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Reolysin®

Oncolytic Virus

Human reovirus-based cancer therapy

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Abstract

Oncolytic viruses infect, replicate in and lyse tumor cells while sparing normal cells. In addition to direct lysis, viruses induce antitumor immunity and some viruses express cytotoxic proteins. Oncolytic viruses can be divided into three categories: 1) naturally occurring viruses that selectively replicate in tumor cells; 2) virus mutants in which some genes essential for replication in normal cells but unnecessary in cancer cells have been deleted; and 3) virus mutants modified by the introduction of tissue-specific transcriptional elements that drive viral genes. Preclinical studies have shown that reovirus proliferates only in tumor cells with activated genes of the *RAS* family or its pathway. Activated *RAS* or its pathway can be found in 60-80% of human malignancies. Oncolytics Biotech is currently conducting clinical studies with the human reovirus-based cancer therapy Reolysin®. Four completed studies in cancer patients demonstrated that intratumoral (including intracranial and intravenous) application of Reolysin® is well tolerated.

Background

Despite the advances in cancer screening, diagnostics and treatment that have decreased mortality in patients, cancer remains one of the main causes of death in all developed countries, and its incidence is on the rise. Current efforts to improve commonly applied cancer therapies are aimed at enhancing drug efficacy while maintaining acceptable levels of toxicity. In order to succeed, innovative therapeutics have been designed to target tumor-specific attributes to permit higher doses with less side effects. One such approach utilizes oncolytic viruses (OVs) that infect, replicate in and lyse tumor cells while sparing normal cells. The possibility of using viruses as oncolytic agents was originally recognized in cases of unintentional exposure. Virus-induced remissions occurring either naturally (1) or induced by vaccination (2) stimulated research on the oncolytic activity of a variety of viruses.

OVs were developed to infect tumor cells, reproduce inside their host, induce cell death, release offspring viral particles and spread across human tumors. Replication-competent viruses “self-amplify”, which potentially leads to maximized dose at the desired site of action, while the absence of replication in normal tissues can result in reduced side effects. Selective replication within tumor tissue can increase their therapeutic index. However, physical barriers such as necrotic areas, stromal cells, extracellular matrix or basal membrane may further limit the distribution of the virus. The significance of diffused virus application was clearly demonstrated in mathematical models of viral replication (3).

The ideal OV should be confined exclusively and specifically to tumor cells, even when delivered systemically, in order to act directly on target cancer cells. The ideal virus should replicate quickly in both dividing and quiescent cancer cells. Furthermore, it should disseminate throughout the tumor mass, destroying its host, and at the same time not be harmful to normal tissues. The perfect virus must also be able to replicate efficiently in the context of innate antiviral immune responses. This may require expression of viral proteins that are involved in the suppression of antiviral immunity. The virus should cause a minimal immune reaction and should 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 (4).

Much work over the last three decades has been performed with the aim of producing such a virus. OVs that have been tested as anticancer drugs have either been naturally selected or have been genetically engineered to grow specifically in and kill cancer cells. OVs can be divided into three categories: 1) naturally occurring viruses (*e.g.*, Newcastle disease virus, vesicular stomatitis virus, autonomous parvoviruses, some measles virus strains, reovirus [5]) that selectively replicate in tumor cells; 2) virus mutants in which some genes essential for

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replication in normal cells but unnecessary in cancer cells have been deleted (*e.g.*, the adenovirus ONYX-015, which replicates only in cells with mutant *p53*, or the herpesvirus G207, which requires the presence of ribonucleotide reductase [*ICP* gene]) (6); and 3) virus mutants with tissue-specific transcriptional elements that drive viral genes (*e.g.*, the adenovirus CV706, which has prostate-specific antigen [PSA]-restricted expression of *E1A* and *E1B*, and the adenovirus adMycTK, which selectively binds myc protein) (7). All of these viruses are tumor-selective *in vitro* and/or *in vivo*. Many of these agents have already been clinically tested using intratumoral, intraperitoneal (i.p.) and/or intravenous (i.v.) routes of administration.

OVs can mediate the destruction of tumor cells by mechanisms other than direct lysis. OVs may effectively induce adaptive antitumor immunity that comprises both antibody and T cell responses targeting tumor-associated antigens (TAAs) (8-10). Some viruses express proteins that are cytotoxic to cancer cells (*e.g.*, adenoviruses express cytotoxic proteins E3 and E4ORF4) (10), and importantly, Schulz *et al*. showed that cells infected with virusues were more effective at delivering nonviral antigens for cross-priming of antigen-presenting cells (APCs) *in vivo* (11).

Viral infection of cells induces an immune response that consists of cytokine production (interferons α, β and γ, tumor necrosis factor α [TNF-α] and several interleukins) and infiltration of cytotoxic cells, such as macrophages, neutrophils and natural killer (NK) cells. Since activation of apoptotic pathways in cancer cells is not the main mode of the destruction induced by OVs, cross-resistance with standard chemotherapeutics or radiotherapy is not frequent. However, an immune response is also likely to destroy replicating virions, thus limiting their effects (5). Although neutralizing antibodies and the complement system do not limit therapeutic efficacy in the case of intratumoral injection of OVs, they may significantly restrict systemic therapy (6). Immunosuppression by corticosteroids decreased the efficiency of G207 (double mutant of the herpes simplex virus type 1 [HSV-1] with deletions at both γ 34.5 (RL1) loci and a *lacZ* gene insertion inactivating the *ICP6* gene) in transplanted human tumors (8). On the other hand, Hirasawa *et al*. (9) found increased efficiency of a reovirus in mouse tumors after the application of ciclosporin and anti-CD4 and anti-CD8 antibodies. It remains to be determined which mechanisms are involved in antiviral immunity and which contribute to the anticancer effect.

Reovirus (an acronym for Respiratory Enteric Orphan viruses) is one of the replication-competent, naturally occurring viruses that preferentially kill tumor cells (12). Research into the mechanism of the tumor cell selectivity of reovirus revealed that it replicates favorably in the presence of activated Ras signaling, which is common in cancer cells (13).

Reovirus replicates in the cytoplasm and comprises two concentric icosahedral protein capsids with trimeric σ1 proteins that protrude from vertices. The capsid surrounds the genome consisting of 10 segments (large L1, L2 and L3, medium M1, M2 and M3, and small S1, S2, S3 and S4) of double-stranded (ds) RNA (14). Each dsRNA segment encodes a single protein, except for the S1 gene segment, which is bicistronic. Proteins are denoted λ, μ and σ according to the RNA segment from which they are transcribed. Reovirus encodes its own polymerases essential for replication of the viral genome and therefore is not dependent on the S phase of the host as some DNA viruses are (4).

Reoviruses are ubiquitous viruses that have been isolated from a wide variety of mammalian species, including humans. In humans, reoviruses are commonly isolated from the respiratory and gastrointestinal tract, but they are not associated with any known disease and are therefore considered to be nonpathogenic (15). Thus, they were classified as orphan viruses (a virus which is not associated with any known disease). According to their hemagglutination activity, three serotypes of reovirus have been described. Laboratory strains of each serotype were isolated and designated serotype 1 Lang, serotype 2 Jones, serotype 3 Abney and serotype 3 Dearing (T3D). All three serotypes of reovirus are found ubiquitously in the environment, particularly in still water or sewage water. As many as 50% of adults aged 20-30 years have already been exposed to reovirus and carry antibodies against the virus, and seropositivity has been detected in 70-100% of older individuals (16, 17).

The lytic cycle of reovirus is complex and consists of many steps. It begins with the attachment of the virion to the receptor of the host cell via σ1 protein and the virus enters the cell by receptor-mediated endocytosis. The σ1 protein is a fibrous trimer composed of an elongated tail domain inserted into the virion and a globular head domain that sticks out from the virion surface (18). The head and tail regions of T3D σ1 contain receptor-binding domains. The domain in the tail binds α-linked sialic acid (19, 20), whereas the domain in the head binds junctional adhesion molecule 1 (JAM-1) (21). σ1 protein of T3D can be dissociated by intestinal proteases such as trypsin or chymotrypsin (22). Reovirus attaches to cells via an adhesion-strengthening mechanism by which initial low-affinity binding to sialic acid facilitates secondary higher affinity binding to JAM-1. The capacity of T3D reovirus to bind sialic acid influences infection of cultured cells (23). However, although ligation of sialic acid and JAM-1 is necessary for reovirus-induced cell death, viral attachment to the cell surface alone is not enough. Inhibitors of acid-dependent viral disassembly block apoptosis activated by reovirus, which indicates the requirement for post-attachment entry steps (24). Within the endosome, proteolysis of viral outer capsid proteins gives rise to an intermediate subviral particle (ISVP). Receptor binding and disassembly must occur within the same cellular compartment to elicit an apoptotic response. A critical component of the signaling cascade that leads to apoptosis of reovirus-infected cells is the transcription factor NF-κB (25). Reovirus also activates c-Jun *N*-terminal kinase (JNK) and extracellular signal-regulated kinase

(ERK) (26), but the involvement of these signaling molecules in NF-κB activation and apoptosis induction is not understood. Triggered ISVPs penetrate through the endosomal membrane. Subsequently, the transcription of 10 RNA segments mediated by viral dsRNA-dependent RNA polymerase proceeds. Later, the synthesis of RNA minus-strand occurs and secondary transcription of late viral mRNAs begins. Final composition of the outer capsid yields viral particles that induce cell lysis (27). Moreover, viral transcription is not indispensable, as inhibitors of viral RNA synthesis do not diminish the capacity of reovirus to induce apoptosis (24, 28).

Reovirus does not replicate in normal mouse fibroblasts, but fibroblasts transfected with activated Ras, epidermal growth factor receptor (EGFR, ErbB-1) or V-erbB are lysed by uninhibited reovirus replication (12). The mechanism of preferential reoviral tropism in *RAS*-transformed cells has not been fully defined, but a defective cellular antiviral response triggered in these cells is obvious. In normal cells, reoviral dsRNAs activate PKR (protein kinase RNA-activated), which in turn phosphorylates the α subunit of initiation factor 2 (eIF-2-α). This phosphorylation shuts off any further protein translation and thus inhibits the initiation of translation of viral transcripts. In contrast, in cells with constitutive Ras activation, the phosphorylation of eIF-2-α is inhibited, resulting in viral translation and subsequent entry into the viral lytic cycle (13). Furthermore, recent evidence suggests that Ras transformation enhances viral uncoating, infectivity and virion release (29), and makes cells more sensitive to virus-induced apoptosis (30). Since activating mutations of the proto-oncogene *RAS* occur in about 60-80% of all human tumors, *e.g.*, pancreatic (90%), sporadic colorectal (50%) and lung (40%) carcinomas and myeloid leukemia (30%) (31), reovirus appears to be a good tool for inhibiting such cell populations.

Generally speaking, reovirus induces cell cycle arrest at G1 and G2/M and apoptosis, and selectively activates mitogen-activated protein kinase (MAPK) cascades. Reovirus-induced apoptosis involves members of the TNF-related apoptosis-inducing ligand (TRAIL) family and is associated with the activation of both death receptor- and mitochondrial-associated caspases (32). However, it is still not known what gene product(s) of reovirus are responsible for these properties.

In addition, reovirus may also activate the host immune system to enhance antitumor activity. Errington *et al*. showed that reovirus induced phenotypic dendritic cell (DC) maturation and the production of inflammatory cytokines, and that infected DCs could in turn elicit NK and T cell-mediated innate antitumor activity (33). In other experiments, they showed that the inflammatory response generated by reovirus-infected melanoma cells caused bystander toxicity against reovirus-resistant tumor cells and activated human myeloid dendritic cells *in vitro* (34).

Since reovirus, a dsRNA virus, is an efficient inducer of interferon α and β, it is believed that a host interferon response may play an important role in oncolysis (35). On the contrary, resistance to reovirus was demonstrated. In an *in vitro* study using a human fibrosarcoma-derived cell line that carried *RAS* mutation resistance to reovirus associated with persistent reovirus infection, elevated PKR phosphorylation and decreased cathepsin B activity were documented (36).

Preclinical Pharmacology

In an *in vitro* study, all primary glioma cultures from patients and 20 of 24 established glioma cell lines treated with reovirus were destroyed, whereas all meningioma primary cultures were spared. In *in vivo* experiments, reovirus therapy prolonged survival in two orthotopic intracerebral mouse glioma models and caused significant cytoreduction and tumor regression in two subcutaneous immunodeficient mouse models of malignant glioma (37, 38). Direct intracerebral injection of reovirus appeared to be safe in mice, rats and cynomolgus monkeys. Survival of nude mice with medulloblastoma cell lines implanted orthotopically was prolonged after intratumoral injection of live reovirus compared to injection of an inactivated reovirus (39). In an orthotopic medulloblastoma model in nude mice, multiple intratumoral reovirus injections were given. As expected, all control animals treated with inactivated virus developed spinal cord or leptomeningeal metastases, whereas none of the animals treated with live virus had detectable metastases (40). The investigators suggested that metastatic tumor cells were selected for high Ras activity and thus presented a favorable target for reovirus. This study also suggested that reovirus therapy might prevent both local invasion and metastatic tumor spread.

In another study, human breast cancer xenografts were inoculated in both the left and right hind flanks and reovirus was injected into one flank tumor only. Reovirus replication and tumor regression were observed at both injected and noninjected contralateral sites. In addition, reovirus could replicate not only in breast cancer cell lines, but also in surgical specimens from breast cancer patients (41). Furthermore, the ability of reovirus to treat breast tumors established in the brain was evaluated. Intracranial reovirus administration prolonged survival in nude mice, as did intrathecal reovirus administration in immunocompetent rat models. Both types of administration prevented local tumor invasion of breast cancer and metastases. Reovirus did not cause mortality when administered intracranially at doses up to 1×10^8 plaque-forming units (pfu) in *nu/nu* mice (42).

Reovirus also demonstrated activity against mouse Lewis lung cancer metastasis following i.v. administration, 65-80% of the tested mice showing regression of their tumors (43).

Reovirus was able to infect all five human pancreatic cancer cell lines tested *in vitro*. Elevated Ras activity was confirmed in these cell lines. Using two pancreatic cancer cell lines in a unilateral tumor xenograft model in nude mice, tumor growth was suppressed by intratumoral injection of reovirus. In addition, local injection of reovirus had

systemic antitumor effects in a bilateral xenograft model, with regression of both injected and uninjected xenografts being observed (44).

Reovirus exhibited significant antitumor activity against all four pediatric sarcoma (Ewing's sarcoma and rhabdomyosarcoma) xenografts tested (45). Reovirus also killed both human melanoma cell lines and freshly resected tumors, and intratumoral administration caused regression of melanoma in a xenograft model *in vivo* (34).

Reoviral irrigation, both immediate and delayed, of squamous cancer-contaminated wounds in SCID mice resulted in a significant reduction in tumor recurrence. Squamous cancer is susceptible to reovirus *in vitro*, and therefore this seems to be a suitable model of local adjuvant therapy after surgically induced complete remission (46).

Reovirus is not inactivated by clinically relevant doses of irradiation and radiation facilitates its cytotoxicity in tumor cell lines (head and neck, colorectal and breast cancer) *in vitro* and in syngeneic tumor xenografts (34, 47, 48). Clinical studies with reovirus in combination with radiotherapy have started based on these experiments. Treatment of human colon cancer cell lines with reovirus and gemcitabine resulted in *in vitro* and *in vivo* synergy (tumor was injected into the flanks of nude mice) (49). Intratumoral inoculation of reovirus in subcutaneous tumors induced in mice by human papillomavirus type 16 (HPV-16)-transformed TC-1 cells resulted in only a small decrease in tumor growth, but never in complete cure. When using cyclophosphamide in combination with viral treatment, a synergistic effect resulting in tumor suppression was observed. The best results were obtained when repeated cyclophosphamide administration was followed by reovirus treatment. A synergistic effect for reovirus and cyclophosphamide (coadministered with an S9 fraction, which is necessary for cyclophosphamide activation) was also found *in vitro* (manuscript submitted for publication). Similar effects of reovirus combined with cyclophosphamide were described in different *in vivo* models (50). Combined reovirus and radiotherapy led to statistically significant increases in cytotoxicity both *in vitro* and *in vivo*, particularly in those cell lines with moderate susceptibility to reovirus alone. The enhanced cytotoxicity of the combination occurred independently of treatment schedule (48).

Immunodeficient mice with tumors derived from the Burkitt's lymphoma Raji cell line were cured by i.v. reovirus, while inactivated reovirus was ineffective (51). Primary lymphoma samples collected from patients were tested *in vitro* and 100% of chronic B cell lymphoid leukemias and 100% of diffuse large B cell lymphomas were sensitive to reovirus, but the majority of follicular lymphomas were resistant (51, 52). Reovirus did not affect $CD34^+$ stem cells or their clonogenic potential. *Ex vivo* use of oncolytic virus seems to be promising for purging autologous hematopoietic stem cell harvests, as shown in *in vitro* studies with experimentally contaminated peripheral blood apheresis products (52). Mice with L1210 leukemia were treated with BCNU and subsequently with reovirus. Complete remission of tumor was observed in 80% of mice, and cured animals were resistant to challenge with L1210 leukemia, although they remained susceptible to challenge with heterologous tumor (53). One may speculate that reovirus potentiates the immune response to tumor antigens.

Clinical Studies

The positive preclinical findings led to the development of Reolysin® (reovirus serotype 3 strain Dearing), which is now being used in clinical trials as a powerful anticancer agent against tumors with an activated *RAS* oncogene or Ras pathway. Oncolytics Biotech has been issued patents that cover the pharmaceutical use of reovirus in the treatment of *RAS*-mediated cancers and the manufacture of reovirus in animal component-free media (54). A survey of clinical trials with Reolysin® is presented in Table I.

Intratumoral reovirus administration in a phase I study in patients with recurrent subcutaneous tumors demonstrated that dose escalation up to 10^{10} pfu did not cause any serious adverse events and no dose-limiting toxicities were found. The only adverse events were headache and transient flu-like symptoms. As a secondary endpoint in the study, tumor responses were also evaluated. Eleven of 18 patients (61%) demonstrated some response to viral therapy, with 32-100% tumor regression, and partial regression was documented in noninjected tumors in some patients (55).

Preliminary results of a clinical study showed that the combination of intratumoral Reolysin® administration and radiation was well tolerated. Three of 8 patients (esophageal, squamous skin and colorectal carcinoma) had significant partial responses. In addition, a patient with metastatic esophageal cancer also had tumor reduction in nonirradiated mediastinal disease (47, 56).

In another study, a total of 33 patients were treated i.v. with Reolysin®. PSA decreased by 50% in a patient with metastatic prostate cancer, with evidence of tumor necrosis on CT scan. Two patients with metastatic colorectal cancer had carcinoembryonic antigen (CEA) reductions of 60% and 27%. One patient with metastatic bladder cancer had a minor response. Reolysin® was well tolerated, with minimal toxicity, and the maximum tolerated dose was not reached (57, 58).

Twelve patients with recurrent malignant gliomas were treated with intratumoral Reolysin®. There were no grade 3 or 4 adverse events related to the administration of reovirus. One patient has stable disease and 11 patients had progressive disease (59, 60).

A prostate cancer trial was designed to evaluate the safety and efficacy of intratumoral administration of Reolysin® for the treatment of cancer restricted to stage T2. Patients received an intratumoral injection of Reolysin® and thereafter were monitored for 3 weeks, and at that time the prostate was removed. The primary efficacy endpoint was the response rate, as measured by pathological examination of the tumor. The pathological data showed evidence of apoptosis in 4 of 6 patients. Results of the

Table I: Clinical trials with Reolysin® (adapted from www.oncolyticsbiotech.com).

Cancer	Trial program	Phase	Status
Advanced pancreatic, lung, ovarian	I.v. administration with cyclophosphamide	I/II	Approved
Metastatic melanoma	I.v. administration as monotherapy	II	Protocol field
Ovarian	I.v. and i.p. administration	I/II	Pending
Sarcomas metastasized to lung	I.v. administration as monotherapy	II	Ongoing
Advanced pancreatic, lung, ovarian	I.v. administration with gemcitabine	I/II	Ongoing
Advanced bladder, prostate, lung, upper gastrointestinal	I.v. administration with docetaxel	I/II	Ongoing
Advanced melanoma, lung, ovarian	I.v. administration with paclitaxel and carboplatin	I/II	Ongoing
Metastatic cancers including head and neck	Local therapy in combination with radiation	II	Ongoing
Various metastatic cancers	Local therapy in combination with radiation	I	Phase Ia complete; phase Ib ongoing
Recurrent malignant glioma	Intratumoral infusion as monotherapy	I/II	Ongoing
Various metastatic cancers	I.v. administration as monotherapy	I	Complete*
Various metastatic cancers	I.v. administration as monotherapy	I	Complete**
T2 prostate cancer	Local administration	I	Complete***
Various subcutaneous tumors	Local administration	I	Complete#

*Of the 18 patients treated, 8 demonstrated stable disease, including a patient with progressive breast cancer who experienced a 28.5% shrinkage in tumor volume. **Reolysin® was well tolerated by patients, with several notable changes in stabilization of disease, as well as some minor tumor regressions in patients who had failed all previous treatments. ***Evidence of apoptotic tumor cell death in 4 of 6 patients, with no safety concerns; 1 patient experienced a PSA drop of 53% and prostate gland shrinkage of 67%. #None of 18 patients receiving Reolysin® experienced any serious adverse events related to the virus, nor were there any dose-limiting toxicities; evidence of viral activity was detected in 11 of 18 patients, with the tumour regression ranging from 32% to 100%.

phase I Systemic Administration Trial showed that Reolysin® delivered systemically was well tolerated and active in patients with colorectal, bladder, prostate, pancreatic, endometrial and non-small cell lung (NSCLC) cancers. A phase II trial to evaluate i.v. Reolysin® in patients with various sarcomas that had metastasized to the lung demonstrated that at least 1 patient of the first 38 patients treated experienced a response and stable disease for longer than 6 months (54).

During a phase I trial of i.v. Reolysin® pretreatment of patients with advanced cancers, a detailed analysis of the immune effects was conducted by collecting samples for analysis of peripheral blood lymphocytes. $CD3^+CD4^+$ cells (helper T lymphocytes) were reduced in most patients, but after reovirus therapy their numbers increased in 47.6% of patients. Most patients had high baseline $CD3^+CD8^+$ cell (cytotoxic/suppressor T lymphocyte) levels, with 33% showing incremental increases after therapy. Most patients had high numbers of circulating $CD3^-CD56^+$ cells (NK cells) before therapy and in 28.6% this increased with treatment. $CD3^+CD4^+CD25^+$ (regulatory T cells that suppress antitumor immunity) were largely unaffected by the therapy. These data confirm that even heavily pretreated patients are capable of mounting dynamic immune responses during treatment with Reolysin® (61).

Several clinical trials, which included over a hundred patients, demonstrated that intratumoral (including intracranial and intravenous) administration of Reolysin® was well tolerated (47, 54-60).

Future directions

The study of the interactions of reovirus with chemo- and/or radiotherapy will be a necessary step in its therapeutic development. In addition to the drug combination used, the timing of drug and virus aplication also appears to be important. Our unpublished results showed that reovirus applied after cyclophosphamide is much more effective than reovirus injected before cyclophosphamide in syngeneic tumor models. Additionally, it will be very interesting to ascertain whether combination therapy with multiple OVs will improve therapeutic efficiency. Since OVs act through different cellular targets, it is probable that tumors resistant to one virus may be sensitive to others. Also, antiviral immunity inhibiting one type of OV could be overcome by using another type of virus (27). The production of neutralizing antibodies could be suppressed by administration of anti-CD20 antibodies (rituximab) prior to the oncolytic virotherapy, or they may be eliminated by plasmapheresis (6). Complement could be transiently neutralized by administration of cobra venom factor or cyclophosphamide (62).

In conclusion, reovirus appears to be a safe and promising anticancer therapy in tumors with activated *RAS* and may become a part of combination therapy.

Disclaimer

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Source

Oncolytics Biotech, Inc. (CA).

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