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Review Article

The use of immunohistochemistry to determine oncolytic reovirus distribution and replication in-human tumors

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ABSTRACT

An oncolytic virus is considered a targeted cancer therapy due to its ability to specifically target, replicate in and lyse cancer cells while leaving normal cells unharmed. Over the last few years several tumor selective oncolytic viruses have been developed. These include certain DNA viruses such as adenovirus that have been genetically manipulated to target specific cancer cells by exerting restrictions on the virus at the level of cell entry, viral gene transcription and viral protein translation. There are a variety of RNA viruses being studied as possible cancer therapies including reovirus. Reovirus is intrinsically oncolytic without the need for any genetic manipulation. The inherent oncolytic properties of this virus are derived from the fact that it specifically targets cells with an activated Ras pathway found in many cancer cells. REOLYSIN® is a proprietary formulation of human reovirus type 3 Dearing developed by Oncolytics Biotech Inc. and is the only therapeutic reovirus in clinical development. This review provides an overview of the development of REOLYSIN as a potential cancer therapeutic and the growing role of *in situ* based molecular pathology methods in providing clinical proof of concept and in guiding clinical development.

1. Reovirus and transformed cells

REOLYSIN® is an isolate of the replication competent type 3 Dearing reovirus, a non-enveloped virus containing 10 segments of double-stranded RNA as its genome. Community-acquired reovirus infections in-humans are mild and are restricted to the upper respiratory and gastrointestinal tract; they are usually asymptomatic. The reovirus infection cycle consists of viral attachment, endocytic uptake, outer shell uncoating and transmembrane penetration into the cytoplasm where viral replication occurs [1]. Reovirus outer capsid protein σ1 mediates cell attachment and receptor-mediated endocytosis is mediated via sialic acid [2] and junctional adhesion molecule-1 (JAM-1) [3]. The human reovirus possesses an innate ability to replicate specifically in transformed cells possessing an activated Ras signaling pathway, a situation often found in malignant cells [4,5].

The preferential lysis of cells with activated Ras by reovirus appears to be due to the inhibition of double-stranded RNA-activated protein kinase (PKR) in these cells [6]. In non-Ras activated cells, the presence of viral transcripts causes PKR autophosphorylation, which then causes inhibition of viral protein synthesis, thereby preventing viral replication. Ras activated cells inhibit the autophosphorylation of PKR, keeping it in an inactive state and allowing viral translation, replication and oncolysis to take place. This causes virus-mediated cancer cell death. Tumor antigens exposed by viral oncolysis may cause an immune response against the exposed cells. The net result is a highly targeted anti-cancer effect with few adverse effects.

The Ras family of proteins consists of three isoforms, H-, K-, and N-Ras, and has been implicated in the development of cancer. Mutated Ras proteins, particularly mutated K-ras, stimulate cell division and proliferation in the absence of growth factors, that are often missing in various cancers. Approximately one-third of human cancers have activating mutations in the Ras gene itself [7,8]. It is conceivable that >50% have an activated Ras signaling pathway because of activating mutations in genes upstream or downstream of Ras; therefore it is possible that reovirus could be used to treat a high proportion of cancers [9].

Pre-clinical testing of reovirus demonstrated the virus' ability to form self-replicating viral factories within the tumor cells that could potentially have ongoing therapeutic effect. Additional critical features of REOLYSIN therapy are evidence of synergy and/or additive effects with standard chemotherapies or radiation, as well as evidence that reovirus may play a role in overcoming drug resistance [10,11]. Pre-clinical and clinical data suggests that the use of immune modulating chemotherapeutic drugs in combination with reovirus may, in fact, enhance the anti-cancer effects of reovirus by causing moderate ablation of the antibody response, thus allowing replication and circulation of the virus in patients to occur to a

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greater extent and/or for a longer period of time [12,13]. The clinical data also indicate that using REOLYSIN in patients with immune modulation due to prior or concurrent chemotherapy does not change the adverse event profile of REOLYSIN. Likewise, the addition of REOLYSIN does not appear to enhance either the frequency or severity of the adverse effects of the chemotherapeutic agents with which it has been combined [14–16].

2. Pre-clinical studies

Several *in vitro* studies have demonstrated reovirus predilection for replication in Ras activated cells. NIH3T3 cells are naturally resistant to reovirus infection. NIH3T3 cells when transformed with *v-erb* oncogene, a truncated EGFR lacking ligand binding extracellular domain but containing a constitutively active tyrosine kinase cytoplasmic domain which activates the Ras signaling pathway, became highly permissive to reovirus infection [17]. Severe combined immune deficient mice bearing tumors established from *v-erbB*-transformed murine NIH3T3 cells or human U87 glioblastoma cells were treated with a single intratumoral injection of reovirus. Reovirus treatment resulted in more than 80% tumor regression in approximately 65–80% of these mice [18].

A possible limitation of SCID mice models in oncolytic viral studies is that they do not allow assessment of the impact of the host's immune system has upon the therapy. To this aim, immune competent C3H mice were implanted with *ras*-transformed C3H-10R1/2 cells to form tumor allografts. Once palpable tumors were established, these mice were given a series of intratumoral injections of reovirus. Complete regressions of treated tumors was seen in six of the nine reovirus-treated mice; however, it is important to note that more injections were required in this allograft model than in the SCID mouse xenograft studies. To assess the impact pre-existing antibodies have upon reovirus therapy, C3H mice were inoculated with reovirus prior to tumor implantation. After 2 weeks, anti-reovirus antibodies were detected in all reovirus injected animals. Perhaps surprisingly, it was noted that animals with previous exposure to reovirus had comparable tumor regressions to those with no previous exposure, suggesting that the development of neutralizing antibodies does not impair the oncolytic activity of reovirus when administered intratumorally [18]. This was a key finding, as the majority of humans have been previously exposed to reovirus and have anti-reovirus antibodies, and therefore even patients with anti-reovirus antibodies could benefit from reovirus' oncolytic activity [18,19]. Several pre-clinical reports that demonstrated the effects of reovirus as an anti-cancer agent in a variety of cancer types followed; these provided the rationale for the commencement of the first in-human studies of REOLYSIN in North America and Europe examining both local and systemic administration.

3. Immune system

The ultimate goal of oncolytic viral therapy is to extend it beyond the arena of local administration to systemic delivery. To realize this goal, emphasis must be paid to the role that innate and adaptive immune responses exert upon viral delivery to, and replication and spread within, tumors following intravenous administration.

While it was demonstrated that intratumoral reovirus therapy could be effective in animals with pre-existing immunity to the virus [18], systemic delivery proved to be more challenging and required interventions to modulate the immune system to make the therapy effective [12,13,20,21,22].

Hirasawa and his colleagues were the first to examine systemic reovirus therapy in an immune competent host with and without immune intervention [12]. It was demonstrated that not only could intravenously delivered virus inhibit the growth of metastatic disease, but that it could also produce improved animal survival. Importantly, these oncolytic effects could be augmented by the addition of immune suppressing agents, suggesting that immune antagonism to oncolytic effectiveness does occur. Unlike intratumoral studies of reovirus oncolysis, pre-existing immunity to the virus diminished the effectiveness of systemically delivered reovirus. The researchers were able to demonstrate that this immune barrier could be overcome with a variety of immune modulating agents including cyclosporine A (CyA), cyclophosphamide (CyP), or anti-CD4/anti-CD8 antibodies. Notably, preimmunized animals treated with either CyA or CyP demonstrated a marked decrease in neutralizing anti-reovirus antibodies (NARA), and these titers were comparable to animals that were not previously exposed to the virus. The general immune suppression of these two agents prevents a complete understanding of the role that B- and T-cell suppression plays in oncolytic effectiveness and tolerability of this intervention.

Subsequent work focusing on the combination of cyclophosphamide and reovirus has perhaps cast some light on the double-edged role that NARA may play in antagonizing oncolytic activity while simultaneously enhancing the specificity of reovirus to tumor tissue [13,21]. C57Bl/6 mice were implanted with subcutaneous B16 tumors and treated with intravenous reovirus and different regimens of intraperitoneal CyP. In this system, it was demonstrated that CyP was able to increase intratumoral viral replication and subsequent tumor regression as compared to monotherapy reovirus. Importantly, the high dose CyP regimen resulted in ablation of the NARA response and was associated with severe toxicities and replication of virus in normal organs [13]. These toxicities included cardiotoxity associated with diffuse myocarditis and calcification. Histopathology of animals treated with either reovirus or CyP did not demonstrate these toxicities, which demonstrated that the pathology was related to the combination. A number of the animals receiving the high dose CyP and reovirus combination were noted to have tails that turned black, consistent with a vaculitis and "black foot syndrome" previously reported in SCID mice models used to investigate intratumoral delivery of the virus [18]. These severe toxicities have also been reported in tumor-bearing B-cell knockout mice [13]. It has been postulated that these toxicities are due to the continuous release of progeny virus from the infected tumor and not to the input virus. The rationale for this comes from the observation that high dose CyP/reovirus combinations did not cause toxicities in metastatic models where tumor burdens are smaller than subcutaneous models and resultant viral replication and shed is diminished [13]. These results clearly define the duality of NARA's role in reovirus activity. In one instance induction of NARA clearly impedes the activity of the virus yet it plays an import role in preventing progeny virus produced in infected tumor tissues from disseminating systemically to non-target organs and in preventing the manifestation of toxicity. Clearly the goal is to modulate the NARA response without ablating it in the clinical situation.

More recent investigations have focused on combining immune modulation with strategies to promote virus extravasation at the site of the tumor [21]. Preconditioning C57Bl/6 mice bearing subcutaneous B16 tumors with PC-61 (which depleted Treg cells *in vivo)* and IL-2 injections enhanced the intratumoral delivery of intravenously delivered reovirus by 2–3 logs compared to mice treated with reovirus alone [21].

Clearly the interaction of an oncolytic virus and the host's immune response is multifaceted. There are even suggestions that oncolytic reovirus treatment may result in immune interactions that are cytotoxic to the tumor itself. A recent study proposes that reovirus may trigger innate antitumor activity that enhances reovirus' oncolytic effects. Reovirus was able to activate dendritic cell