ISSN 0026-8933, Molecular Biology, 2012, Vol. 46, No. 5, pp. 639–650. © Pleiades Publishing, Inc., 2012.
Original Russian Text © P.M. Chumakov, V.V. Morozova, I.V. Babkin, I.K. Baikov, S.V. Netesov, N.V. Tikunova, 2012, published in Molekulyarnaya Biologiya, 2012, Vol. 46, No. 5, pp. 712–725.

REVIEWS

UDC 578.5

Oncolytic Enteroviruses

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Received February 17, 2012

Abstract—The growing body of knowledge concerning the molecular biology of viruses and virus–cell interactions provides possibilities to use viruses as a tool in an effort to treat malignant tumors. As a rule, tumor cells are highly sensitive to viruses, which can be used in cancer therapy. At the same time, the application of viral oncolysis in cancer treatment requires that the highest possible safety be ensured for both the patient and environment. Human enteroviruses are a convenient source for obtaining oncolytic virus strains, since many of them are nonpathogenic for humans or cause mild disease. The current progress in genetic engineering enables the development of attenuated enterovirus variants characterized with high safety and selectivity. This review focuses on the main members of the *Enterovirus* genus, such as ECHO, coxsackievirus, and vaccine strains of poliovirus as a promising source for the development of oncolytic agents applicable for cancer therapy. We have summarized the data concerning recently developed and tested oncolytic variants of enteroviruses and discusses the perspectives of their application in cancer therapy, as well as problems associated with their improvement and practical use.

DOI: 10.1134/S0026893312050032

Keywords: oncolytic enteroviruses, viral cancer therapy, Coxsackieviruses, Echoviruses, Polioviruses

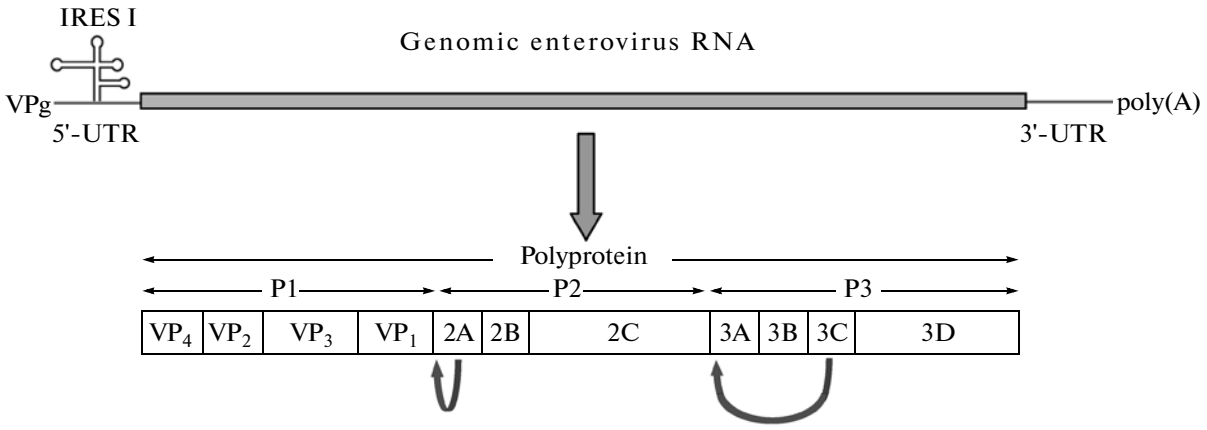
INTRODUCTION

Despite the doubtless considerable progress in the understanding of malignant transformation, tumor therapy still constitutes an extremely difficult problem. Until the beginning of the 20th century, the only available possibility was surgical resection of the tumor. Following the discovery of X-rays and radioactivity, high hopes were associated with radiotherapy; however, there was no major breakthrough [1]. Chemotherapy began to develop in the end of 1940s; for a number of tumors, it significantly increased patients' survival rates, especially in the case of leukemias [2, 3]. However, an important shortcoming of both radio- and chemotherapy is their relatively low specificity. In recent years, the development of chemotherapeutic drugs and antibodies more specific to certain types of cancer cells has become a topical issue [4–6]. An alternative research approach is focused on potential applications of oncolytic viruses.

The possibility to use viruses in anticancer therapy is based on the selectivity of the cytolytic action that nonpathogenic or mildly pathogenic human viruses effect on cancer cells. As early as in the 1900s, an unexpected improvement was observed in some cancer patients following vaccination or a viral disease. For instance, the reported cases include the sound remission of leukemia after an influenza-like disease [7], and tumor regression in a cervical cancer patient after an anti-rabies vaccination [8].

Soon after the discovery of viruses, a search for the optimal cultivation models began. It was found that tumor cells commonly exhibit increased sensitivity to many viruses. In the 1940s, Levaditi and Nicolau [9–11] showed that many infectious viruses were oncotropic. It was found that viruses could proliferate selectively in animal tumors, and, in some cases, oncotropism was accompanied by oncolytic activity [11]. These observations suggested that viral oncolysis could theoretically be used in anticancer therapy.

The oncolytic activity of viruses was further investigated in the 1950s–1970s [12]. In particular, it was experimentally shown that measles induced temporary remission in patients with Hodgkin's disease and Burkitt's lymphoma [13, 14]. Oncolytic activity was observed for influenza viruses [15], enteroviruses [12, 16], and some other types of viruses [17, 18]. There have been reports of successful applications of adenoviruses in cervical cancer [19–21] and enteroviruses in gastrointestinal tumors [22].



Structure of enterovirus genome and encoded proteins. IRES I, type-I internal ribosome binding site; 5'UTR and 3'UTR, 5'- and 3'-untranslated regions. Arrows at the bottom indicate the positions where two viral proteases, 2A and 3C, cleave the enterovirus polyprotein to three fragments, P1, P2, and P3.

In spite of these inspiring results, the use of virus-based anticancer treatment encountered substantial difficulties. First, there have been cases of uncontrollable viral infection drastically deteriorating the patients' state. Second, in most cases viral infection provoked due virus-specific immune response, which weakened considerably the oncolytic effect. Next, the clinical application of these approaches was hampered by the lack of understanding of the mechanisms underlying the antitumor properties of viruses. Ethical criteria of trials concerning the antitumor activity of the virus were also lacking, and some clinical trials that failed to follow appropriate guidelines seriously undermined the idea of employing the phenomenon of viral oncolysis in anticancer therapy. For all of these reasons, research into the anticancer potential and efficiency of viruses was suspended for several decades [23].

The next step in the study of oncolytic viruses was associated with advances in molecular biology and virology, as well as with progress in genetic engineering techniques. It became possible to design recombinant viruses that infect tumor cells with increased selectivity or induce the production of antitumor compounds in infected cells [24, 25]. Modern approaches to increasing virus selectivity to tumor cells have are directed at two major tasks. The first approach, transduction targeting, implies the modification of virion surface proteins so as to promote their preferential binding to the receptors characteristic mainly for the tumor cell surface [26]. The second strategy, nontransductional targeting, is aimed at increasing the selectivity of virus replication in tumor cells [24, 27]. Each of these approaches enhances oncotropism; however, the strongest effect can be achieved by combining both of them [28]. Experimental oncolytic strains have been developed based on viruses of several families, the most promising certainly being the viruses whose prototypes are not associated with serious human diseases.

ENTEROVIRUSES: CHARACTERIZATION AND CLASSIFICATION

Enteroviruses belong to the *Enterovirus* genus of the Picornaviridae family. Thanks to their resistance to the acidic environment of the stomach, they replicate mainly in the gastrointestinal tract. They are also resistant to many detergents and disinfectants, and retain viability at room temperature for a long time. Enteroviruses are ubiquitous; the genus comprises at least ten species, which are subdivided in different genotypes and serotypes. Among enterovirus species, there are both those that are pathogenic for humans, as well as those with no proven connection to any disease [29]. In particular, the genus includes the poliovirus, the agent of poliomyelitis.

Enteroviruses are non-enveloped viruses with icosahedric virions 28–30 nm in diameter. Their capsid is built of four structural proteins, VP1–VP4 [30]; the outer surface is formed by VP1–VP3, and VP4 is located within the capsid. An enterovirus genome is a single-stranded (+)RNA molecule 7.2–8.5 kb long, which is infectious when introduced into a cell. Genomic RNA contains a single open reading frame (ORF), 5'- and 3' noncoding regions, and a poly(A) sequence at the 3'-end (figure). The 5'-end of the genome is covalently bound to the VPg protein [31, 32]. The 5'-untranslated region (UTR) is highly structured; it contains regions required for genome replication, as well as an internal ribosome-binding site (IRES) used for the cap-independent translation of viral RNA [33, 34]. The 3'-UTR of the enterovirus genome is required for the initiation of (–)RNA strand synthesis [35]. Viral RNA lacking the poly(A) sequence loses its infectivity [36]. Genomic RNA of enteroviruses can recombine with relatively high frequencies [37]; the probability of recombination is higher for RNA molecules with higher homology [30].

The replication of enterovirus RNA, as well as virion assembly, occurs in the cytoplasm of an infected

cell. In the cytoplasm, viral genomic RNA is translated to a single precursor protein [38]. Next, the precursor is cleaved by viral proteases, first to three fragments, and then to mature viral proteins (figure). In addition to the structural proteins of the virion, the precursor protein comprises the viral RNA-dependent RNA polymerase and a number of other nonstructural proteins involved in genome replication. These proteins enable the synthesis of the (–)RNA intermediate and the subsequent production of new genomic (+)RNAs. Following the synthesis and accumulation of capsid proteins, new virion particles are produced.

To infect the target cell, enteroviruses make use of different cell surface proteins as receptors and coreceptors. Many enteroviruses bind to the CD55/DAF factor, which is one of the complement cascade components. However, for most enteroviruses, interactions with CD55 are insufficient for successful cell infection. For example, some ECHO viruses require β_2 microglobulin as a coreceptor [39], while most group A Coxsackieviruses interact simultaneously with CD55 and the ICAM-1 integrin [40, 41]. Group B Coxsackieviruses use mainly the so-called Coxsackie and adenovirus receptor (CAR), a 46-kDa protein of the immunoglobulin superfamily [42–44], while polioviruses enter the cell using the CD155 glycoprotein [45].

Enteroviruses enter the organism through the mucosa of upper respiratory pathways or the gastrointestinal tract; following that, they proliferate mainly in the lymphoid tissues of the rhinopharynx (adenoids and tonsils) and the small intestine (Peyer's glands). Normally, the infection is mild and asymptomatic; sometimes it causes some moderate disease symptoms, such as fever, headaches, nausea, abdominal pain, and vomiting [29]. However, if an enterovirus infection spreads outside the gastrointestinal tract, it may cause a more severe disease [30]. A very grave enterovirus infection is poliomyelitis. Most often, polioviruses replicate nearly asymptomatically in the gastrointestinal tract, but sometimes the infection spreads to the central nervous system and affects anterior horn cells. Poliomyelitis patients commonly continue to suffer from the lasting paresis or paralysis of different muscle groups, which sometimes leads to a lifelong disability. Coxsackieviruses A and B, ECHO viruses, and other enteroviruses are usually less dangerous, although they may also cause serious conditions, such as aseptic serous meningitis, meningoencephalitis, acute myocarditis, and hemorrhagic syndrome of the newborn [46, 47]. In addition, they may cause acute respiratory infections, diarrhea, hemorrhagic conjunctivitis, and Bornholm disease (epidemic pleurodynia). Some Coxsackieviruses A (serotypes 4–6, 9, 10, and 16) and B (serotypes 2 and 5), as well as enterovirus 7, are often identified as the causative agents of enteroviral stomatitis, which sometimes takes the course of enteroviral exanthema of hands, feet, and mouth [30]. ECHO-11 and ECHO-19 can cause enteroviral uveitis, frequently resulting in blindness [48, 49]. They can also provoke hemorrhagic syndrome in newborns [50, 51]. Although the list of possible enterovirus-associated diseases looks impressive, more than 90% of poliovirus infections and at least 50% of other enterovirus infections actually take a subclinical or asymptomatic course; consequently, some enteroviruses can be classified as apathogenic viral saprophytes [52–54].

ONCOTROPIC AND ONCOLYTIC PROPERTIES OF ENTEROVIRUSES

Oncolytic properties of different enteroviruses were first reported in the 1950s [55, 56]. Manifold investigations of the oncolytic activity of enteroviruses were performed in both animal models of solid tumors and human volunteers. Most comprehensive studies have been performed with ECHO viruses (serotypes 1, 7, and 12) [53, 57–59], type-1 poliovirus [60–63], and Coxsackie virus A21 [64–66].

Due to the considerable variations in the pathogenicity of different enteroviruses, there are two principal approaches to the research of their oncolytic activity. Apathogenic and low-pathogenic variants can be investigated without any modifications. In contrast, pathogenic viruses must be attenuated; in particular, genetic engineering techniques can be used to produce recombinant variants with increased selectivity to tumor cells and reduced proliferation in healthy cells.

ECHO viruses were among the first enteroviruses to be investigated as antitumor agents. The name "enteric cytopathic human orphan" implies that ECHO viruses have a cytopathyc effect in cell cultures, but, as orphans, are not associated with any human diseases. Although this connection was discovered later for some ECHO viruses, the term was firmly established. Several nonpathogenic ECHO viruses and some Coxsackievirus strains were isolated from healthy children in the course of mass anti-polio vaccinations in the late 1950s, as the reasons underlying the absence of immunity to poliovirus vaccine in some children were investigated. It was found that concurrent asymptomatic enterovirus infection prevented the colonization of the intestines by vaccine poliovirus due to interference effects.

Live Enterovirus Vaccines

It was found that nonpathogenic strains of ECHO viruses and Coxsackieviruses obtained from healthy children can prevent not only the colonization of the intestine by vaccine poliovirus strains, but also some viral diseases due to the interference phenomenon. Based on the collection of nonpathogenic enterovirus strains obtained from the feces of healthy newborns and infants, live enterovirus vaccines (LEV) were obtained by the group of Dr. Voroshilova in the Institute of Poliomyelitis and Viral Encephalitis in 1960–

1970s [67]. Following a thorough investigation, GS–ECHO-1 and L572-ECHO-12 [68, 69] were classified as vaccine strains and used to produce LEVs: LEV-4 and LEV-7, respectively [52, 53, 70]. It was initially planned to use LEVs to control enterovirus infections, as competing nonpathogenic symbionts would replace pathogenic viruses. Further, it was shown that LEVs can restrict infections caused not only by pathogenic enteroviruses, but also by influenza virus, agents of other acute respiratory infections, herpes virus, and some others [52, 53, 67], probably because of the interference effect due to interferon production stimulated by the replication of nonpathogenic viruses. LEVs were also tested as a treatment against some chronic diseases, such as herpetic lesions, multiple sclerosis, lateral amyotrophic sclerosis, and progredient tick-borne encephalitis, as well as malignant tumors [52, 53].

In 1958–1968, three strains of live poliomyelitis vaccines and several LEVs were tested in several hospitals in 1452 patients with advanced stages of cancer who had been resistant to conventional treatment; in a portion of cases, a positive clinical effect was attained [53]. In particular, improvements of the general condition tumor reduction or stabilization, were observed in 58% of cases in the test group of 248 patients, which enables subsequent surgical intervention [53]. The best results were observed for gastrointestinal tumors [71]. It was found that nonpathogenic enteroviruses can proliferate in tumor cells [72], act as potent interferon inducers and activators of T-cell immunity, support leukopoesis, have a radioprotective effect, and can be used in combination with other methods of anticancer treatment [53, 73, 74].

Rigvir: An Enterovirus-Based Oncolytic Drug

Oncolytic activity of ECHO viruses was also studied from the beginning of the 1960s by the group of Dr. Muceniece in the Kirchenstein Institute of Microbiology [59]. The oncolytic activity of natural enterovirus strains was increased by multiple passaging in human tumor cell cultures. Clinical trials of five attenuated oncolytic ECHO enterovirus strains began in 1968. The trials were performed in stage-IV cancer patient volunteers, when the conventional therapy had proved inefficient. Viral preparations were administered by intramuscular injections. In some patients, a portion of tumor cells were destroyed that show characteristic cytopathic signs, but the overall therapeutic efficiency was low, supposedly because of the large bulk of the tumor and the rapid development of antiviral immunity. Accordingly, the suggested treatment strategy included radical surgery with subsequent virotherapy for the eradication of residual tumor cells and metastases and the stimulation of antitumor immunity [75].

Based on the results of the trial, ECHO virus strain ECHO-7, which showed the most pronounced oncolytic properties, was selected for further research and named Rigvir [76, 77]. In 1988, phase-III clinical trials began, which compared the efficiency of Rigvir with that of surgery and radio- and chemotherapy. In 2004, a patent was issued for Rigvir, and it was officially registered in Latvia, becoming the first enterovirus medication worldwide to complete the full cycle of clinical trials and to be applied in cancer therapy. Since 2008, Rigvir has been available in Latvia as a prescription medication. The information on its properties and usage is available at the site of the Latvian Virotherapy Center (www.viroterapija.lv).

Oncolytic Properties of ECHO Viruses

The progress in molecular and cell biology stimulated investigation of oncolytic enteroviruses on a new level. For several years, the enterovirus ECHO-1 Farouk strain has been used as a model enterovirus at the University of Newcastle (Australia) [57, 58, 78]. In particular, it was found that ECHO-1 could cause cell lysis in all eight cell lines of ovarian cancer studied; it significantly suppressed the growth and dissemination of tumors cultured in vivo in immunocompromised mice [78]. Oncolytic properties of ECHO-1 were also confirmed in a model of mouse xenografts of human prostate cancer cells [57]. To investigate the oncolytic properties of the virus, the growth and lysis of xenografts of metastasing gastric cancer cell lines were monitored using the bioluminiscence of luciferase-expressing cells [58]. The one-time intratumor injection of ECHO-1 significantly slowed tumor growth and dissemination. An important advantage of oncolytic viruses used as an anticancer treatment is their long-term persistence in the organism due to their constant proliferation in tumor cells, which eliminates the need for repetitive administration [58].

The mechanism of ECHO-1 selectivity to tumor cells is also being actively studied. This virus enters target cells by binding to the VLA-2 receptor (integrin $\alpha_2\beta_1$) [79]. Viral surface proteins interact with domain I of the α_2 subunit [80]. Integrin $\alpha_2\beta_1$ is produced in large quantities by tumors of the ovaries, stomach, prostate, and some other organs, which partially explains the increased tropism of ECHO viruses to these types of cancer cells [57, 58, 81]. Integrin $\alpha_2\beta_1$ interacts with extracellular matrix proteins, e.g., type-I collagen and laminin. Its overexpression may facilitate tumor expansion, in particular, metastatic dissemination into the abdominal cavity [82–85]. Presumably, interaction of integrin $\alpha_2\beta_1$-positive cells with ECHO-1 might prevent tumor dissemination because of the virus competing with extracellular matrix proteins for integrin binding [58]. It should be noted that the mechanisms of the ECHO-1 cytolytic activity itself are so far insufficiently understood. It was found that integrin $\alpha_2\beta_1$ is not the only cellular protein required for the successful proliferation of the virus, which suggests that additional mechanisms of cell sensitivity

exist [58]. Unlike some other viruses, ECHO-1 binds to the inactive conformation of integrin $\alpha_2\beta_1$. This results in the clusterization of integrin on the cell surface and the activation of signaling pathways that facilitate the invasion of the virus by endocytosis; importantly, the mechanism of integrin $\alpha_2\beta_1$ interaction with the virus differs from the mechanism of its interaction with the extracellular matrix, resulting in the activation of different signaling pathways [86]. The details of this process still remain to be elucidated.

Oncolytic Properties of Coxsackie Viruses

Coxsackie enteroviruses are divided in groups A and B. The first data concerning the oncolytic activity of some Coxsackieviruses B were obtained more than 50 years ago [12]. However, it was only recently that researchers began more comprehensive investigations of the oncolytic potential of Coxsackieviruses using the model of group A virus, mainly Coxsackie A21 (strain Kuykendall) [64–66].

Coxsackievirus A21 simultaneously requires two receptor molecules, i.e., CD55/DAF and the integrin ICAM-1 [40, 41]. These receptors are present in moderate quantities on the surfaces of normal respiratory epithelial cells [87]. At the same time, ICAM-1 and DAF are often abundant on the cell surface of many tumor lines, making these cell more sensitive to Coxsackievirus A21. The same molecules contribute to tumor malignancy, since large amounts of DAF protect tumor cells from cytotoxic complement action [88], while ICAM-1 promotes tumor dissemination by interacting with lymphocyte function-associated antigen, LFA-1 [89].

The oncolytic activity of coxsackievirus A21 was demonstrated in different types of tumors both in vitro and in animal models. This strain proved to be an efficient oncolytic in melanoma [60, 90, 91], multiple myeloma [64], breast cancer [66], and prostate cancer [57] cells.

Tumor cell sensitivity to Coxsackieviruses depends not only on the presence of the necessary surface receptor molecules, but also on a number of other factors, including the relationship between the rates of virus replication and tumor growth. Mathematical modeling predicts that viral oncolysis would be less efficient in rapidly growing tumors if the increment in tumor cell proliferation exceeded the number of dying cells [92]. This prediction was verified by comparing the efficiency of oncolysis in two morphologically similar melanoma lines that differ in their growth rates in immunocompromised mice. SK-Mel-28 (slow growth) and ME4405 (rapid growth) cell lines express similar levels of ICAM-1 and DAF and are equally able to sustain Coxsackievirus A21 replication. The dynamics of virus-induced oncolysis was better in the slowly growing line SK-Mel-28. However, this does not imply that oncolytic therapy is inappropriate for rapidly growing tumors, while, despite the different dynamics of tumor cell destruction, a one-time Coxsackie A21 administration, either intratumoral or intraperitoneal resulted in a significant reduction of both SK-Mel-28, and ME4405 tumors, and sometimes even in their complete elimination [65].

The oncolytic activity of the Coxsackievirus A21 was also studied in vitro in multiple myeloma cells [64]. Following the injection of CV-A21 into bone marrow biopsy specimens from patients with multiple myeloma, 98.7% of CD138+ plasmatic cells were destroyed. This result suggests that virotherapy with CV-A21 can be performed immediately before the autologic stem cell transplantation to eliminate malignant plasmatic cells ex vivo.

Breast cancer cells were also shown to be sensitive to Coxsackievirus A21 [66]. High-multiplicity viral infection produced a rapid cytopathic effect in eight of the nine breast cancer cell lines investigated, while no cytopathic action was observed in the control cell line of normal breast cells. The oncolytic efficiency of Coxsackievirus A21 was evaluated in the model of bioluminiscent xenografts of breast cancer tumors in immunocompromised mice (tumor cell lines T47D and MDA-MB-231). By day 42 following a one-time intravenous administration of the virus, metastases in test animals had disappeared and the bulk of the tumor reduced to one-third of its initial size, whereas in the control group the tumor bulk reached 300–5000% of the initial size. It was observed that, in some animals, the virus persisted until the end of the experiment because residual tumor cells were sustaining the replication of the virus. However, this long-term persistence of the virus does not truly reflect the reality, since the experiment involved immunocompromised mice that lacked antiviral immunity. In fact, both the developing immune reaction to the virus or the existing immunity to a previously experienced asymptomatic infection may reduce the therapeutic effect. For these reasons, it seems promising to perform several sequential courses of virotherapy with Coxsackieviruses of different serotypes; in particular because several of them (e.g., serotypes A13, A15, A18, and A21) have similar oncolytic efficiencies [55].

Since different tumors may show varying sensitivity to the virus, the efficiency of the treatment can be improved by combining the oncolytic administration of the virus with chemotherapy. This possibility was investigated in cell lines derived from breast cancer (MDA-MB-231 and T47D), pancreatic (PANC-1), and colorectal cancer (DLD-1) [91]. All of these cell lines express high levels of ICAM-1, but PANC-1 and DLD-1 cells expressed low levels of DAF. A combination of Coxsackievirus A21 and doxorubicin had a synergistic effect in all cell lines studied in vitro and considerably accelerated cell death. Xenografts of luciferase-labeled MDA-MB-231 cells also were sensitive to the combination of Coxsackievirus A21 and doxorubicin; moreover, the therapeutic effect was provided with doxorubicin doses significantly lower than

those used in monotherapy. When considering the promising oncolytic potential of the virus, stage I and II clinical trials of a Coxsackievirus A21-based drug CAVATAK™ (www.viralytics.com) have been conducted in Australia since 2009 in patients with head and neck cancer, malignant melanoma, breast cancer, and pancreatic cancer.

Oncolytic Properties of Attenuated Poliovirus Strains

Polioviruses are agents of poliomyelitis, an infectious disease that affects spinal motor neurons. There are three serotypes of pathogenic wild-type poliovirus strains; for this reason, the prophylaxis of poliomyelitis employs a live oral vaccine that consists of attenuated vaccine poliovirus strains of serotypes I, II, and III, which were obtained by A. Sabin in 1950s [93]. These strains lack neurovirulence, although they still can proliferate in the intestines and cause stable lifelong immunity. Vaccine poliovirus strains are typical nonpathogenic enteroviruses suitable for testing their oncolytic properties. The oncolytic activity of polio vaccine strains was shown in some early studies [52, 53, 72]. In recent years, genetic engineering techniques have been actively used to produce recombinant poliovirus variants, in particular with increased oncolytic potential [94, 95].

The only cell receptor of polioviruses is the CD155 glycoprotein of the immunoglobulin superfamily [45]. Similar to other enterovirus receptors, this protein is abundantly exposed on the surface of tumor cells of different origins, including epidermal and osteogenic carcinomas, breast and colorectal cancer, neuroblastoma, and some others [96–98]. High levels of CD155 production are commonly observed in malignant tumors of neuroectodermal origin, i.e., astrocytoma, oligodendroglioma, and glioblastoma multiforme [96–98]. At the same time, the level of CD155 expression in untransformed cells is extremely low, which apparently makes them insensitive to polioviruses [96]. It was found that increased CD155 presentation on many tumor cells is determined by the activity of the Sonic hedgehog (Shh) morphogen and the transcription factors Gli1 and Gli3 [99]. The Shh signaling pathway is normally active only in embryogenesis; however, it is pathologically activated in many tumors [100]. Consequently, CD155 is a marker of different cancer cell lines, and CD155-expressing tumors are potential targets for poliovirus-driven oncolysis.

Since the use of large therapeutic doses of polioviruses in oncology is associated with the hazard of potential virus reversal to the wild type, the initial research steps were largely focused on safety issues. Genetic engineering techniques were used to construct so-called antitumor replicon vectors based on the type-I poliovirus Mahoney strain [101, 102]. In these vectors, the gene that encodes the capsid protein VP1 was substituted with the *gag* gene of human immunodeficiency virus type 1 (HIV-1). Complete virus particles were assembled in cells concurrently infected with the modified poliovirus and a helper vaccinia virus strain carrying the VP1-encoding gene. The resulting recombinant poliovirus replicons were able to infect target cells and amplify in them, but no further infection of other cells occurred [103, 104]. The replicons could amplify in different primary tissue cultures of central nervous system tumors (malignant glioma, astroctoma, gliosarcoma, neuroblastoma, meningioma, and anaplastic glioma) [102]. The infection produced characteristic cytophatic effects, such as vacuole formation, cell rounding, and cell-membrane rupture. Next, the oncolytic activity of polioviruses was tested in tumor cell lines of different origin, in particular in breast cancer (BT20), colorectal cancer (DLD-1), cervical cancer (A-431), and melanoma (SK-MEL-2, SK-MEL-21, and SK-MEL-28) cells. Cytopathic action of varying intensity was observed in all tumor cell lines studied, except for the Burkitt's lymphoma strain Raji, which does not express CD155 [105]. D54-MG glioma xenografts in immunocompromised mice were also sensitive to poliovirus replicons in vivo. Virotherapy significantly increased survival rates in mice with transplanted tumors [102].

The safety of poliovirus replicons was tested in transgenic mice expressing the poliovirus receptor. These mice are normally highly sensitive to infection with the wild-type poliovirus. Intraspinal administration of poliovirus replicon doses 10000-fold exceeding the lethal dose for the wild-type poliovirus did not produce any observable adverse effects [102]. Based on these data, it is considered reasonable to use poliovirus replicons in virotherapy aimed at destruction of CNS micrometastases after surgical removal of the primary tumor [102].

Oncolytic virotherapy is one of the most promising approaches in the treatment of CNS tumors. At present, the dissemination of the tumor to the CNS implies the advent of the terminal stage of the disease with mean survival rates of less than 1 year. The prognosis is poor, in particular because the risk of radiation necrosis of the brain limits the possibilities of radiotherapy, while drug delivery to the brain and spinal cord is hampered by the blood–brain barrier [100].

The genetically modified PV-RIPO strain (variants PV1-RIPO and PVS-RIPO) [96], derived form the Sabin's type-I polio vaccine strain is considered a promising oncolytic poliovirus variant for CNS tumors. This virus recombinant carries IRES of human rhinovirus type 2 (HRV2). PV-RIPO cannot proliferate in normal cells of neurogenic origin and, unlike the original vaccine strain, does not cause meningitis in CD155-expressing transgenic mice [106].

The investigation of the molecular basis of PV-RIPO interaction with neurons showed that HRV2 IRES interacts with a double-stranded RNA binding protein (DRBP76) heterodimer and the nuclear factor of activated T cells (NF45). The DRBP76 heterodimer

is neuron-specific; it is located primarily in the cytoplasm and participates in protein translation, and its binding inhibits the initiation of viral protein translation in neurons by suppressing the HRV2 IRES [107, 108]. It was found that the IRES substitution in RV-RIPO did not affect its high oncolytic activity. The increased activity of the HRV2 IRES in rapidly growing malignant cells suggests that there are some important differences in the regulation of translation rates in tumor cells that make them sensitive to the oncolytic action of PV-RIPO. The details of the molecular mechanisms that underlie the oncolytic action of viruses are still insufficiently studied, since they involve a large number of regulatory proteins, canonical and noncanonical translation factors, IRES-binding proteins, etc. [59].

The oncolytic activity of PV-RIPO was investigated not only in primary nervous system tumors, but also in brain metastases of breast cancer, in vitro and in animal models. The experimental breast cancer cells expressed high levels of CD155, which made them highly sensitive to poliovirus in vitro. The oncolytic effect of PV-RIPO on breast cancer metastases to the subarachnoid space and the brain parenchyma was studied in immunocompromised athymic rats. Both intraspinal and subpial PV-RIPO administration proved highly efficient [100]. The potential of PV-RIPO in the therapy of malignant meningitis accompanying glioblastoma multiforme was also evaluated. This study was performed in CD155-expressing transgenic mice and in athymic rats. The intraspinal administration of the virus produced no signs of toxicity; at the same time, the life expectancy of experimental mice increased significantly, and some rats experienced a long-term remission up to the complete eradication of the transplanted tumor [109].

Since the use of attenuated viruses is associated with the risk of genetic reversal and the restoration of pathogenicity, the genetic stability of the PV-RIPO recombinant was confirmed by serial passages on HTB-15 glioblastoma multiforme xenografts with subsequent analysis of the strain's genetic and phenotypic traits. The virus was shown to retain all the features of the initial strain, as well as its oncolytic properties [60]. At the same time, 10 days after an intratumor injection, the virus quantities present in the animal were very low, and in 28 days, it was entirely absent. Thus, the virus was unable to persist in the organism after the disappearance of the viable tumor cells sustaining its replication [60].

The chimeric PV-RIPO strain is currently at the final stage of animal model tests, and will soon enter clinical trials [106].

Another promising area of oncolytic poliovirus application may be the treatment of neuroblastomas, which are solid tumors that occur relatively frequently in children. Neuroblastomas respond poorly to conventional therapy, and the prognosis is usually unfavorable.

The oncolytic effect of attenuated polioviruses on neuroblastoma cells was demonstrated both in neuroblastoma cells in vitro and in xenografts in immunocompromised mice [62, 63]. Mice with SJ-N-JF tumor xenografts received intratumor injections of attenuated poliovirus, which resulted in rapid destruction of tumor cells and complete tumor eradication. In vitro experiments showed that live attenuated poliovirus caused cell death in 27 of 29 neuroblastoma lines studied [63].

To ensure the highest possible safety of virotherapy, a recombinant strain A_{133}Gmono-*cre*PV exhibiting an oncolytic action on neuroblastoma was developed based on the type I poliovirus Mahoney strain [95]. In this strain, the spacer of the 5' UTR of the viral genome carried a point mutation [95] that reduced neurovirulence more than 1000-fold, probably by decreasing the level of viral RNA translation [110]. To prevent the possibility of the strain reverting to the wild-type virus, the genomic element *cre*, which is necessary for virus replication and is initially located within the gene encoding the viral protein $2C^{ATPase}$, was inserted within the 5'-UTR spacer [111, 112]. The resulting attenuated virus strain was stable and could efficiently replicate in neuroblastoma cells, causing their destruction.

The use of oncolytic virotherapy can be hampered by the pre-existing immunity to the virus employed, or rapidly developing immune response to the introduced oncolytic virus. At the first stage, attenuated and recombinant polioviruses were tested in immunocompromised mice, which provided no possibility to evaluate the restrictions associated with antiviral immunity [61, 62]. For this reason, the oncolytic activity of A_{133}Gmono-*cre*PV was studied in immunocompetent CD155tgA/J transgenic mice expressing human CD155. This mouse strain is highly sensitive to poliovirus and can be used as an animal model of poliomyelitis [95]. To model the pre-existing antiviral immunity, mice were immunized with the mono-*cre*PV poliovirus recombinant; subsequent transplantation of Neuro-$2a^{CD155}$ cells caused neuroblastoma formation. Next, a course of intratumor injections of the A_{133}Gmono-*cre*PV virus recombinant was performed. In nine of 11 mice, the tumors disappeared completely, and no signs of neurotoxicity were observed. In two animals, the tumor relapsed in several months after virotherapy, and the relapsing tumors were resistant to repeated A_{133}Gmono-*cre*PV therapy. It was found that, in this case, tumor cells expressed low levels of CD155. These results indicate the need for combination therapy for neuroblastomas, since monotherapy with oncolytic viruses may be insufficient for a complete cure. The combination of oncolytic virotherapy, chemotherapy, and radiotherapy can have a lower general toxicity, which reduces the risk of cardiopulmonary complications, kidney dysfunction, or endocrine problems.

It is especially worth mentioning that mice completely cured of neuroblastoma using the A_{133}Gmono-*cre*PV oncolytic virus became resistant to the subsequent reintroduction of the tumor with Neuro-2a^{CD155} cells. Apparently, antigen-presenting cells take up tumor antigens of lysed cells, which leads to the specific peptide presentation and activation of cytotoxic T-lymphocytes. These events accelerate further tumor degradation and induce antitumor immunity. Similar observations were previously made in experiments with other oncolytic viruses [113–115]. A recent publication reported the acquisition of immune resistance to neuroblastoma as a result of virotherapy with A_{133}Gmono-*cre*PV. It was found that transplantation of splenocytes or CD8+ T-cells from a mouse cured with poliovirus therapy arrested tumor growth in the recipient mouse [63]. These results suggest that a combination of virotherapy with A_{133}Gmono-*cre*PV and immunotherapy with poliovirus oncolysates may be a promising treatment strategy in neuroblastoma.

APPLICATION OF ENTEROVIRUSES AS ONCOLYTIC DRUGS: PROBLEMS AND PROSPECTS

Taking into account the wide range of cell receptors used by enteroviruses to enter the cell, corresponding oncolytic virus-based drugs can be efficient against a variety of tumors. The subsequent application of several oncolytic strains that represent different types of enteroviruses may have a cumulative therapeutic effect [53]. It should be pointed out that the current projects seem promising and highly efficient [60, 95].

At the same time, certain problems remain that are associated with enterovirus biology. First of all, we should not forget that many enteroviruses are potentially pathogenic for humans, and their use may cause different complications. For this reason, oncolytic preparations should be based either on initially nonpathogenic enterovirus strains, or on recombinant attenuated variants. However, the use of initially nonpathogenic strains does not guarantee their safety, since enteroviruses have high variations. It should also be taken into account that these viruses are apt to recombination, which may cause them reverse to the wild type, or produce a new pathogenic variant; for these reasons, the stability of all enterovirus-based oncolytic preparations must be thoroughly controlled. Finally, the antitumor potential of viruses is limited, so it is reasonable to combine virotherapy with other types of treatment. The surgical removal of the bulk of the tumor would be an efficient way to reduce the probability of generating virus-resistant clones. In this case, virotherapy might be appropriate for the elimination of residual tumor cells and metastases inaccessible by surgery.

In spite of the above limitations, the further development of enterovirus-based oncolytic drugs might produce a valuable complement to the existent methods of anticancer therapy. An intelligent combination of surgical, chemotherapeutic, radiological, and biological methods of antitumor treatment will be able to significantly reduce the number of incurable cases.

ACKNOWLEDGMENTS

This study was supported by the State Program of Support of Leading Research Schools of the Russian Federation (project no. Nsh-2996.2012.4), State Contract no. 02.740.11.0767 "Identification of Viral Infectious Agents Important for Healthcare in West Siberia (Hepatitis, Gastroenteritis, Serous Meningitis) and Investigation of Their Genetic Diversity for Development and Improvement of Diagnostic Systems," Contract no. 11.G34.31.0034 between Novosibirsk University and the Ministry for Science and Education "Novel Approaches to Drug Development: Search, Selection, and Design of Nonpathogenic Virus Strains as Prospective Oncolytic Preparations," the Molecular and Cell Biology Program of the Presidium of the Russian Academy of Sciences, and the Russian Foundation for Basic Research (project no. 11-04-00410).

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