PERSISTENCE OF ONCOLYTIC COXSACKIE VIRUS A7 IN SUBCUTANEOUS HUMAN GLIOBLASTOMA XENOGRAFTS IN MICE IN THE CONTEXT OF EXPERIMENTAL THERAPY

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Natural non-pathogenic and vaccine strains of human enteroviruses are currently considered as promising agents capable of treating various kinds of cancer, including glioblastoma multiforme, the most aggressive brain tumor with so far no effective therapy. Enteroviruses can selectively replicate in cancer cells and cause tumor lysis. However, the ability of enteroviruses to persist in tumor tissue for a long period of time and to replicate in several successive cycles while spreading from cell to cell remains largely unclear. This study aimed to determine the possibility of completely destroying subcutaneous mouse xenografts of human glioblastomas through a single intravenous administration of virus-carrying peripheral blood leukocytes, as well as to find out the duration of persistence of the virus in the body of experimental animals in the context of viral therapy. Neurospheres were formed *in vitro* by incubating fragments of patients-derived glioblastomas and used to initiate subcutaneous tumors in immunodeficient mice. It was established that human peripheral blood leukocytes infected *in vitro* can effectively deliver Coxsackie A7 virus to the tumor cells. A single injection of 2×10^4 virus-infected leukocytes led to a gradual regression of tumors, while the virus presence was constantly detectable in the blood of mice, up to the complete regression of the tumors. The study allows to make the conclusion that blood leukocytes can effectively deliver Coxsackie A7 virus to the tumor. In the absence of a full-fledged immune response in mice, the viruses persist in tumors leading to their complete destruction.

Keywords: glioblastoma multiforme, Coxsackie A7 virus, viral oncolysis, mice models, tumor xenografts, experimental therapy, oncolytic viruses

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ПЕРСИСТИРОВАНИЕ ОНКОЛИТИЧЕСКОГО ЭНТЕРОВИРУСА КОКСАКИ А7 В ПОДКОЖНЫХ МЫШИНЫХ КСЕНОТРАНСПЛАНТАТАХ ГЛИОБЛАСТОМ ЧЕЛОВЕКА ПРИ ЭКСПЕРИМЕНТАЛЬНОЙ ТЕРАПИИ

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Природные непатогенные и вакцинные штаммы энтеровирусов человека в настоящее время рассмариваются в качестве перспективных средств для лечения онкологических заболеваний, в том числе мультиформной глиобластомы — наиболее агрессивной опухоли головного мозга, для которой не существует эффективных средств терапии. Энтеровирусы могут избирательно реплицироваться в клетках опухоли, вызывая их лизис. Однако способность энтеровирусов длительно присутствовать в опухолевой ткани и совершать несколько последовательных циклов репликации с распространением от клетки к клетке плохо изучена. Целью исследования было установление возможности полного уничтожения подкожных ксенотрансплантатов глиобластом человека при однократном введении вируса внутривенной доставкой с помощью лейкоцитов периферической крови, а также длительность присутствия (персистирования) вируса в организме экспериментальных животных в процессе вирусной терапии. В качестве опухолевых клеток использовали нейросферы, полученные *in vitro* путем инкубации фрагментов удаленных у пациентов опухолей. Установлено, что лейкоциты периферическойкрови человека, инфицированные *in vitro*, способны осуществлять эффективную доставку в клетки опухоли вируса Коксаки А7. Однократное введение 2×10^4 зараженных вирусом лейкоцитов приводило к постепенной регрессии опухолей при постоянно определяющемся присутствии вируса в крови мыши. По результатам исследования сделан вывод, что доставка энтеровируса Коксаки А7 в опухоль может быть эффективно осуществлена с помощью лейкоцитов крови. В отсутствие полноценного иммунного ответа в опухолях у мышей наблюдается персистирование вирусов, заканчивающееся их полным уничтожением.

Ключевые слова: мультиформная глиобластома, вирус Коксаки А7, вирусный онколиз, мышиные модели, ксенотрансплантаты опухолей, экспериментальная терапия, онколитические вирусы

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Therapy of brain tumors, especially of glioblastoma multiforme (GBM) still remains an unresolved problem [1, 2]. The search for new alternative approaches to their treatment is of particular importance. The main problem lies in the resistance of tumor-initiating stem cells to therapy, which inevitably leads to relapses. Many oncolytic viruses can effectively destroy glioblastoma stem cells and prevent the relapses [3–8]. As the tumor develops, its cells acquire a number of characteristic properties that can serve as specific targets for therapy [9]. Besides, they lose many specific functions the cells serve in a multicellular organism [10–12]. One of those functions is protection from viruses [13–15]. Infected with viruses, tumor cells generally neither are capable of inducing type 1 interferons, nor acquire immunity to reinfection with viruses after interferon treatment [16–20]. This is why tumor cells display the increased sensitivity to infection by many different viruses, and justify the development of oncolytic viruses for cancer therapy [21–24]. Oncolytic viruses not only selectively infect and destroy cancer cells but also significantly activate antitumor immunity and modify tumor microenvironment. They stimulate both innate and adaptive immunity, which results in an extended antitumor effect even after the virus is no longer present in the tumor [25–28]. Various oncolytic viruses make use of the above mechanisms in their own way. It is convenient to study direct viral oncolysis caused by direct replication of viruses in a model of tumor xenografts implanted either to immune deficient athymic mice [29] or to mice with severe combined immunodeficiency (SCID) [30]. These models also allow refining virus delivery approaches. Virus administration should result in the successful infection of some tumor cells with subsequent initiation of viral replication cycles, release of virus progeny and further expansion of viral infection to remaining tumor cells located in the same or distant tumor nodes. The process is easily launched once the virus is directly injected to the virus–sensitive tumor. However, in most cases of metastatic cancer tumor sites are not accessible to such injections. Systemic administration of the virus through intravenous or intramuscular injections is also often ineffective, as the virus quickly leaves the circulation being absorbed by endothelial cells, or destroyed by some nonspecific protective factors in the blood. A promising alternative to such systemic delivery is the use of virus-sensitive carrier cells, infected *in vitro* and introduced to the bloodstream [31–33]. In such cells, the virus replicates as they circulate through the body, and then the virus particles are released in distant parts of the body, including tumors. In this study, we used the model of subcutaneous human glioblastoma tumor xenografts. Immune deficient mice were injected with cultured neurospheres derived from glioblastoma tumors of two patients. Neurospheres are dense clusters of cells developed through the culturing of tumor explants under conditions preventing the attachment of cells to culture plates. The culturing takes place in a special medium containing epidermal growth factor and fibroblast growth factor (EGF, bFGF, respectively) [34, 35]. Like other spheroids derived from human tumors, neurospheres are rich in tumor-initiating stem cells [36] and therefore have increased tumorigenisity [37, 38]. This study aimed to refine the delivery of oncolytic enteroviruses with the help of peripheral blood leukocytes in the model of subcutaneous tumor xenografts in mice, as well as to establish the duration of persistence of the virus in the body of experimental animals in the context of viral therapy.

METHODS

Cells culture for viruses titration

The Vero cell culture (immortalized kidney cells of the African green monkey) was grown in DMEM medium (PanEco, Russia) supplemented with 10% fetal bovine serum (FBS), 100 mg/ml penicillin and 100 mg/ml streptomycin. The cells were grown in 10 cm plastic culture dishes in a humidified atmosphere containing 5% CO_2 at a temperature of 37 °C; then, they were dispersed every 3 days in the ratio of 1:4–1:6.

Neurospheric tumor-forming glioblastoma cell cultures

Obtaining cell cultures from patients with glioblastoma (GM-3564 and GM-3876) has been described previously [39]. To boost tumor development, we used SCID/Beige immunodeficient mice as experimental animals (obtained from the Novosibirsk SPF vivarium and maintained in the laboratory); the mice received subcutaneous administration of neurospheres. The glioblastoma neurospheres used were only passed twice and kept at the nitrogen liquification temperature. They were defrosted immediately before the start of the experiments. The medium they were plated on was DMEM + F12 medium (PanEco, Russia) containing 20 ng/ml EGF and 10 ng/ml bFGF, and placed in an incubator with 5% CO_2 at 37 °C. When the neurospheres developed (in 7–10 days), they were washed twice with PBS, counted, carefully pipetted until the disappearance of large cell aggregates and injected subcutaneously into SCID/Beige mice, 500 spheroids per insertion point. The tumors appeared in 3 weeks. The tumors about 10 mm in diameter were excised, dispersed through a sterile nylon mesh with a pore diameter of 50 μm, treated with collagenase (PanEco, Moscow) to obtain a cell suspension, washed twice with PBS; the resulting suspension was subcutaneously administered to SCID/Beige mice in the amount of 2×10^5 cells per injection point, the goal being to obtain tumors to test the oncolytic activity of the virus. Preliminary adaptation of the neurospheres to growth as tumors in mice resulted in boosted tumorigenicity and an increase in the number of tumors developed after repeated administration.

Oncolytic virus strain

We used the LEV8 strain of Coxsackie A7 enterovirus [40, 41] that can effectively replicate in GM-3564 and GM-3876 cells [39]. Titration of the infectious activity of viral preparations was done with the help of the final dilution method and Vero cell culture using 96-well plates.

Delivering the virus with peripheral blood leukocytes

The peripheral blood leukocyte fraction was obtained from the freshly harvested heparinized human blood by centrifugation in a Ficoll-Paque solution (PanEco, Russia) following the standard protocol [42]. The leukocytes, washed twice in DMEM medium, were counted and a suspension with a density of 10^6 cells/ml prepared. The suspension was incubated with Coxsackie A7 virus (10 infectious units per cell) at 37 °C for 1 hour. Then the leukocytes were washed 3 times with 10 ml of PSB (0.14 M NaCl) and centrifuged at 800 g for 5 min. The infected leukocytes (2×10^4 cells) were injected into the the tail vein of SCID/Beige mice in a volume of 0.1 ml; they bore about 400–600 μl to the tumor. The tumor size was measured every third day. To register presence of the virus in the mice's blood, we took a drop of it from the tail vein and titrated on Vero cells applying the final serial dilutions method and using 96-well plates.

RESULTS

Earlier, we found that GM-3564 and GM-3876 cell cultures obtained from the tumor material of two glioblastoma patients

are highly sensitive to the Coxsackie A7 virus [39]. In the context of this study, we used this strain to find out if it is possible to deliver it to the tumor with the help of a cell carrier, as well as to determine if the virus can persist for a long period of time and thus make the oncolytic effect stable. To achieve the goals set, we implanted GM-3564 and GM-3876 neurospheric cultures xenografts subcutaneously to SCID/Beige mice. After subcutaneous administration of the neurospheres, the tumors grew to 400–600 μl in 10 days. We divided the mice into 2 groups of 10 animals each for our experiments, one for each type of tumor cells (total of 4 groups): one group received virus-infected leukocytes injections (tail vein), another — non-infected leukocytes (control). Figure 1 shows the dynamics of the tumor size changes (in mm^3); the measurements were taken on every third day for 27 days. In the control group, which had non-infected leukocytes injected to the tail vein, the tumors continued to grow; the mice were euthanized when the tumors in them reached the size of 1500 mm^3. Generally, it happened sometime between days 9 and 15 after injection of the leukocytes. In the treatment groups, where the mice received leukocytes infected with Coxsackie A7 virus (injected into the tail vein), the tumors continued to grow for 3 more days and then rapidly collapsed. The effect was the same for both GM-3564 and GM-3876 cells. In 18–21 days after the injections, it was already impossible to measure the tumors; only a subtle subcutaneous scar tissue was found in their place. At the same time, the virus titer was detected in the mice's blood every three days (Table).

The first encounter of the virus in the blood of mice occurred on the 3rd day after the injection; on the 6th day, its quantity peaked and then began to decrease, same as the size of the tumor. From days 18–21 and on, the virus could not be registered anymore and the mice were practically free from tumors.

DISCUSSION

We used the human glioblastoma xenografts model in SCID/Beige line mice and found that it is possible to deliver the oncolytic Coxsacki A7 virus to the tumor in human peripheral blood leukocytes infected *in vitro* and injected into the tail vein. This method of administration made the virus detectable in blood on the 3rd day and ensured its presence there until the tumors disappeared. Thus, the virus persisted in mice as long as there were virus-sensitive tumor cells. Destruction of such cells lead to disappearance of the virus. Previously, we have observed extended persistence of type 1 poliovirus in mice with A172 glioblastoma xenografts; in that experiment, tumors and virus disappeared from the mice's organisms simultaneously [43]. However, in contrast to the present study that research implied injecting mice intravenously with large doses of free virus. The Coxsackie A7 virus enters cells with the help of LIMP-2 protein encoded by SCARB2 gene [44]. LIMP-2 expresses on the surface of many types of human cells, including leukocytes; it seems that the protein contributes to the spread of virus throughout the body and participates in the expansion of enterovirus infections caused by some pathogenic strains of Coxsackie A. The virus delivery method we applied has a number of advantages over systemic administration of free virions: being inside the cell, the virus is protected from antibodies and other factors that can inactivate it. We presume that the virus is capable of a limited replication within leukocytes, which accounts for its appearance in the remote areas of the body, including tumors. Also, the delivery with leukocytes allows significant reduction of the initial amount of virus needed for therapy. Further studies should be aimed at finding out the applicability of this method to treatment of cancer patients.

CONCLUSIONS

We have found that intravenous injection of leukocytes carrying an oncolytic strain of the Coxsackie A7 virus to immunodeficient SCID/Beige line mice leads to a rapid collapse and subsequent disappearance of subcutaneous tumor xenografts obtained from glioblastoma cells of two different patients. The virus actively multiplied in mice while there were virus-sensitive tumor

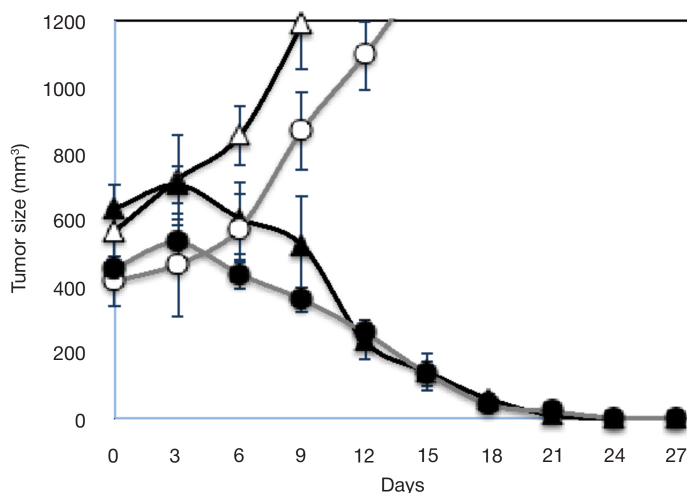

Fig. 1. Size of the GM-3564 and GM-3876 glioblastoma subcutaneous tumor xenografts: dynamics of change, control (uninfected human leukocytes) and treatment (leukocytes carrying Coxsackie A7 virus) groups. Δ — leukocytes injected to mice with GM-3564 tumors; ▲ — infected leukocytes injected to mice with GM-3564 tumors ; ○ — leukocytes injected to mice with GM-3876 tumors; ● — infected leukocytes injected to mice with GM-3876 tumors

Table. Coxsackie A7 virus titers found in the blood of the treatment group mice 0–27 days after the injection of virus-infected leukocytes

Days	0	3	6	9	12	15	18	21	24	27
GM-3564	n.	1.5×10^2	1.7×10^4	3.2×10^3	2.6×10^3	6×10^2	1.9×10^2	n.	n.	n.
GM-3876	n.	n.	1.0×10^2	5.2×10^3	5×10^3	1.2×10^3	2.6×10^2	n.	n.	n.

Note: n. — virus not detected.

cells in their bodies. The results of this study show that even in the absence of T-cell immunity, oncolytic enterovirus can destroy glioblastoma tumors in athymic mice through direct cytolytic action. Also, we have found that using leukocytes as virus carriers is an effective method of delivering the latter to tumors.

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