Reovirus – possible therapy of cancer*
Minireview

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Oncolytic viruses infect, replicate in, and eventually lyse tumor cells but spare normal ones. In addition to direct lysis, a result of viral replicative cycle, viruses also mediate tumor cell destruction by inducing nonspecific and specific antitumor immunity. Some viruses express proteins that are cytotoxic to tumor cells. Viruses recognized as oncolytic agents can therefore be divided into three categories: 1/ naturally occurring viruses (e.g. Newcastle disease virus, vesicular stomatitis virus, autonomous parvoviruses, some measles virus strains, reovirus) that selectively replicate in tumor cells, in some instances owing to their relative resistance to interferon action; 2/ virus mutants in which some genes essential for replication in normal cells but evitable in cancer cells have been deleted (e.g.adenovirus ONYX 015 that replicates only in cells with defected p53 or herpes virus G207 which exacts the presence of ribonucleotide reductase); 3/ virus mutants modified by the introduction of tissue-specific transcriptional elements that drive viral genes (e.g.adenovirus CV706 that has PSA restricted expression of E1A and E1B and adenovirus adMycTK that binds selectively on myc protein).

Reovirus is prevalent in the human population but not associated with any known human disease. Studies have shown that reovirus multiplicate preferentially in tumor cells with activated gene of *ras* family or *ras-signaling pathway* while sparing normal cells. Activated *ras* or its pathway could be found in as many as 60–80% of human malignancies. In our studies we used cell lines that demonstrably express activated *ras*. We showed the cytopathic effect of reovirus (serotype 3 strain Dearing) on medulloblastoma cell lines and compared it with its acting on normal human fibroblasts. Oncolytics Biotech Inc. is currently guiding three Phase I or Phase I/II Reolysin studies, and has completed two clinical studies and concluded enrolment in a third one.

Key words: oncolytic viruses, reovirus, clinical trials

Malignant tumors remain one of the main causes of death in all developed countries and their incidence is still rising. Fortunately, progress has been made in the overall survival of cancer patients after introduction of improved imaging and diagnostic techniques; elucidation of the molecular processes that cause cancer, and further comprehension of treatment using combined chemo- and radiotherapy. However, survival has not improved substantially with current chemotherapy and radiotherapy in patients diagnosed with metastatic disease and certain high-incidence tumors such as brain tumors, pancreatic, colorectal, and liver carcinomas. Surgery and radiation therapy afford only local control, therefore are not effective in metastatic diseases and chemotherapy is limited by toxicity and by primary or secondary chemoresistance to the drugs in use. This incepts usually due to tumor cells developing different mechanisms that override cell death caused by chemotherapy and radiotherapy. As a result, resistance to treatment through clonal expansion of genetically resistant tumor cells occurs. Much effort has been directed toward finding alternate pathways that would complement therapeutic induction of apoptosis, overcome multidrug resistance, and ultimately improve overall cure rates. In view of this, several new classes of anticancer agents are being promoted as potential supplements to current anticancer therapy. They include monoclonal antibodies, biological response modifiers, angiogenesis inhibitors, modulators of signal transduction, gene therapy including antisense oligonucletides, telomerase and kinase inhibitors. An additional group of agents includes viruses that infect, replicate in, and eventu-

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ally lyse tumor cells but spare normal ones. The possibility of using viruses as oncolytic agents was originally recognized in cases of unintentional exposure. The virus-induced remissions occurring either naturally [1] or induced by vaccination [2] stimulated research on the oncolytic activity of a variety of viruses.

Oncolytic viruses

Revolutionary advances in molecular biology and genetics have led to a fundamental understanding of the replication and pathogenicity of viruses and the carcinogenesis. These advances have allowed novel agents to be engineered to enhance the antitumoral potency as well as safety of oncolytic viruses. Oncolytic viruses were evolved to infect cells, replicate inside the host, induce cell death, release the viral particles, and finally to spread in human tissues. Replicating viruses "self-amplify" that potentially leads to maximized dosing at the desired site of action, while a lack of replication in normal tissues can result in efficient clearance and reduced toxicity. Selective replication within tumor tissue can theoretically increase the therapeutic index of these agents enormously. Furthermore, oncolytic viruses can mediate the destruction of tumor cells by several mechanisms. In addition to direct lysis, a result of viral replicative cycle, viruses also mediate tumor cell destruction by inducing nonspecific and specific antitumor immunity. Some viruses express proteins that are cytotoxic to tumor cells (adenoviruses express cytotoxic proteins E3 and E4ORF4) [3]. Viral infection of cells elicits an immune response that consists of cytokine generation (interferons α, β and γ, TNFα, and several interleukins) and infiltration of macrophages, neutrophils, and NK cells. Therefore, since activation of classical apoptotic pathways in the cancer cell is not the exclusive mode of killing, cross-resistance with standard chemotherapeutics or radiotherapy is much less likely to occur. On the other hand, the effect of immune response is also likely to destroy replicating virions and so limit the direct lytic effect [4]. TODA et al [5] showed that treatment of tumors in mice with genetically modified oncolytic herpes virus G207 also elicited systemic immunity against other tumors in which virus was not detected through a cytotoxic T cell response. Immunosuppresion by corticosteroids decreased efficiency of G207 in transplanted human tumor [6]. On the other hand HIRASAWA et al [7] found increased efficiency of reovirus in mice tumor after co-administration of cyclosporine A and anti CD4 and anti CD8 antibodies. It remains to be determined which mechanisms are involved in antiviral immunity and which in anticancer immunity.

As with any anticancer therapy, the cytotoxic effects of the treatment upon the normal tissue surrounding the tumor should be minimized. The ideal oncolytic virus would express such high specificity for tumor cells even when delivered systemically; it would localize to act directly on cancer cells. Additionally, the virus would replicate quickly in dividing as well as quiescent cancer cells to high titers. Further would disseminate throughout the tumor mass, destroying cells directly or sensitizing them to the action of other therapeutic agents, but would still remain non-dangerous to surrounding normal tissue. The ideal virus must also be able to replicate efficiently in the context of a developing, or even a pre-existing antiviral immune response. This may require expression of viral proteins that are involved in suppression of the antiviral immune response. Virus would therefore cause minimal immunological reaction, and would be well tolerated by patients. Furthermore, infection with the virus should stimulate an effective antitumor immune response that would lead to the destruction of metastases [8]. Much work over the last three decades has been performed with the aim of producing such an ideal virus.

Oncolytic viruses, which have been tested as cancer therapeutics, have either been naturally selected or have been genetically engineered to grow specifically in and kill tumor cells. Viruses recognized as oncolytic agents can therefore be divided into three categories: 1/ naturally occurring viruses (such as Newcastle disease virus, vesicular stomatitis virus, autonomous parvoviruses, some measles virus strains, reovirus [9]) that selectively replicate in tumor cells, in some instances owing to their relative resistance to interferon action [8]; 2/ virus mutants in which some genes essential for replication in normal cells but evitable in cancer cells have been deleted (e.g.adenovirus ONYX 015 that replicates only in cells with defected p53 or herpes virus G207 which exacts the presence of ribonucleotide reductase) [4]; 3/ virus mutants modified by the introduction of tissue-specific transcriptional elements that drive viral genes (e.g. adenovirus CV706 that has PSA restricted expression of E1A and E1B and adenovirus adMycTK that binds selectively on myc protein) [10]. Each of these agents has shown tumor selectivity *in vitro* and/or *in vivo*, with many of these agents following intratumoral, intraperitoneal and/or intravenous routes of administration. Overview of the most crucial oncololytic viruses shows Table 1.

There is now clear evidence in pre-clinical models that oncolytic viruses have great potential to become important new therapeutics. Results from Phase I and II intratumoral trials are beginning to supervene and it seems that the current oncolytic viruses are safe and have reduced acute side effects when compared with many other conventional cancer therapeutics [11, 12]. The first virus studied in clinical trials is the adenovirus ONYX-015, which has been the subject of 18 phase I and II clinical trials with published results, starting in 1996. To date, more than 250 and 170 patients have been treated with ONYX-015 and Newcastle disease virus respectively [13]. Indeed evidence from both pre-clinical and clinical studies suggests that combining replication-competent viruses with standard anticancer treatments such as chemotherapy and radiotherapy may result in greater therapeutic benefit [14–17]. ONYX-015 became the first virus combined with chemotherapy to undergo clinical trials [18].

Table 1. Overview of oncolytic viruses in clinical trials (modified according Kirn DH Replication-selective microbiological agents: fighting cancer with targeted germ warfare. J Clin Invest 2000; 105: 837–839)

Virus family	Oncolytic virus	Specificity	Genetic alterations
Adenovirus	ONYX-015	cells lacing p53 function	E1B-55kD, E3b deletion
	CV 706	prostate cells	E1A expression driven by PSA element, deletion E3
	CV 787	prostate cells	E1B expression driven by PSA element
	Ad5-CD/tk-rep	cells lacing p53 function	E1B-55kD deletion
	adMycTK	myc expressing cells	Myc-Max binding motif
Herpes simplex	G207	proliferating cells	ribonucleotide reductase disruption and deletion of gamma 34.5
	NV1020	proliferating cells	deletion of gamma 34.5
	1716	proliferating cells	deletion of gamma 34.5
Vaccinia	wild type +/– GM-CSF	unknown	wild type
Newcastle disease v.	73-T, PV 701, Ulster strain, MTH-68/N	unknown	wild type
Autonomous parvoviruses	H-1	transformed cells-↑proliferation, ↓differentiation, ras, p53 mutation	wild type
Reovirus	Reolysin	ras-pathway activation	wild type

Table 2. Clinical trials with Reovirus (modified according www.oncolyticsbiotech.com)

Clinical Study/Trial	Application	Objective	Results	Cancer type	Therapy
Phase I Study	intratumoral	safety, maximum tolerated dose	no serious adverse events related to the virus	progressing solid tumors	momotherapy
T2 Prostate Cancer Trial Phase I	intratumoral	safety, histopathology	evidence of apoptosis tumor cell in 4 of 6 patients, no safety concerns	prostatic cancer	monotherapy
Phase I/II Recurrent Malignant Glioma Trial	intratumoral	safety	well tolerated	recurrent malignant glioma	monotherapy
		safety	not finished		with chemotherapeutics and radiation therapy
Phase I Systemic Administration Trial	intravenous	safety	not finished	advanced primary or metastatic solid tumors	monotherapy
		tumor and immune response	not finished		
Phase I Combination Reolysin/Radiation Therapy Trial	intratumoral	feasibility, safety and anti-tumor effects	not finished	advanced cancer	with radiation
		evidence of any anti-tumor activity			
Phase I Systemic Delivery Trial	intravenous	maximum tolerated dose, dose limiting toxicity,	not finished	advanced or metastatic tumors	monotherapy
		viral replication, immune response, any evidence of antitumor activity	not finished		
Phase I/II Recurrent Malignant Gliomas Trial	infusion	maximum tolerated dose, dose limiting toxicity, safety	not finished	malignant gliomas	monotherapy
		viral replication, immune response, antitumor activity	not finished		

In some cases virus therapy in combination with chemotherapeutics has provided enough evidence of efficacy to warrant proceeding to phase III trials [18–21]. The majority of clinical studies to date involve intratumoral treatments. Systemic treatment of cancer using oncolytic viruses is clearly the next key step for broader applicability. Recently, intravenous treatment of advanced cancer patients using oncolytic viruses has included results of studies with PV701 for maximum tolerated dose determination [23] and of trial with ONYX-015 [21]. Adenovirus ONYX-015 was also administered by hepatic artery infusion in patients with gastrointestinal carcinoma metastatic dissemination to the liver [22]. However more studies with humans need to be initiated or more fully developed for both locoregional and systemic treatment approaches. We need to know how reliable preclinical models predict outcomes in humans. It is likely that questions concerning viremia, virus clearance, humoral and cellular immune responses, tumor to tumor spread, and virus stability can only be answered by testing in humans. Fine tuning and optimization of viral therapeutics will best be done in a Phase I setting.

Knowledge of mechanisms affecting efficiency of oncolytic viruses and of potentiation of their efficiency by cytostatics and/or radiotherapy is important for their use in therapeutic protocols. Therefore we started preclinical experiments with reovirus. We intend to study immunological

mechanisms which may potentiate its efficiency; however, antibodies may neutralize the virus.

Reovirus

Reovirus (an acronym for *r*espiratory *e*nteric *o*rphan) is highly prevalent in the human population but not associated with any known human disease [24]. It has been isolated from the respiratory and gastrointestinal tract and is considered an orphan virus, because it lacks clinical symptoms [25]. It is found naturally in sewage and water supplies. By the age of 12 years, half of all children show evidence of reovirus exposure and by adulthood, most people have been exposed. As mentioned above, reovirus is non-pathogenic, meaning there are typically no symptoms from infections. The link to its cancer-killing ability was established after the reovirus was discovered to reproduce well in various cancer cell lines. Serotype 3 Dearing strain is under clinical investigation in its natural, non-mutated form.

Taxonomically, it is a member of the *Reoviridae* family. These are non-enveloped viruses with icosahedron shape and size ranging from 70 to 85 nm. In addition to the inner core (size 60–70 nm), they posses an outer capsid structure. Their genome is segmented and contains 10–12 pieces of double-stranded RNA and its size is 24 kb. Reovirus represents one of the *Reoviridae* genera that infect human beings [24].

The reovirus lytic cycle begins with attachment of a virion to sialic acid residues on the cell surface via the trimeric σ1 cell attachment protein, which protrudes from the 12 vertices of the icosahedral capsid [26]. Following attachment, clathrin-coated pits form and the virus enters by receptor-mediated endocytosis. Within the resulting endosome/lysosomes, acid-dependent proteolysis of viral outer capsid proteins σ3 and μ1/μ1c begins, generating an intermediate subviral particle (ISVP). Later on, degradation of σ3 occurs, which theoretically exposes μ1/μ1c, allowing for penetration of the ISVP across the lysosomal membrane. μ1/μ1c has been shown to be capable of disrupting membrane bilayers *in vitro* [27]. μ1/μ1c is also myristoylated which may aid in ISVP/membrane fusion [28]. Following this step, primary transcription of 10 capped, full-length transcripts takes place, mediated by the viruses' double-stranded RNA-dependent RNA polymerase. Primary transcripts are translated using host machinery and subsequently associate with primary translation products to form RNA assortment complexes.

Final synthesis of minus strand genomic RNA occurs within these nascent particles and secondary transcription of late viral mRNAs begins. The synthesis of viral mRNA within the virus particle is the characteristic feature of reoviruses' replication. Late viral protein synthesis from secondary transcripts often coincides with a decrease in host protein synthesis [29]. Final assembly of the outer capsid yields progeny reovirus particles, leading to cell lysis and death. In the infected culture the maximal virus yield is achieved 15–18 hrs post infection, with 200–2000 plaque forming units per cell. Quite typical for the harvested virus population is a high ratio between physical and infectious particles (1:100 to 1:1000). This occurs most probably due to the predominant presence of incomplete, defective particles arising in the course of virus replication. Reoviruses are stable over a long period of time and are resistant to exposure to high ionic strength, relatively high temperature (exceeding 50 °C) and extreme pH values.

Recent studies have shown that Reovirus propagates preferentially in tumor cells with activated gene of *ras* family or *ras-signaling pathway* while sparing normal cells [30]. Activated *ras* or its pathway could be found in as many as 60–80% of human malignancies [31]. Studies have shown that reovirus fails to productively infect NIH-3T3 cells unless they express activated *ras* [31, 32]. The reason why cells with activated *ras* pathway can be productively infected by reovirus is associated with the disruption of the cell defense against viral infection. In non-transformed, reovirus infected cells, after primary transcription, the double-stranded RNA-dependent protein kinase (denoted PKR) is activated. By phosphorylating the initiation factor eIF2-α, PKR shuts off viral protein synthesis. This phosphorylation is inhibited when the *ras* signaling pathway is activated, resulting in viral translation and subsequent entrance into the viral lytic cycle. In tumor cells with an activated *ras* pathway, reovirus is able to freely replicate and eventually kill host tumor cells. As cell death occurs, progeny virus particles are free to infect surrounding cancer cells. This cycle of infection, replication and cell death is believed to be repeated until there are no longer any tumor cells carrying an activated *ras* pathway available. The activation of the *ras* pathway can be mimicked in normal cells by treating these cells with 2-aminopurine (2-AP) which prevents the activation of PKR [32].

In our studies we used cell lines that demonstrably express activated *ras*. We showed the cytopathic effect of reovirus (serotype 3 strain Dearing) on medulloblastoma derived cell lines and compared it with its acting on normal human fibroblasts that are believed to have their *ras cascade* inactivated. The cytopathic effect on medulloblastoma occurred within four days, while fibroblasts remained untouched (Fig. 1). Reovirus significantly potentiates effect of cisplatin on medulloblastoma and glioblastoma derived cell lines [33]. Reovirus we used is identical with REOLYSIN® produced by Oncolytics Biotech Inc.

This company is currently guiding three Phase I or Phase I/II Reolysin studies in the United Kingdom and the United States, and has completed two clinical studies and concluded enrolment in a third study in Canada. The recent clinical program for Reolysin addresses various human cancers and uses various modes of administration including local delivery, systemic delivery and delivery in combination with radiation therapy. Phase I/II recurrent malignant glioma study in the United States is in current state of preparation (www.oncolyticsbiotech.com).

It has been the failure of conventional anticancer treatment

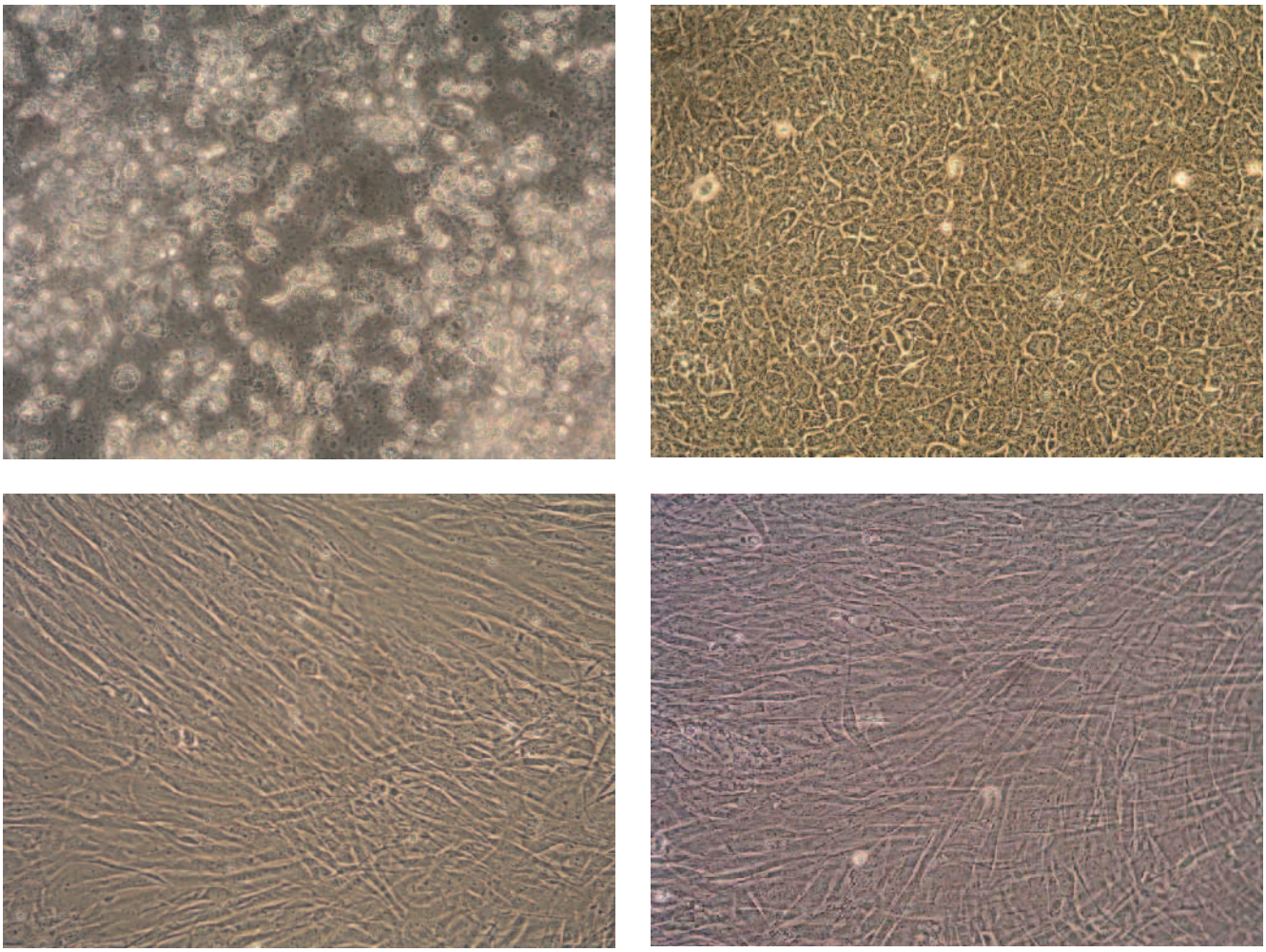

Figure 1. Effect of reovirus on medulloblastoma derived cell line ATCC HTB 186 (Daoy) and normal human fibroblasts.
Right – 96 hours after reovirus infection, left – controls 96 hours without virus.
Upper row – medulloblastoma derived cell line ATCC HTB 186 (Daoy).
Bottom row – normal human fibroblasts.

that has inspired researchers all over the world to look for new drugs which could efficiently kill even the chemoresistant tumor cells. From all different groups of current agents discovered, replication competent viruses seem promising for cancer treatment mainly because of their ability to amplify themselves and spread throughout the tumor mass. Additionally, they can possibly express foreign proteins that fortify their own innate cytolytic potential. Significant progress has been made in targeting viruses to particular cell types, but a real tumor-specific virus is yet to be constructed. However it still seems a little ironic, that viruses might be used to combat neoplasms, since approximately 15% of the incidence of human cancer is attributable to virus infection [34]. It is probable that in the future an extent group of viruses that are able to target different cells will suit for use as anticancer agents. As many viruses lyse the cells in which they replicate, the suggestion that viruses might potentially be used to destroy specific cell populations is not altogether surprising.

Conclusion

Oncolytic viruses represent a rapidly expanding novel therapeutic platform for cancer. Hundreds of viruses are now being tested preclinically, and approval has been sought and/or testing in humans has been initiated in at least ten ones. Only a few therapeutic areas within biotechnology have ever expanded so quickly.

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