A. V. Soboleva et al /J. Pharm. Sci. & Res. Vol. 10(4), 2018, 936-939

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Glioblastoma Multiforme stem cells are highly sensitive to some human non-pathogenic enteroviruses

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Abstract

Glioblastoma multiforme (GBM) is the most severe and still incurable form of brain tumors. Following surgical removal of the tumor, subsequent chemotherapy and radiation therapy, a relapse of the disease inevitably occurs, which is resistant to further treatment. The cause of relapse is tumor stem cells that can penetrate deep from the resectable tumor and display a high resistance to radio- and chemotherapy. Oncolytic viruses can be a promising alternative for the treatment of GBM. We used a set of four nonpathogenic human enteroviruses (Sabin live vaccine strain of Poliovirus Type 1, Echovirus 12, Coxsackievirus A7, Coxsackievirus B5) to test the sensitivity of GBM cells obtained from five patients. The presence of stem cells in the cell population was evaluated by the formation of neurospheres in a specialized serum-free medium. Neurospheres are enriched with the GBM-initiating stem cells, while the GBM cell cultures maintained in the presence of fetal bovine serum form monolayers of attached differentiated cells that contain little or no stem cells. We compared the sensitivity to viruses of differentiated GBM cells and the corresponding neurosphere cultures. The monolayer cultures of GBM have demonstrated generally high but variable sensitivity to the enteroviruses. Each of the cell cultures tested was highly sensitive to at least four enterovirus strains. The same strains that demonstrated good replication in monolayer cultures were also active in spheroid cultures originated from the same patient suggesting that the viruses can efficiently kill GBM stem cells. Based on the results of the study, it can be concluded that the potential use of oncolytic enteroviruses for glioblastoma therapy is promising.

Key words: glioblastoma multiforme, stem cells, stem cell markers, resistance to therapy, oncolytic enteroviruses.

INTRODUCTION

GBM is the most aggressive and still incurable form of malignant brain gliomas [1]. Cancer stem cells (CSC) contribute significantly to the invasiveness and the aggressive growth of malignant tumors [2], as they penetrate deeply from the site of a primary tumor and usually inaccessible for a surgical removal [3]. In addition, GBM stem cells are remarkably resistant to radio and chemotherapy, which is apparently partly due to the ability to effectively repair DNA damage [4], as well as increased expression of membrane proteins of the ABC family of transporters that successfully pump out therapeutic drugs from the cells [5, 6]. For these reasons, after any course of therapy, recurrence of the disease is almost inevitable [7]. Therefore, it is especially important to search for therapeutic effects that can effectively destroy glioblastoma stem cells without showing significant toxicity to normal cells and tissues [8-10]. In this capacity, oncolytic viruses seem to hold promise, as several cases of long-term remissions and even complete cure have already been described after the experimental virotherapy [11-14]. The use of human enteroviruses as potential oncolytic agents capable of killing stem cells with gliomas also seems promising [15]. Enteroviruses circulate widely among the population, multiplying in the digestive tract, mainly in the cells of lymphoid organs - tonsils and Peyer's plaques. They are small (diameter about 25-30 nm) RNA-containing non-enveloped viruses with a single-stranded genome of positive polarity and belong to the family of picornaviruses [16]. Enteroviruses are divided into several groups and serotypes, which have traditionally been divided into ECHO, Coxsackie A and Coxsackie B viruses and polioviruses, but the more modern classification includes nine types of enteroviruses, denoted by letters from A to J [17]. To interact with the cell, enteroviruses use various surface proteins, such as CD55 / DAF, CD155 / PVR, CXADR / CAR, ICAM-1, VLA-2, SCARB2 and possibly some others [18] as receptors. After a penetration into the cell through endocytosis, the virion is internalized [19], and the replication cycle is being started. With the spread of enteroviruses beyond the gastrointestinal tract, they can cause diseases of varying severity. The most serious enterovirus infection is poliomyelitis. Coxsackie viruses of groups A and B, ECHO viruses and many other enteroviruses, as a rule, are not so dangerous, although they can sometimes cause diseases such as arachnoiditis, meningoencephalitis, myocarditis and some others. However, in most cases the infection proceeds asymptomatically, which allows considering enteroviruses as nonpathogenic saprophytic viruses [20]. Such variants of enteroviruses are the most promising candidates as oncolytic strains, which is confirmed by a number of experimental results [16]. In this study, we used six strains of nonpathogenic enteroviruses isolated from healthy individuals, as well as three live vaccine strains of polioviruses to test their ability to kill GBM stem cells.

MATERIALS AND METHODS.

Cell lines.

Human rhabdomyosarcoma cell line RD (ATCC CCL-136) that is highly sensitive for the enterovirus strains used in the study and human glioblastoma cell line U87MG (ATCC HTB-14) were grown in Dulbecco-modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum and 100 mg/ml each of penicillin and streptomycin.

Isolation of patient-derived glioblastoma cells.

Samples of tumors from surgical material were obtained from N.N. Burdenko Institute of Neurosurgery, after receiving informed consent of patients according to the approved protocol of the ethical committee. The diagnosis of Glioblastoma Multiforme was confirmed histologically and on the basis of clinical manifestations. Surgical material was taken mainly from the outside edge regions of tumors, in a volume of 2-5 ml. The samples were placed in sterile tubes containing chilled sterile DMEM, and within 2-3 hours were delivered to the laboratory. Tumor tissue was washed twice in sterile phosphate buffered saline (PBS, PanEco, Moscow) supplemented with 500 μg / ml penicillin and streptomycin and then placed in a sterile Petri dish in a small amount of PBS. Blood vessels and clots, necrosis masses and normal brain tissue were removed under the control of a stereomicroscope. The tumor masses were then crushed through a sterile nylon mesh, 0.1 mm pore size (SPL Lifesciences). The suspension was adjusted with a sterile DMEM medium to a volume of 10 ml, and then washed three times with 10 ml of DMEM medium and centrifugation for 4 minutes at 160 g, room temperature. For the long-term preservation of viable cells obtained from tumors and prior to culturing the washed dispersed tumor samples were brought to 7% dimethylsulfoxide, 10 mg/ml bovine serum albumin and stored in the liquid nitrogen vapor phase.

Growing of neurospheres.

For preparation of neurospheres enriched with glioblastoma stem cells [21-23], the pellet of the freshly-processed glioblastoma masses was washed with 10 ml PBS, suspended in 0.25% trypsin-EDTA solution and incubated for 15 min at 37^{o}C. The digestion was stopped by the addition of BSA (ThermoFisher) to 1 mg/ml, gently pipetted and pelleted by the low-speed centrifugation. The cells were then suspended in 15 ml of Neurosphere medium that constituted Neurobasal Medium (Life Technologies) supplemented with 1× B27 without vitamin A (Thermo Fisher), 2 mM glutamine, 1 mM sodium pyruvate, 10 ng/ml basic fibroblast growth factor (bFGF), and 10 ng/ml of epidermal growth factor (EGF, PanEco, Moscow) and 100 μg / ml of penicillin and streptomycin, the number of cells was counted, adjusted to 4 x 10^5 cells / ml and dispersed in 1.5 ml per well in 6-well plates. The medium was half-changed every two to three days until a growth of neurospheres became prominent. At day 10-14 the neurospheres were collected, washed with PBS and dispersed to single-cells by 0.25% trypsin-EDTA digestion at 37^{o}C for 10-20 min. The single-cell suspension was used for neurosphere passaging, for a preparation of differentiated glioblastoma cell cultures, or for storage at the temperature of liquid nitrogen.

Growing differentiated glioblastoma cells.

The single-cell suspension obtained by the digestion of neurospheres was plated at a density of 5 x 10^4 cells per ml in DMED supplemented with 10% fetal bovine serum, 100 μg / ml of penicillin and streptomycin, in 60 mm tissue culture treated plates. The cells were split 1:3 - 1:4 every 3-5 days after a digestion with 0.25% trypsin-EDTA. The conditions of growth in the presence of serum, without the additional growth factors resulted in the formation of monolayers of differentiated glioblastoma cells with very low expression levels of the stem cell marker CD133 [22].

Testing sensitivity of glioblastoma cell cultures to enteroviruses.

The following enterovirus strains were used in the study: vaccine Sabin strain of poliovirus type 1 (PVS1), and four human enterovirus strains isolated from healthy individuals [16, 20] - Coxsackievirus A7 (LEV8 strain), Coxsackievirus B5 (LEV14 strain), Echovirus 12 (LEV7 strain) were propagated in RD cells and stores as clarified cell culture supernatants at -20^{o}C. Virus titers were determined by infecting monolayers of RD cells with serial dilutions of the virus stock in 96-well plates. Cultures of differentiated glioblastoma cells (four parallel samples for each point) were infected at a multiplicity of infection (MOI) of 0.1 (0.1 infectious units per one cell) in a serum-free medium. After a one-hour absorption at 37^{o}C the inoculum was removed, and the cells were washed three times with PBS. Mock infection and a treatment with 100 □M temozolomide (TMZ) were used as controls. The cells were then incubated in DMEM supplemented with 2% fetal bovine serum and antibiotics for four days at 37^{o}C in a humidified 5% CO_2 atmosphere. Parallel samples were scored daily for cell viability (standard MTT test) and for virus titer in the supernatants after three cycles of freezing and thawing. For virus titration the cultures were subjected to three cycles of freezing and thawing, the supernatants were clarified by low-speed centrifugation, and virus titers were determined by titration in RD cells as described [24].

Testing the sensitivity of glioblastoma stem cells in neurospheres to enterovirus strains. Neurospheres for the virus sensitivity tests were obtained by plating 5 x 10^4 cells obtained after digestion of neurospheres in 6-well plates in a volume of 1.5 ml of serum-free Neurosphere medium (see above). The medium was half-changed every 3 days, and by day 10 the cultures containing neurospheres were ready for virus infections. The neurospheres were washed in the fresh medium and incubated at MOI=5 with individual enterovirus strains. Mock infection and a treatment with 100 □M temozolomide (TMZ) were used as controls. The infected neurospheres were washed three times after a one-hour adsorption period, resuspended in the fresh Neurosphere medium and incubated for 48 hours in CO_2 incubator at 37^{o}C. The neurospheres, despite the cytopathic effect, were washed three times in PBS containing specific sheep antibodies to the appropriate enteroviruses capable of neutralizing up to 10^7 TCID50 of viruses, and then incubated in the Neurosphere medium containing the neutralizing antibodies for 24 hours in CO_2 incubator. The samples were then collected, digested with trypsin-EDTA, the reaction was stopped with the addition of 1 mg/ml of BSA, and the cells were plated for neurospheres formation to 6-well plates with the Neurosphere medium containing virus-neutralizing antibodies. The number of neurospheres was calculated at day 14 after the plating.

RESULTS AND DISCUSSION

Tumor samples from 45 confirmed cases of GBM were plated for a neurosphere formation. We obtained a total of 15 cultures showing growth of neurospheres (the success rate was 34%). Because of the variable growth rate of neurospheres we were able to select 5 neurosphere cultures suitable for further analysis. Neurospheres were easily converted to differentiated glioblastoma cell monolayers by plating to the medium containing 10% fetal bovine serum, without the specific additives required for the maintenance of the stem-like state of

glioblastoma cells. Table 1 shows that when 2 x 10^5 cells of each pair of cells obtained by digestion of neurospheres and differentiated monolayers were plated in Neurosphere medium to 60 mm plates the number of neurospheres formed by day 14 has differed dramatically suggesting that the monolayer cultures were substantially devoid of stem-like cells capable of producing neurospheres.

We then tested the sensitivity of differentiated glioblastoma cell cultures to infection with different non-pathogenic strains of human enteroviruses. Glioblastoma cells were passaged for 14 days in DMEM supplemented with 10% FBS to allow their differentiation. The cells were then seeded to 96-well plates. Parallel 96-well plates were infected at MOI=0.1 with enteroviruses, washed after one-hour adsorption, and kept in DMEM plus 2% FBS for three days.

With one-day intervals the viability of cells (MTT test, Figure 1A), and virus titers (Figure 1B) were determined. Cytopathic manifestations were emerging within 18-24 hours in some cultures, but the dynamics and final toxicity by the end of day 3 were variable. The cytopathic changes correlated with the titers of viruses accumulated at different time points. From the results presented in Figure 1 we conclude that the glioblastoma cultures have revealed very different spectrums of their sensitivity to different human enteroviruses and titers of newly produced viruses. However, with the exception of one culture (GB461) that was totally resistant to Echovirus 12/LEV7, all other combinations resulted in a complete lysis of cells and a variable production of newly replicated viruses.

The cultures were also treated with 100 □M of Temozolomide (TMZ), which resulted in a nearly complete death of all glioblastoma cultures within 3 days (Figure 2) suggesting that differentiated cultures of glioblastoma cells are sensitive to TMZ.

To test the sensitivity of neurospheres that are rich in the glioblastoma-initiating stem-like cells we have grown five of the patient-derived glioblastoma cultures in the Neurosphere medium for two weeks, collected and washed neurospheres, normalized their volumes by measuring the number of cells by digestion of a sample with trypsin, and infected neurosphere samples each representing 5 x 10^5 cells with the set of human non-pathogenic enteroviruses at MOI=5. After the adsorption period the neurospheres were washed and incubated for two days in a CO_2 incubator. To stop further spreading of the newly synthesized viruses the neurospheres were then treated with specific virus-neutralizing antibodies and all further cultivations were performed in the media containing the appropriate antibodies. The neurospheres were collected, dispersed to single cells and seeded to 6-well plates in Neurosphere medium. Medium was changed every 3 days with the addition of fresh antisera. Counting of neurospheres was performed two weeks after the plating. Results of the experiment performed in duplicate 6-well plates are summarized in Table 2.

Table 1. Numbers of neurospheres formed by seeding 2 x 10^5 cells from neurospheres and differentiated cultures and maintained in the Neurosphere medium for 14 days.

Glioblastoma sample	GB421	GB427	GB445	GB458	GB461	U87MG
Neurosphere	98/81	67/54	38/49	112/78	29/45	128/136
Differentiated	1/0	0/0	3/0	0/4	1/3	2/3

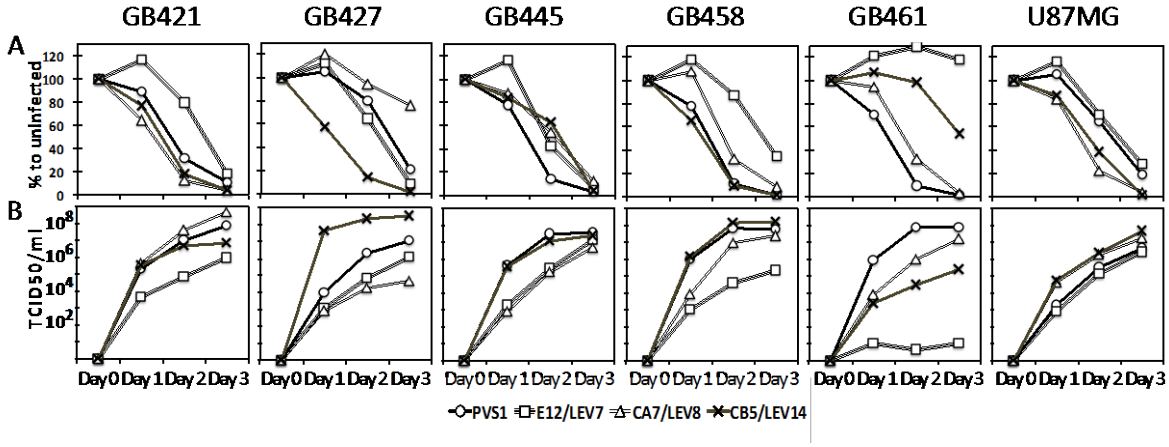

Figure 1. The patient-derived differentiated glioblastoma cell cultures are differentially sensitive to a set of non-pathogenic enteroviruses. (A) Time-course of cell viability following infection with different viruses measured in MTT assay; (B) Time-course of virus accumulation in different glioblastoma cultures. Virus titers are expressed in TCID50/ml of supernatants.

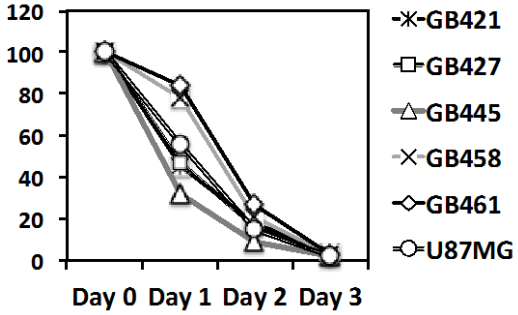

Figure 2. Time course cytotoxicity induced by 100 □M of Temozolomide (TMZ) assay.

Table 2. The number of neurospheres formed after the treatment of samples of neurospheres each representing 5 x 10^5 cells with enteroviruses at MOI=5 for two days, and incubation of single-cell suspensions prepared from the treated neurospheres in Neurosphere medium containing virus-neutralizing antibodies for 14 days. Treatment with 100 □M of TMZ for two days was us

Neurosphere treatment	GB421	GB427	GB445	GB458	GB461	U87MG
Mock-infection	108/96	87/94	68/55	218/178	47/69	132/156
100 □M TMZ	36/21	12/10	21/11	38/44	8/14	21/17
PVS1	0/0	0/2	0/0	0/0	0/0	0/1
E12/LEV7	0/0	0/0	0/0	2/4	23/48	1/3
CA7/LEV8	0/0	4/6	0/0	0/0	0/0	0/0
CB5/LEV14	0/0	0/0	0/0	0/0	7/9	0/0

The results indicate that there is good correlation between the ability of a virus to replicate in a particular differentiated glioblastoma cell culture and the sensitivity of neurospheres to the virus. For the virus-cell pairs that have demonstrated a complete degeneration of differentiated cell culture, there was a complete inhibition of neurospheres formation. However, for some combinations, such as GB427 cells/CA7 virus, or GB461 cells/CB5 virus, the effect was partial, and a limited quantity of neurospheres was detected. For the virus-resistant pair GB461/E12 the inhibition of neurospheres formation was minimal. We conclude that most of the enterovirus strains used were capable of producing strong inhibition of neurosphere formation, which is in contrast to a relatively weak effect of TMZ treatment. Our results suggest that human enteroviruses may possess a promising therapeutic potential for the treatment of recurrent glioblastoma cases.

CONCLUSION

We conclude that GBM cells maintained either as differentiated monolayer cultures or as spheroids enriched in stem cells display generally high but variable sensitivity to either of the four enterovirus strains used in the study. The results suggest that human enteroviruses are active in destroying GBM stem cells making them promising candidates for the treatment of glioblastoma patients. However, more studies are required for finding specific biomarkers that would predict individual responses of tumors to a particular set of oncolytic enteroviruses.

ACKNOWLEDGEMENT

The study was supported by the Ministry of Science and Education of Russia, project code RFMEFI60714X0014

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