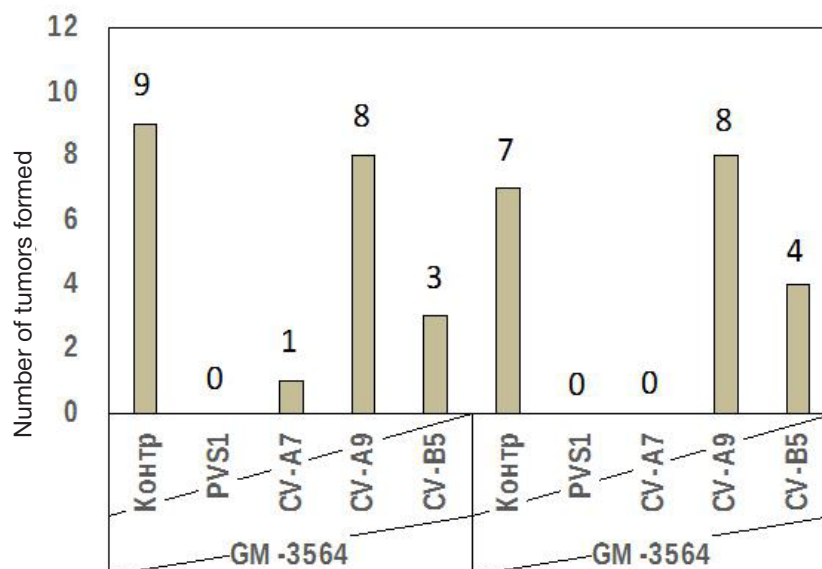


Fig. 2. Tumorigenicity-inhibition assay with glioblastoma neurospheres pretreated with each of the four viruses before being injected subcutaneously into nude mice. Bars reflect number of tumors formed in each treated and untreated (Contr) group of mice. PVS1 — Type 1 Poliovirus; CV-A7 — Coxsackie virus A7; CV-A9 — Coxsackie virus A9; CV-B5 — Coxsackie virus B5

we analyzed the ability of four non-pathogenic oncolytic enteroviruses to prevent the formation of subcutaneous tumor xenografts obtained through injecting neurospheres that were developed from the tumor material of two glioblastoma patients. The results of the study indicate that two viral strains, type 1 poliovirus and Coxsackie virus A7 were the most effective. However, neurospheres from the two different patients reacted to those viruses differently, which points to the need for a personalized selection of the most effective therapeutic strains after preliminary analysis of biopsy or surgical material taken from the patients' tumors. The recently published results of clinical trials of recombinant poliovirus PVSR1PO prove its ability to initiate prolonged remissions in 21% of glioblastoma patients, while the remaining patients saw no therapeutic effect from the virus [42]. Adding more therapeutic virus strains to the range available will significantly increase the percentage of successfully treated glioblastoma cases.

CONCLUSIONS

Based on the results of the study, we conclude that each of the four strains of non-pathogenic oncolytic enteroviruses produces cytotoxic effect on monolayer cell cultures of human glioblastomas, and that the effect correlates well with the ability of the same strains to prevent tumor formation in xenografts transplanted to immunodeficient mice. Thus, there is a clear relationship between the effects produced by the viruses *in vitro* and their oncolytic activity *in vivo*. Besides, it was found that viruses that can efficiently replicate in monolayer glioblastoma cell cultures can effectively kill tumor-initiating glioblastoma stem cells, which signals the high oncolytic potential of the viruses in preventing tumor relapses. The differences in reaction to viruses that tumor cells from two patients have shown indicate the need for personalized selection of therapeutic strains.

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