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Reovirus Therapy of Tumors with Activated Ras Pathway

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Human reovirus requires an activated Ras signaling pathway for infection of cultured cells. To investigate whether this property can be exploited for cancer therapy, severe combined immune deficient mice bearing tumors established from v-*erbB*–transformed murine NIH 3T3 cells or human U87 glioblastoma cells were treated with the virus. A single intratumoral injection of virus resulted in regression of tumors in 65 to 80 percent of the mice. Treatment of immune-competent C3H mice bearing tumors established from *ras*-transformed C3H-10T1/2 cells also resulted in tumor regression, although a series of injections were required. These results suggest that, with further work, reovirus may have applicability in the treatment of cancer.

Activating mutations of the proto-oncogene Ras occur in about 30% of all human tumors (*1*), primarily in pancreatic (90%), sporadic colorectal (50%), and lung (40%) carcinomas and myeloid leukemia (30%). Because Ras is a key regulator of mitogenic signals, aberrant function of upstream elements such as receptor tyrosine kinases (RTKs) can also result in Ras activation in the absence of mutations in Ras itself (*2*). Indeed, overexpression of RTKs such as HER2/Neu/ErbB2 or the epidermal growth factor receptor (EGFR) is common in breast cancer (25 to 30%) (*3*), and overexpression of platelet-derived growth factor receptor (PDGFR) or of wild-type or truncated EGFR is prevalent in gliomas and glioblastomas (40 to 50%), tumor types in which Ras mutations are rare (*4*–*6*). Attempts to target the Ras signaling pathway for cancer therapy have focused on drugs such as farnesyltransferase inhibitors that down-regulate the pathway (*7*). In contrast, the possibility of exploiting the activated Ras pathway itself as an anticancer therapy has not been explored.

The human reovirus is a ubiquitous, nonenveloped virus containing 10 segments of double-stranded RNA as its genome (*8*). Reovirus infections in humans are believed to be mild and restricted to the upper respiratory and gastrointestinal (GI) tracts, but in general they are asymptomatic (*8*). Recent in vitro studies have shown that mouse fibroblasts that are resistant to reovirus infection become susceptible after transfection with the gene encoding EGFR (*9*) or with the v-*erbB* oncogene (*10*). Transformation of the reovirus-resistant NIH 3T3 cells with activated Sos or Ras also results in enhanced infection (*11*), indicating that an activated Ras signaling pathway is exploited by reovirus. Restriction of reovirus replication in untransformed NIH 3T3 cells is due to activation of the double-stranded RNA-activated protein kinase (PKR) by early viral transcripts, which in turn inhibits the translation of these transcripts (*11*). Activated Ras (or an activated element of the Ras pathway) presumably inhibits (or reverses) PKR activation, thereby allowing viral protein synthesis. The selective replication of reovirus in cells with an activated Ras signaling pathway (*11*), coupled with the relatively nonpathogenic nature of this virus in humans (*8*), makes it attractive as a potential oncolytic agent.

To test the efficacy of reovirus as a tumor therapy, we implanted severe combined immune deficient (SCID) mice with v-*erbB*–transformed NIH 3T3 cells (designated THC-11), which support reovirus replication in vitro (*10*). Tumor cells were introduced subcutaneously and unilaterally into the hind flank of the mice. Palpable tumors (mean area of 0.31 cm^2) were established after 2 weeks, and eight mice, each with one tumor, were given a single intratumoral injection of 1.0×10^7 plaque-forming units (PFUs) of reovirus serotype 3 (strain Dearing) in phosphate-buffered saline (PBS). Control tumors ($n = 10$ mice) were injected with equivalent amounts of ultraviolet (UV)–inactivated virus. The single dose of reovirus resulted in marked (~80%) repression of tumor growth in six of eight animals by day 12 (Wilcoxon test: $P = 0.0062$) (Fig. 1) when tumors in the control animals exceeded the acceptable tumor burden.

We next assessed the efficacy of reovirus against human tumor cells. We tested the human glioblastoma U87 cell line because it overexpresses the PDGFR (*5*, *12*) and thus has increased levels of activated Ras (*6*). These cells were susceptible to reovirus infection in vitro as evidenced by the synthesis of viral proteins and shutoff of host protein synthesis within 24 hours after infection (Fig. 2A) and widespread cytopathic effects by 48 hours (Fig. 2B). The U87 cells were then implanted as xenografts into the hind flank of SCID mice.

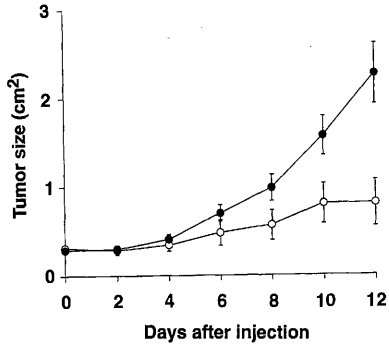

Fig. 1. Effect of reovirus on murine THC-11 tumors (v-*erbB*–transformed NIH 3T3 cells) grown subcutaneously in SCID mice (*22*). Each mouse received a single implant. Two weeks after implantation, tumors were injected with 1.0×10^7 PFUs of reovirus (open circles; $n = 8$ tumors) or an equivalent amount of UV-inactivated reovirus (filled circles; $n = 10$ tumors), and tumor growth was followed for 12 days. The experiment was repeated two additional times with similar results (mean ± SEM).

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Once tumors reached a mean area of 0.21 cm^2, a single intratumoral injection of reovirus was given. Tumor growth was substantially suppressed, and tumor regression was observed in four of five mice by the fourth week after treatment (Wilcoxon test: $P = 0.008$) (Fig. 3A). Hematoxylin-eosin (HE)–staining revealed that, after reovirus treatment, the remaining tumor mass consisted largely of normal stroma without detectable tumor cells, nor was there any evidence of tumor cell infiltration into the underlying skeletal muscle (Fig. 3B). Necrosis of tumor cells was due to direct lysis by the virus, the mechanism by which the virus kills the cells in cultures. To determine whether the virus spread beyond the tumor mass, we analyzed the tumor and adjoining tissue by immunofluorescent microscopy with antibodies to total reovirus proteins. Reovirus-specific proteins were confined to the tumor mass; no viral staining was detected in the underlying skeletal muscle (Fig. 3B). As expected, viral proteins were not present in tumors injected with the UV-inactivated virus. These results suggest that reovirus replication in these mice is restricted to the target U87 tumor cells.

One of the greatest challenges in viral therapeutics is the host immune system, which could ultimately inactivate the therapy. To test the effect of reovirus on tumors grown in immune-competent animals, we used a *ras*-transformed C3H-10T1/2 fibroblast [designated C3 (*13*)] that forms tumors in the syngeneic immune-competent C3H mice. The susceptibility of the cells to reovirus infection in vitro was confirmed by analysis of cytopathic effect (*14*) and viral protein synthesis (Fig. 4A). The cells were then implanted as a tumor allograft into C3H mice at sites overlying the left hind flank, and palpable tumors were established (mean area of 0.22 cm^2) after 2 weeks. Because of the presence of a competent immune system, the mice were given a series of intratumoral injections of reovirus over 21 days (six injections of 1.0×10^8 PFUs each for the first 9 days, followed by injections of 1.0×10^7 PFUs every 48 hours for the next 12 days). The control mice were injected with equivalent amounts of UV-inactivated reovirus. Tumor growth was measured for the duration of the treatment. Complete regression of the tumors was seen in six of nine reovirus-treated mice (Kruskal-Wallis test: $P = 0.0023$) (Fig. 4B). Because the reovirus-treated and control animals had comparable amounts of antibodies to reovirus (*14*), tumor regression did not appear to be due to the immune response. Surviving animals with complete tumor regression exhibited no morbidity or reoccurrence of tumor growth for the 4 months after the cessation of the treatment.

To determine whether preexisting antibodies to reovirus would abrogate the oncolytic effect of reovirus in vivo, we challenged C3H mice with an intramuscular injection of reovirus (1.0×10^7 PFUs). After 2 weeks, antibodies to reovirus could be detected in all the injected animals. These animals, along with unchallenged animals, were then implanted with C3 cells. Tumors were established after 2 weeks, and the mice were given the same series of intratumoral reovirus injections as before. The results (Fig. 4C) showed that previous exposure to reovirus did not restrict the ability of the virus to destroy the tumors (Kruskal-Wallis test: $P = 0.004$).

This study illustrates the potential application of reovirus as an anticancer agent. Although only about one-third of human cancers have activating mutations in the Ras gene itself, it is conceivable that >50% have an activated Ras signaling pathway because of activating mutations in genes upstream or downstream of Ras (*2*). Thus, the proportion of cancers potentially treatable by reovirus is high. Indeed, of the 25 human cancer cell lines we have examined to date, 20 (80%) are susceptible to reovirus infection in vitro (*15*). There is also a strong correlation between in-

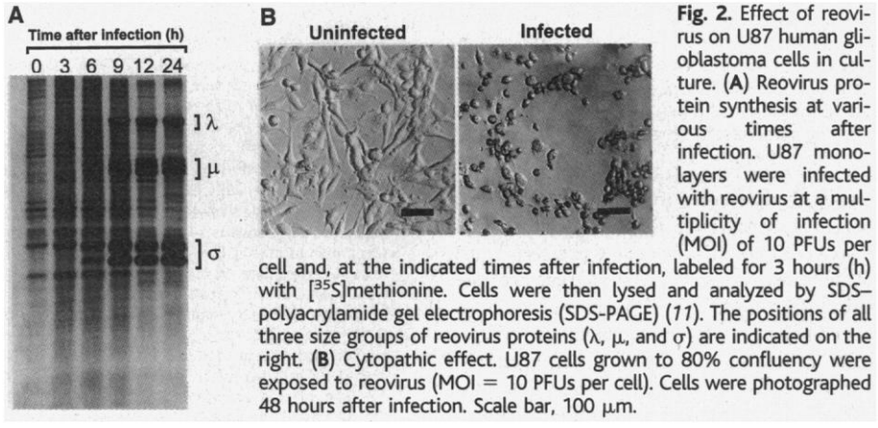

Fig. 2. Effect of reovirus on U87 human glioblastoma cells in culture. (**A**) Reovirus protein synthesis at various times after infection. U87 monolayers were infected with reovirus at a multiplicity of infection (MOI) of 10 PFUs per cell and, at the indicated times after infection, labeled for 3 hours (h) with [^{35}S]methionine. Cells were then lysed and analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (*11*). The positions of all three size groups of reovirus proteins (λ, μ, and σ) are indicated on the right. (**B**) Cytopathic effect. U87 cells grown to 80% confluency were exposed to reovirus (MOI = 10 PFUs per cell). Cells were photographed 48 hours after infection. Scale bar, 100 μm.

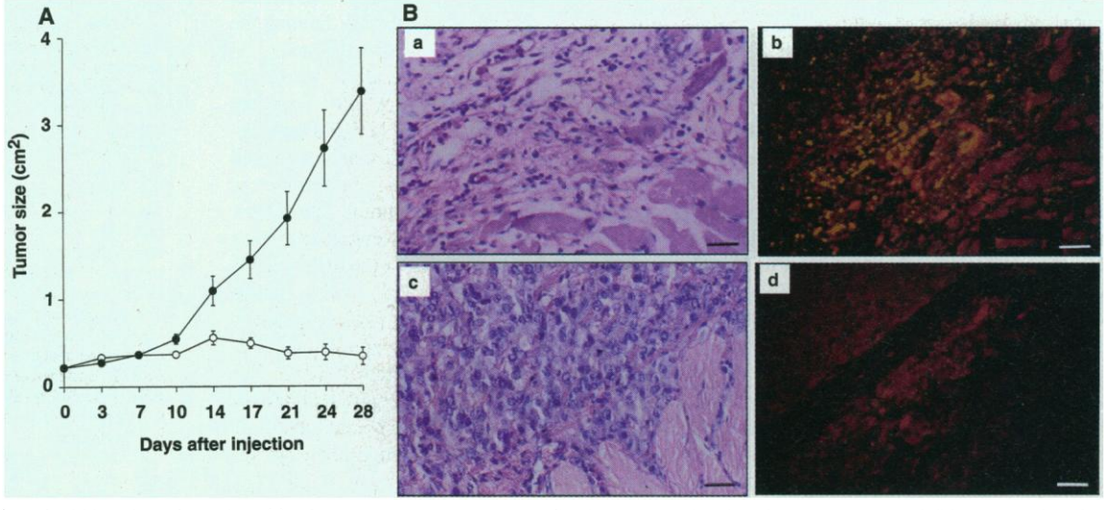

Fig. 3. Effect of reovirus on U87 human tumor xenografts grown subcutaneously in SCID mice. (**A**) Mouse received a single implant. Two weeks after implantation, tumors were injected with reovirus (open circles; $n = 5$ tumors) or UV-inactivated reovirus (filled circles; $n = 5$ tumors), and tumor growth was followed for a period of 4 weeks. This experiment was repeated three additional times with similar results (mean ± SEM). (**B**) HE staining of the remaining tumor mass 4 weeks after treatment with reovirus (a) and UV-inactivated reovirus (c). There was no evidence of viable tumor cells or tumor infiltration into the underlying skeletal muscle in the reovirus-treated tumors. Immunofluorescence analysis of paraffin sections of reovirus-treated tumor (*23*) with antibodies to reovirus shows that reovirus replication was restricted to the tumor mass and did not spread to the underlying normal tissue (b). Reovirus proteins were not detectable in the tumor treated with UV-inactivated virus (d). Black scale bars, 50 μm; white scale bars, 75 μm.

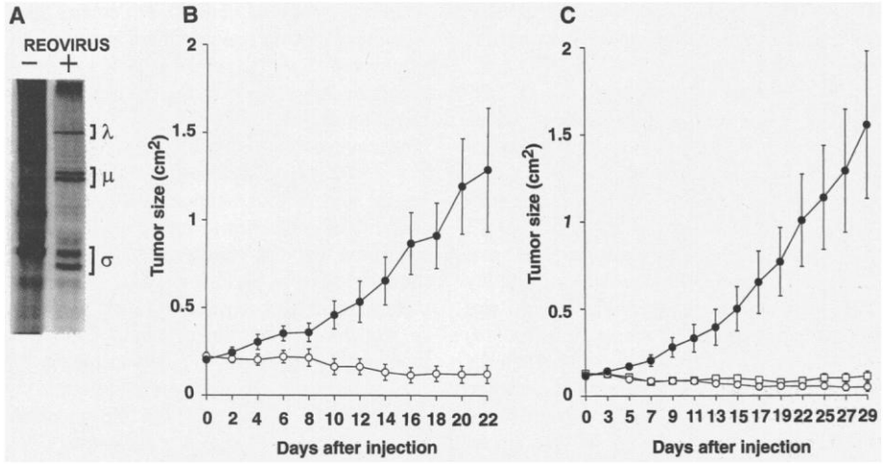

Fig. 4. Effect of reovirus on murine C3 cells grown in culture and in C3H mice. **(A)** Reovirus protein synthesis in C3 cells at 48 hours after infection. C3 cells grown to 80% confluency were infected with reovirus (MOI = 10 PFUs per cell). Infected (+) and mock-infected (−) cells were labeled at 48 hours after infection with [^{35}S]methionine for 2 hours. Cells were then lysed, and proteins were analyzed by SDS-PAGE. Reovirus proteins (λ, μ, and σ) are indicated on the right. **(B)** Immune-competent C3H mice were each implanted with a single C3 allograft. After tumor establishment, animals were given a series of intratumoral injections of reovirus (open circle; n = 9 tumors) or UV-inactivated reovirus (filled circle; n = 9 tumors). This experiment was repeated three times with similar results (mean ± SEM). **(C)** Oncolytic effect of reovirus in mice with previous exposure to the virus. C3H mice were given a single intramuscular injection of reovirus, and antibodies to reovirus were detectable within 2 weeks. Mice were then each implanted with a single C3 allograft and treated with the same regimen of reovirus as described above: Tumors in mice with no previous exposure to reovirus were injected with reovirus (open circles; n = 5 tumors) or with UV-inactivated reovirus (filled circles; n = 5 tumors); tumors in animals with antibodies to reovirus antibodies were injected with reovirus (open squares; n = 6 tumors). This experiment was repeated two additional times with the same results (mean ± SEM).

fectibility and high basal level of mitogen-activated protein kinase (MAPK) activity (*15*), the latter being a good indication of Ras pathway activation (*16*). Interestingly, our attempts to establish palpable tumors in SCID mice with the reovirus-resistant cell lines (all have low basal MAPK activity) have not been successful. This is compatible with the notion that reovirus specifically targets malignant tumors with a highly activated Ras signaling pathway. It is interesting to note in this regard that undifferentiated and mitotically active crypt cells of the intestinal epithelium have very high basal levels of MAPK activity (*17*), which may explain why the GI tract is the normal target of this virus.

Although reovirus is not associated with any major human diseases, it is pathogenic in neonatal mice (*8*) and SCID mice (*18*). The SCID mouse is therefore not an ideal model for reovirus-induced tumor regression studies. Indeed, we found that 50 to 60% of reovirus-treated animals eventually developed hind limb necrosis and died. The immunodeficiency of the SCID mice probably accounts for the high mortality rate of these mice upon exposure to reovirus. However, the ability of reovirus to infect murine tumor cells allowed us to assess the efficacy of reovirus treatment in an immune-competent host. This is particularly important because most human adults have neutralizing reovirus antibodies (*8*). The results from the C3H mouse model are therefore promising in that they suggest the feasibility of using this treatment in humans. Although the presence of a competent immune system demands more frequent reovirus injections to achieve the same regression seen in the SCID mouse model, it does alleviate the side effects seen in the latter. Previous exposure to reovirus did not prevent regression of solid tumors, and it is likely that any differences observed could be eliminated by increasing virus dosage in animals that have neutralizing antibodies before treatment. However, the presence of these antibodies may preclude administration through the systemic route, thereby limiting the number of tumor types that can be treated.

The idea of using viruses as antitumor agents has been discussed for some time, but serious attempts have been initiated only recently. Of note are the E1B gene-attenuated adenovirus ONYX-015, which targets cancer cells lacking functional tumor suppressor protein p53 (*19*), the avian Newcastle disease virus, which also appears to target the N-ras oncogene in tumor cells (*20*), and a genetically altered herpes simplex virus designed to target cancer cells with a dysfunctional p16/pRB tumor suppressor pathway (*21*). Our study suggests that reovirus, with its relatively mild pathogenicity and potent oncolytic activity, also warrants investigation as an anticancer therapeutic.

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22. NIH 3T3 cells transformed with the v-*erbB* oncogene (THC-11) and the U87 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Reovirus serotype 3 (strain Dearing) was grown as in (*11*). Five- to 8-week-old male SCID mice (Charles River, St. Constant, Quebec, Canada) were treated according to a protocol approved by the University of Calgary Animal Care Committee. Actively growing THC-11 or U87 cells were harvested, washed, and resuspended in sterile PBS at a density of 2×10^7 cells per milliliter. Cells (2.0×10^6 in 100 μl) were injected subcutaneously at a site overlying the hind flank. Implanted tumors were allowed to grow for 2 to 3 weeks until palpable tumors of 0.5 cm 0.5 cm were obtained, at which time they were injected with 1.0×10^7 PFUs of reovirus or UV-inactivated reovirus in 20 μl of sterile PBS. Tumor size was measured twice weekly for 2 to 4 weeks. All animals were killed when the control mice showed severe morbidity due to excessive tumor burden.
23. Immunofluorescence analysis was carried out on formalin-fixed, paraffin-embedded tumor sections mounted on cover slips. After removal of paraffin by xylene, the sections were rehydrated and exposed to the primary antibody (rabbit polyclonal antiserum to reovirus type 3 diluted 1/100 in PBS) for 2 hours at room temperature. After three washes with PBS, the sections were exposed to the secondary antibody (goat antibody to rabbit immunoglobulin G, coupled to fluorescein isothiocyanate conjugate, diluted 1/100 in PBS containing 10% goat serum and 0.005% Evan's Blue counterstain) for 1 hour at room temperature. Finally, the fixed and treated sections were washed three more times with PBS and then once with water. The sections were dried, mounted on slides in 90% glycerol containing 0.1% phenylenediamine, and viewed with a Zeiss Axiophot microscope.

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