CHANGES IN THE SENSITIVITY OF HUMAN GLIOBLASTOMA CELLS TO ONCOLYTIC ENTEROVIRUSES INDUCED BY PASSAGING

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Existing therapies for glioblastoma multiforme do not ensure patient's recovery. Oncolytic viruses (OV) represent a promising alternative as they can destroy glioblastoma-initiating stem cells, the major cause of relapses. However, while individual OV strains are effective for some patients, they could be ineffective for others. To achieve a predictable therapeutic effect, live tumor cells of the patient need to be tested for their sensitivity to different viruses. The aim of this study was to assess how sensitivity of tumor cells to viruses changes with passaging in the cell culture. Primary glioblastoma cell cultures were prepared from excised tumors. We compared the sensitivity of the cells to four non-pathogenic enteroviruses (type 1 poliovirus, Coxsackie virus A7, Echoviruses 1 and 12) for freshly explanted primary tumor cell cultures and for those that had undergone 700 divisions during passaging. Cell sensitivity was assessed by the MTT assay based on the proportion of viable cells 72 hours after the cells were inoculated with serial 10-fold dilutions of virus preparations. Cells isolated from the tumors of 3 patients exhibited varying sensitivity to the used viral strains. Differences in the lowest virus dose required for the successful infection of the cell cultures were as high as 10^5. Passaging induced sensitivity shifts, such as increased or decreased sensitivity to individual viruses. Differences in the sensitivity correlated with the ability of the infected cells to produce the virus. Based on our findings, we conclude that the sensitivity of cancer cells to viruses should be tested at very early stages of passaging, preferably in primary cultures.

Keywords: oncolytic viruses, non-pathogenic human enteroviruses, glioblastoma multiforme, cell culture, viral infection, sensitivity to viruses, viral oncolysis, virotherapy

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ИЗМЕНЕНИЯ ЧУВСТВИТЕЛЬНОСТИ КЛЕТОК ГЛИОБЛАСТОМ ЧЕЛОВЕКА К ОНКОЛИТИЧЕСКИМ ЭНТЕРОВИРУСАМ ПРИ ПАССИРОВАНИИ В КУЛЬТУРЕ

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Современная терапия мультиформных глиобластом не приводит к излечению пациентов. В качестве альтернативы перспективны онколитические вирусы (ОВ): они способны уничтожать опухолевые стволовые клетки, источники рецидивов. Однако каждый отдельный штамм ОВ эффективен только в ряде случаев. Для подбора подходящего штамма требуется тестирование чувствительности на живых опухолевых клетках пациента. Целью исследования было изучение изменения чувствительности к вирусам в процессе пассирования опухолевых клеток пациента в культуре. Органные и первичные культуры клеток глиобластом получали из операционного материала больных. Проводили сравнение чувствительности к четырем штаммам непатогенных энтеровирусов (вакцинный штамм полиовируса 1 типа, вирус Коксаки А7, Эховирусы 1 и 12) на первичных культурах, и на клетках, прошедших около 700 удвоений при пассировании. Чувствительность к вирусам оценивали по измерению доли жизнеспособных клеток с помощью МТТ теста через 72 ч после заражения серийными десятикратными разведениями вирусных препаратов. Клетки каждого из четырех пациентов имели строго индивидуальные спектры чувствительности к испытанным вирусным штаммам. Различия в минимальной инфекционной дозе, необходимой для заражения культур, составляли до 10^5. При пассировании происходили изменения в чувствительности, которые могли приводить к повышению чувствительности к одному вирусу, и понижению — к другому. Различия в чувствительности коррелировали со способностью зараженных клеток продуцировать инфекционный вирус. На основании полученных данных можно заключить, что испытание индивидуальной чувствительности опухолевых клеток пациентов следует проводить на как можно более ранних этапах пассирования, предпочтительно — на первичных культурах.

Ключевые слова: онколитические вирусы, непатогенные энтеровирусы человека, мультиформная глиобластома, культура клеток, вирусная инфекция, чувствительность к вирусам, вирусный онколиз, виротерапия

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Glioblastoma multiforme is the most aggressive and still incurable form of brain tumors. The median survival time after the diagnosis and treatment is only 15 months [1, 2]. Treatment is complicated by the blood-brain barrier that limits access of chemotherapy drugs to the tumor and by the ability of glioblastoma stem cells to migrate far from the tumor into brain tissues, evading surgical resection. Recently, a hope has emerged that this desperate therapeutic situation can be overcome by using oncolytic viruses. There have been reports of successful treatment outcomes and long-lasting remissions in patients with glioblastomas who received oncolytic virotherapy [3–8]. Moreover, oncolytic viruses have demonstrated the ability to kill glioblastoma stem cells [9–14]. However, virotherapies work only for some patients because the molecular genetic defects in tumors that affect their sensitivity to different viral strains vary between individuals. Therefore, it is wise to use panels of viruses with overlapping specificity against individual tumor cells in order to achieve the desired therapeutic effect. This approach can be more effective if cancer cells of the patient are tested for the sensitivity to a wide range of oncolytic viruses prior to treatment. To run such tests, viable cancer cells from excised tumor fragments are required. Once the cells are obtained, they need to be cultured and passaged to study the underlying cause of their varying sensitivity to different viruses. To test this approach, we optimized protocols for cell culture, cryopreservation and passaging that yielded viable glioblastoma cells. We aimed to determine the extent to which the subcultured cells can retain their original sensitivity to a certain virus type. The cells were cultured from the specimens obtained from different patients. We compared the response of glioblastoma cells from different passages (from primary tissue culture to passage 10) to infection with a few strains of oncolytic enteroviruses.

METHODS

Cell lines of glioblastoma multiforme

Glioblastoma cell lines U87MG and A172 from the American Type Culture Collection (ATCC) were cultured in DMEM (PanEco, Moscow) supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 100 un/ml penicillin, and 100 μg/ml streptomycin at 5% CO_2 and 37 °C. Primary tissue cultures were prepared from fragments of freshly resected tumors.

Tissue and primary glioblastoma cultures

Tumor samples were collected at Burdenko Neurosurgery Research Institute according to the protocol approved by the Institute's Ethics Committee. The samples were collected into sterile tubes filled with DMEM and stored at +4 °C for no more than 24 h. Tissue cultures were prepared from tumor fragments washed in PBS and placed onto sterile culture plates; necrotic tissue and blood vessels were excised using a pair of tweezers and a scalpel. Cell suspensions were prepared from tumor fragments stirred through a sterile nylon mesh with 50-micron pores, purified by centrifugation at 800 g for 5 min 3 times, suspended in the growth medium, and carefully pipetted until a homogeneous suspension of single cells and cell aggregates was obtained. For cryogenic preservation of viable tissue cultures at liquid nitrogen temperature, cell suspensions were placed into DMEM containing 50% serum and 7% dimethyl sulfoxide and aliquoted into cryogenic vials at 1 ml per vial. The vials were kept in a well-insulated container at –80 °C for the first 24 h and then transferred to a liquid nitrogen tank. Dispersed cells were grown to reach the density of 2×10^4 in 1 ml DMEM-F12 (PanEco, Moscow) supplemented with 10% FBS and antibiotics, seeded onto 6-cm plastic culture plates and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was replaced every 3 days. Cell growth was monitored twice a week. When a monolayer of cells was formed on days 6–15 of culturing, the cells were either cryopreserved in liquid nitrogen as described above or passaged further.

Passaging of glioblastoma cultures

About one third of primary cultures demonstrated a stable growth and formed a monolayer. The rest stopped dividing, perhaps due to the lack of favorable conditions. For passaging, monolayers of washed cultured cells were treated with a trypsin solution and plated onto new dishes at a ratio of 1:2–1:3. Passaging was repeated multiple times. With each passage, a portion of cells was cryopreserved.

Strains of oncolytic viruses

In our study we used non-pathogenic strains of human enteroviruses: type 1 poliovirus (Sabin vaccine strain), Coxsackie virus A7 (LEV8), Echovirus 1 (LEV4), and Echovirus 12 (LEV7) [4, 15]. The enteroviruses were propagated in Vero cells (permanent African green monkey kidney cells) at a multiplicity of infection of < 1 PFU per cell and harvested 24 h later. Virus titers were measured using the endpoint dilution assay.

Analysis of viability of infected cells

96-well plates containing primary and continuous cell lines were infected with viruses in a series of 10-fold virus dilutions at a multiplicity of infection from 10^{-5} to 1 PFU per cell in 4 replicates. After one hour of adsorption, the virus-containing fluid was removed, the cells were washed in PBS and cultured in the growth medium supplemented with FBS. In 72 h cell viability was measured by MTT and CellTiter 96® Non-Radioactive Cell Proliferation assays (Promega, USA) according to the manufacturer's protocol.

Analysis of viral replication in the infected cells

Five days after viral infection, we identified the wells in which the cells had been completely lysed by the lowest infectious dose. Following three cycles of freezing and thawing, supernatants were clarified by centrifugation (10 min at 1000 g) and used for virus titration as described above.

RESULTS

In this study we assessed the sensitivity of cancer cells obtained from three patients with glioblastoma and maintained in the culture for different number of passages to a few non-pathogenic strains of human enteroviruses. We compared responses to viral infection between freshly explanted primary glioblastoma cultures and those that had undergone six passages. Passaging lasted for about 2 months and by that time the cells had undergone about 700 division cycles. To determine the lowest effective dose of the viruses, glioblastoma cultures were incubated with serial tenfold dilutions of standard virus preparations; cell viability was measured 72 h after infection. Fig. A shows sensitivity profiles of primary cell cultures prepared from tumor fragments of 3 patients (in the pictures

the profiles are designated as GM-3564-0, GM-3876-0 and GM-3912-0) in response to infection with 4 strains of human enteroviruses. Standard Vero cell cultures routinely used for the propagation of viruses served as the control. Fig. 1B shows the sensitivity of glioblastoma cells from passage 6 (designated in the picture as GM-3564-6, GM-3876-6 and GM-3912-6) to the same viral strains. Vero cells were again used as the control; their sensitivity was measured in a replicate (Vero-2).

The cell sensitivity to 4 enteroviral strains varied significantly between 3 studied primary glioblastoma cell cultures. Culture GM-3564 was most sensitive to Coxsackie virus A7 (the cells were successfully infected using a 10^{-6}-fold dilution of this virus); it was less sensitive to poliovirus (a 10^{-4}-fold dilution was effective) and only slightly sensitive to Echoviruses 1 and 12 (10^{-3}-fold dilutions were effective). Culture GM-3876 was most sensitive to poliovirus (the cells were successfully infected with a 10^{-6}-fold dilution of this virus) and less sensitive to Coxsackie virus A7 (a 10^{-5}-fold dilution), Echovirus 12 (a 10^{-4}-fold dilution) and Echovirus 1 (a 10^{-3}-fold dilution). Culture GM-3912 was most sensitive to Coxsackie virus A7 (a 10^{-7}-fold dilution), poliovirus (a 10^{-5}-fold dilution) and Echoviruses 1 and 12 (a 10^{-4}-fold dilution). Experiments conducted on Vero cells demonstrated that the highest activity was exerted by Echovirus 1 (a 10^{-7}-fold dilution), followed by poliovirus (a 10^{-6}-fold dilution), Coxsackie virus A7 (a 10^{-6}-fold dilution), and Echovirus 12 (a 10^{-5}-fold dilution). Apparently, the passaging affected the sensitivity of cells to certain viruses. GM-3564 cells demonstrated an increased sensitivity to type 1 poliovirus and reduced sensitivity to Coxsackie virus A7, while their sensitivity to Echoviruses 1 and 12 remained unchanged. The sensitivity of GM-3876 cells to poliovirus also increased, while the sensitivity to Coxsackie virus A7 decreased; these cells also exhibited a slight increase in the sensitivity to Echovirus 12, but their sensitivity to Echovirus 1 remained unchanged and low. GM-3912 cells showed a slight increase in the sensitivity to Echovirus 12 and an unchanged sensitivity to the rest 3 viruses.

Different sensitivity of glioblastoma cells to different viruses and the changes induced by passaging may be associated with altered rates of viral replication. To qualitatively assess the replication of viruses, we measured their infectivity titers in the supernatants of cell cultures infected with a penultimate dilution that caused a cytopathic effect. The viral dose in that dilution was about 10 infectious units per each well of a 96-well plate, which is an optimal dose ensuring successful infection of a cell culture. This dose precludes the accumulation of defective interfering particles. The table below presents viral titers in every primary and passaged glioblastoma cell cultures.

Titration results confirm the supposition that changing sensitivity to viral infection during passaging can be associated with a more or less effective replication of a virus. During GM3564 passaging, poliovirus increased its replication over 30-fold, while production of Coxsackie virus A7 decreased 3-fold; replication of Echoviruses 1 and 12 remained unchanged. Different sensitivity ranges were also observed for two other cell cultures.

DISCUSSION

Glioblastoma multiforme is a highly aggressive type of brain tumors. Its genome is very unstable and the cell population is

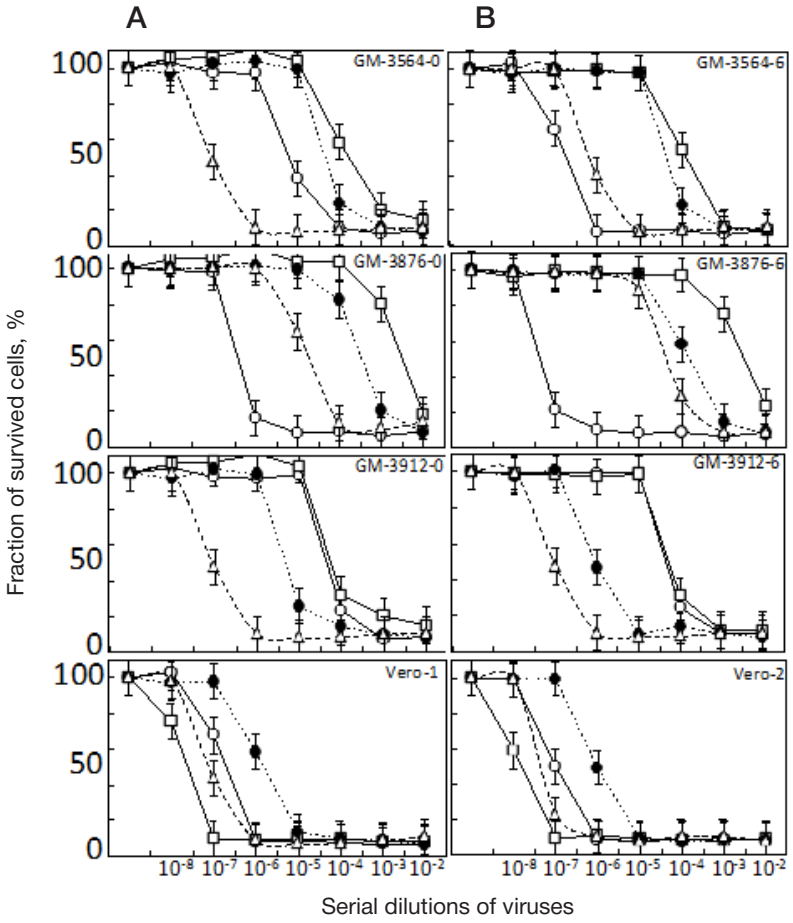

Fig. 1. Sensitivity of primary glioblastoma cell cultures obtained from 3 patients (**A**) and the same cells from passage 6 (**B**) to 4 strains of non-pathogenic oncolytic enteroviruses: —○— — type 1 poliovirus; - -Δ- - — Coxsackie virus A7; —□— — Echovirus 1; ….●…. — Echovirus 12. The horizontal scale (10^{-8}–10^{-2}) shows 10-fold dilutions of virus preparations

Table 1. Infectious titers of viruses produced in primary and passaged glioblastoma cell cultures infected at a low multiplicity of infection (<0.001 infectious units per cell)

Cell culture	Poliovirus 1	Coxsackie virus A7	Echovirus 1	Echovirus 12
GM3564-0	4×10^5	3×10^7	5×10^3	6×10^4
GM3564-6	2×10^7	1×10^7	6×10^3	4×10^4
GM3876-0	1×10^6	2×10^4	1×10^3	3×10^2
GM3876-6	1×10^7	8×10^3	2×10^3	4×10^2
GM3912-0	1×10^4	3×10^7	8×10^3	1×10^4
GM3912-6	1×10^4	4×10^7	8×10^3	8×10^5
Vero-1	5×10^7	8×10^7	2×10^8	5×10^6
Vero-2	4×10^7	1×10^8	1×10^8	5×10^6

heterogenous and continuously changing. Although a certain equilibrium is maintained in the tumor in terms of its cellular composition, supported by local conditions, this balance is shifted when cells are transferred to a culture flask. As a result, cells that are better adapted to *in vitro* conditions may overgrow. Therefore, one can assume that selection of certain cell types can be accompanied by shifts in the sensitivity to oncolytic viruses.

Our findings suggest that passaging of a primary glioblastoma cell culture is accompanied by certain changes leading to an increased or decreased sensitivity to individual viruses. This may be a result of the initial population heterogeneity of cancer cells that, due to a number of causes, have different sensitivity to viruses. Certain cell types tend to thrive excessively in the culture, affecting the overall sensitivity to viruses.

CONCLUSION

We have tested glioblastoma cells obtained from 3 patients for their sensitivity to 4 oncolytic enteroviral strains. We conclude that initial tumor cells differ in their sensitivity to different viruses and passaging may induce qualitative changes in the cell sensitivity to individual viral strains. Our study demonstrates the need for sensitivity tests at the very early stages of *in vitro* cell cultures.

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