Reovirus Prolongs Survival and Reduces the Frequency of Spinal and Leptomeningeal Metastases from Medulloblastoma

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ABSTRACT

Medulloblastoma (MB), the most common pediatric brain tumor, is a highly malignant disease with a 5-year survival rate of only 60%. Tumor cells invade surrounding tissue and disseminate through cerebral spinal fluid, making treatment difficult. Human reovirus type 3 exploits an activated Ras pathway in tumor cells to support productive infection as an oncolytic virus. Here, we examined the ability of human reovirus to kill MB cells lines and surgical specimens in vitro and inhibit tumor growth/ metastases in vivo. Most human MB cell lines tested (five of seven = 71.4%), two MB cell lines derived from spontaneously arising tumors in Patched−/− mice (two of two = 100%), and three MB primary cultures derived from surgical specimens, were susceptible to reovirus infection. Reovirus was internalized and transcribed in both susceptible and resistant cell lines. However, viral protein synthesis was restricted to cell lines with higher levels of activated Ras, suggesting that Ras plays a critical role in reovirus oncolysis in MB. Using an in vivo Daoy orthotopic animal model, we found that a single i.t. injection of reovirus dramatically prolonged survival compared with controls (160 versus 70 days, respectively; P = 0.0003). Repeating this experiment with GFP-labeled Daoy cells and multiple i.t. administrations of reovirus, we again found prolonged survival and a dramatic reduction in spinal and leptomeningeal metastases (66.7% in control injections versus 0.0% in the live virus group). These data suggest that this oncolytic virus may be a potentially effective novel therapy against human MB. Its ability to reduce metastases to the spinal cord could allow a reduction in the dose/field of total neuroaxis cerebral spinal radiotherapy currently used to treat/prevent cerebral spinal fluid dissemination.

INTRODUCTION

MB is a highly malignant brain tumor that occurs predominantly in children and young adults. Despite multimodality therapy, including aggressive surgery, radiation, and chemotherapy, the 5-year survival is only 60% (1–4). The major barriers to treatment are both its invasive nature (rendering it surgically incurable) and its ability to metastasize throughout the cerebral spinal axis (5). The available treatments result in disabling cognitive, growth, and neuroendocrine deficits in the majority of survivors. More effective treatments are desperately needed for this disease. Oncolytic viruses have been evaluated in other brain tumors (6–10) but, with one exception (11), not in MB.

Reovirus (respiratory, enteric, orphan) is a double-stranded RNA virus commonly isolated from the respiratory/gastrointestinal tracts of humans. It is not known to cause disease in humans (hence, the designation orphan) but causes a lethal infection in neonatal (12) and severe combined immunodeficiency mice (13). It does not produce disease in adult or immunocompetent animals, even when administered i.c. (14). It binds to mammalian cells through ubiquitous sialic acid (15, 16) and junction adhesion molecule (17). It preferentially infects and lyses tumor cells but not normal cells (7) by usurping the activated Ras-signaling pathway for replication (18–20). The restriction of reovirus replication in untransformed cells is because of the activation of the double-stranded RNA-activated protein kinase by early viral transcripts, which blocks translation of viral proteins by inhibiting eIF2-α (20). Activated Ras (or an activated element of the Ras pathway) inhibits (or reverses) double-stranded RNA-activated protein kinase activation and allows viral protein synthesis and lytic infection to occur. Since our initial report (7), oncolysis with reovirus has been studied in several cancer models in vivo (10, 21, 22).

Relatively little is known about the molecular genetic events that lead to MB development. Mutations in the p53 gene (23, 24), amplification of c-myc (25) and N-myc (26), up-regulation of different PAX genes (27), down-regulation of retinoblastoma (28), and more recently, mutation of Ptc1 (29–34), have all been implicated in MB pathogenesis. The biological significance of these is not understood but favorable prognosis in MB patients correlates with maximal treatment, high TrkC expression (35–37), and high expression of genes characteristic of cerebellar differentiation or extracellular matrix proteins (38). Little is known about Ras in MB, but signaling through Trk-A or Trk-C activates Ras (29, 30), and Ras/ERK pathways are up-regulated in metastatic MB (41). Hence, Ras might be an important therapeutic target in MB. The purpose of this study was to determine the effectiveness of reovirus as an experimental treatment in MB.

MATERIALS AND METHODS

Cell Lines, MB Surgical Specimens, and Virus. The established MB and glioblastoma cell lines Daoy, D283, D341, U87, and U118 were obtained from the American Type Culture Collection (Manassas, VA). MB lines D425 and D384 were supplied by Dr. Darell Bigner (Duke University); two MB cell lines, SJMM1 and SJMM2, were derived from MB arising in Ptc1−/− mice (30, 31); SJMM1 has low levels of the normal Ptc1 allele and wild-type p53, whereas SJMM2 has high levels of the normal Ptc1 allele and mutated p53 (30, 31).
Fig. 1. Effects of reovirus on established human MB cell lines in vitro. A, cytopathic effect in human MB cell lines exposed to reovirus (MOI = 40 PFUs/cell). Widespread cell killing was found in the U87, Daoy, UW228, ONS76, and D283 but not in the D341 or U118 cell lines forty-eight h after infection (original magnification, ×100). Glioma cells U87 (sensitive) and U118 (resistant) are used as positive and negative controls, respectively. B, viral proteins were detected by immunofluorescence using an antibody to type 3 reovirus visualized using an FITC-conjugated secondary (green) in MB cells treated with LV (MOI = 20 PFUs/cell, 24 h after infection) or DV. All cells were counterstained using Evan’s Blue (red). Susceptible cell lines (U87, Daoy, UW228, ONS76, D283) produced viral proteins, whereas the poorly infectable D341 and U118 cell lines showed very little virus protein (original magnification, ×100). C, only cell lines sensitive to reovirus cell killing synthesized reovirus proteins. Mock- (PBS) and reovirus-infected cells labeled with [35 S]methionine were immunoprecipitated with antireovirus antibodies and analyzed by SDS-PAGE. Reovirus marker (M) showing reovirus proteins (three size groups: λ, μ, and σ) are indicated on the left. Substantial amount of reovirus production was found in susceptible lines (e.g., U87, Daoy, UW228, ONS76 and D283), but very little viral protein was observed in less susceptible lines (e.g., D341 and U118).
Biopsy samples of human MB were obtained during brain tumor surgery from the Alberta Children’s Hospital/Foothills Hospital (Calgary), and short-term cultures established. Signed informal consent was obtained and this protocol was approved by our Institutional Review Board. Briefly, tissue was dissociated with trypsin (0.25%) and harvested (except for suspension cells D283, D341, D425, and D384) by trypsin treatment, and replated in DMEM/F12 containing 10 or 20% FBS. Each cell line was tested routinely for Mycoplasma contamination. All cell lines were characterized for killing by LV, DV, or no virus (i.e., PBS) at increasing virus concentration, and cell viability was measured at 48–72 h by MTT or WST-1 assays (for methods, see below). WST-1 is a derivative of MTT that can be used for both adherent and nonadherent cell lines.

Reovirus infection was performed by incubating cell lysates with 1 mM EDTA and 1 mM GDP ([35S]methionine was used and purified. DNA was prepared by exposing LV to UV light for 45 min. Adenovirus infection was performed by incubating cell lysates with 1 mM EDTA and 1 mM GDP (200 μg/ml), and transfected clones were identified by fluorescent microscopy. GFP expression was >95% as determined by fluorescence-activated cell sorting.

Ras Pull-Down Assay. Ras pull-down assays were performed to detect the active form of Ras. Because only the active form of Ras (GTP-bound-Ras) can bind and stimulate Raf-1 (44), GTP-Ras levels can be determined by using the Ras-binding domain of Raf conjugated to agarose beads (Upstate Biotechnology, Lake Placid, NY). To assess the level of activated Ras in MB cell lines, cell lysates were incubated with Raf-1 antibody (Oncogene Science). For each cell line, negative controls were performed by incubating cell lysates with 1 mM EDTA and 1 mM GDP ([×100) for 15 min at 30°C. Reactions were terminated using 60 mM MgCl2.

Animals. Six to 8-week-old female CD-1 nude mice were purchased from Charles River Canada (Constant, Quebec, Canada). The animals were housed in groups of three to five in a vivarium maintained on a 12-h light/dark schedule with a temperature of 22 ± 1°C and a relative humidity of 50 ± 5%. Food and water were available ad libitum. All procedures were reviewed and approved by the University of Calgary Animal Care Committee.
In Vivo Studies in Nude Mouse Orthotopic i.c. MB Model. The ability of reovirus to cause regression of human MB xenograft transplanted into the cerebellum was tested in female CD-1 nude mice using Daoy MB cells (because they are the most susceptible MB cells and grow reliably in vivo). Actively growing Daoy cells (2.0\times10^6) were injected i.c. into the cerebellum through a 0.5-mm burr hole in the midline 1.5–2-mm posterior to the lambdoid through a scalp incision to a depth of 2–2.5 mm. All stereotactic techniques were described previously (10). Twenty days later when microscopic tumors grew but animals were asymptomatic, a single i.t. injection of 1\times10^7 PFUs of either LV or DV in 2 μl of PBS was administered stereotactically over 20 s. Animals were monitored for 440 days when we arbitrarily terminated the experiment. Animals losing ≥20% of body weight or having trouble ambulating, feeding, or grooming were sacrificed. Animals were anesthetized, perfused intracardially with PBS and then fixed by 4% paraformaldehyde. All of the brains and major organs of these animals were examined histologically.

In Vivo Studies Testing the Efficacy of Multiple i.t. Reovirus Administration. To determine whether we could improve survival by the repeated administration of reovirus i.t. and to monitor tumor dissemination in the...
cerebrospinal fluid, we labeled Daoy cells with GFP before implantation, and mice were imaged with the WBFIS. WBFIS allowed us to visualize and monitor the growth of MB xenografts noninvasively in living animals. Actively growing Daoy-GFP cells (2.0 × 10^6) were injected i.c. into the right putamen of nude mice using a Kopf stereotactic apparatus (Kopf Instruments, Tujunga, CA) with a 5-μl syringe (Hamilton Co., Reno, NV) as we reported previously (10). Twenty days later, LV or DV (1.0 × 10^7 PFUs) was administered i.t. The WBFIS uses a blue light fiber optics (Illumatool Tunable lighting system LT-9500, Encinitas, CA), with a 190-W cool light source, a 470/480-nm optic filter cup, and a 515-nm view filter (45). For the local or high magnification imaging, a Leica MZ-FLIII fluorescence stereomicroscope equipped with 100-W mercury-vapor burner and mounted with a Kodak DC 290 digital camera was used. Images were processed and analyzed by using Photoshop 6.0 and Image-Pro Plus software. With this device, a tumor of 0.5 × 0.5-mm beneath the skin and skull was detectable (45). Animals were monitored individually for tumor growth using the WBFIS twice a week for the first 2 weeks and then every other day. Fluorescent tumors were visualized 3–4 weeks after implantation. Once a fluorescent tumor was visible, the tumor received an additional injection of either LV or DV (i.t.). Reovirus was administered a maximum of seven times. Animals were followed until sacrifice was required or the experiment ended. Major organs as well as brain and spinal cord were removed and examined. Brains and spinal cords were first examined in situ before removal. They then were completely serially sectioned in 8 μm, and the metastatic lesions were examined and confirmed under WBFIS and fluorescent microscopy. Unfortunately, two DV-treated spinal cords were not recovered because of technical difficulties.

Statistical Analyses. Statistical analyses were carried out using Statistical Analysis Software (SAS Institute, Inc., Cary, NC). Survival curves were generated by the Kaplan-Meier method. The log-rank statistic was used to compare the distributions of survival times. All reported P values were two-sided and were considered to be statistically significant at P < 0.05.

RESULTS

Susceptibility of MB Cells to Reovirus Infection in Vitro. We analyzed the susceptibility of seven established human MB cell lines...
(Daoy, UW228, ONS76, D283, D341, D425 and D384; data for D384 and D425 not shown) to reovirus by using cytopathic effect, immunofluorescence and [35S]methionine labeling to measure viral protein synthesis (Fig. 1). Reovirus-susceptible glioma line U87 and resistant line U118 were used as positive and negative controls, respectively (10). We found a spectrum of susceptibility ranging from very susceptible to resistant. Five of seven (71.4%) cell lines [Daoy, UW228, ONS76, D283, and D425 (D425 data not shown)] were susceptible (>90% killed and lysed by reovirus) to LV 48 h after infection (Fig. 1A). Dramatic and widespread cell killing also occurred after a shorter (24 h) exposure to LV in 4 (57.1%) of these MB lines (data not shown). D341 and D384 were resistant to reovirus oncolytic cell killing compared with other MB lines (D384 data not shown). In contrast, cells receiving either DV or no virus (data not shown) remained healthy. Because D341 cells have a longer doubling time than other cell lines, we infected D341 with reovirus and followed cell survival up to 120 h after infection. The results showed that D341 cells were still resistant to killing by reovirus compared with other cell lines (data not shown). Immunofluorescence and [35S]methionine labeling were performed (Fig. 1, B and C) to detect reovirus proteins and confirm that cell killing was the direct result of reovirus infection.

Reovirus Is Transcribed in Both Susceptible and Resistant MB Cells. To determine whether reovirus infection was blocked at the level of transcription (or upstream of this), we infected MB cells with reovirus and performed semiquantitative RT-PCR at various times after reovirus infection. We found that the reovirus s1 transcripts appeared in all cell lines tested, independent of whether they were susceptible or not (Fig. 2). The time points at which mRNA was synthesized varied from 4–12 h after infection (i.e., transcripts were detectable at 4 h in U87 as compared with 12 h in U118). These results showed that reovirus infection was not blocked at the level of viral gene transcription but at the level of viral protein translation. This result is consistent with our previous observation with NIH3T3 and H-Ras-transfected NIH3T3 cells (20).

Susceptibility of MB Cells to Reovirus Infection Is Associated with Levels of Activated Ras. Previous studies have shown that NIH3T3 cells that are resistant to reovirus infection become susceptible when transformed with activated Sos, H-Ras, or v-erbB (19, 20). Little is known about the regulation of Ras in MB, although a recent study found that up-regulation of the Ras/MAPK pathways might predispose MB to dissemination (41). Because Ras activity may be critical for a lytic reovirus infection in MB, we determined the level of Ras activity in several MB cell lines. We found that susceptibility of MB cells to reovirus infection correlated with the levels of activated Ras as detected using the Ras monoclonal antibody (detects H, K, and weakly N-Ras) (Fig. 3A). The quantification (relative Ras-GTP, done by densitometry) of Ras-GTP levels found in Fig. 3A is shown in Fig. 3B. The levels of activated Ras found in ONS-76 were somewhat lower than might be expected on the basis of its susceptibility (i.e., UW228 and D283 were equally susceptible but higher levels of Ras-GTP). We therefore examined N-Ras levels of this cell line by using an N-Ras-specific antibody. ONS76 was shown to express high level of activated N-Ras (Fig. 3C). Fig. 3D shows quantification of Ras-GTP levels found in Fig. 3C. Susceptible lines had high levels of activated H-Ras/K-Ras (e.g., U87, Daoy, UW228, and D283) or N-Ras (ONS76), whereas the less susceptible lines (e.g., D341 and U118) showed relatively low levels of activated Ras (H, K, or N). Western blots for MAPK, Ras, or tubulin were used as protein-loading controls.

Primary MB Cultures Derived from Surgical Specimens and Cell Lines from Spontaneously Arising MBs in Ptc1+/- Mice Are Susceptible to Reovirus Infection and Show High Levels of Activated Ras. Because of concerns that established MB cell lines might not be representative of MBs in vivo, we also tested lines derived from surgical specimens and two MB cell lines (SJMM1, SJMM2) derived from Ptc1+/- mice in terms of Ras activity and susceptibility to reovirus. The clinical samples were identified and confirmed as MB both histologically and by immunohistochemistry (Fig. 4A). We found that primary MB cultures from all three patients were susceptible to killing by reovirus (though less susceptible than Daoy; Fig. 4, B and C). Activated Ras (H/K-Ras-GTP) was also detected in these samples (Fig. 4, D and E). The two mouse cell lines, SJMM1 and SJMM2, were generated from tumors arising in Ptc1+/- mice and continued to express the NORMAL Ptc1 allele and one of them (SJMM1) was p53+/-+. Mutations of Ptc1, a receptor for Shh, are found in basal cell carcinoma and MB (46–49), and 14% of Ptc1+/- mice spontaneously develop MB (30). We also found that both Ptc1+/- cell lines were susceptible to reovirus killing (Fig. 5A) and had moderate levels of activated Ras (Fig. 5B).

Reovirus Prolongs Survival and Produces Long-Term Survival in an Animal Model of MB. We implanted Daoy cells into the cerebellum of nude mice and found a single reovirus administration i.t. dramatically prolonged survival (P = 0.0003; Fig. 6A) and produced some long-term survivors without histological evidence of residual tumor. The median survival of LV-treated animals was 152 days, more than twice as long as DV-treated animals (median survival of 65 days). Furthermore, three of eight (37.5%) mice were long-term survivors and were sacrificed 440 days after tumor implantation (when we decided to terminate the experiment). These three mice remained healthy and gained/maintained body weight. This survival experiment was repeated with similar results (Fig. 6B), although with a smaller proportion of long-term survivors.

Histological analysis showed that all of the DV-treated mice had large tumors in the cerebellum with brain stem invasion. In addition, over half of the animals (four of seven; 57.1%) showed cerebrospinal fluid tumor spread (i.e., in the ventricles remote from tumor inoculation or in the leptomeninges.). Five of eight (62.5%) LV-treated animals had recurrent tumors on histological examination and four of these five (80.0%) had leptomeningeal tumor. However, the three LV-treated long-term survivors (three of eight) had no residual tumor either in the cerebellum or elsewhere. Instead, scar tissue was found in these surviving mice (Fig. 6C). All three mice showed mild dilation of the ventricles with mild subependymal inflammation near the viral inoculation site, around the dorsal third ventricle, lateral ventricles,
and corpus callosum (Fig. 6). There was no evidence of leptomeningeal tumor in these animals, although without labeling Daoy cells, we could not be sure.

**Multiple Injections of Reovirus Prolonged Survival and Reduced Spinal and Leptomeningeal Metastasis in MB in Vivo.** To determine whether multiple i.t. inoculations of reovirus might prolong survival and reduce the incidence of MB dissemination we labeled Daoy cells with GFP to better assess microscopic leptomeningeal tumor and inoculated tumor cells into the right putamen of mice. WBFIS was used to monitor tumor growth in vivo (Fig. 7, A and B). Cells were inoculated into the putamen rather than the cerebellum because repeated administration into the cerebellum was technically difficult. As before, all six of six (100%) DV-treated animals died of recurrent tumor (median survival = 78 days), whereas only three of seven (42.8%) LV-treated animals died (median survival not yet reached) by day 230 ($P = 0.001$; Fig. 8A). Tumor size/growth in the LV-treated group was significantly inhibited by multiple virus injections ($P < 0.0001$; Fig. 8B). Five of seven (71.4%) LV-treated animals required more than one reovirus injection because of recurrence of their tumors, whereas the remaining two animals were tumor free after a single i.t. injection. Tumor growth was significantly delayed in three LV-treated animals that received several reovirus injections, but they eventually died from progressive MB tumor (Fig. 8D). In comparison, the other two mice that received multiple reovirus injections had no evidence of recurrence on WBFIS; these two are still alive and have not yet been examined histologically (Fig. 8C). Furthermore, all DV-treated animals (six of six) had either spinal cord or leptomeningeal metastases, whereas none of the LV-treated animals

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**Fig. 6.** Kaplan-Meier survival analysis from two independent experiments (A and B) for nude mice with an intracerebellar human MB (Daoy) treated with a single i.t. inoculation of reovirus (20 days after tumor implantation, shown as inverted arrow). A, survival was significantly longer in LV- compared with DV-treated mice (log-rank test, $P < 0.0003$). The median survival of the DV group was 65 versus 152 days in the LV group. At 440 days (when we arbitrarily terminated the experiment), three of eight (37.5%) LV-treated mice were alive and appeared healthy. B, we repeated the experiment in A and found similar results ($P < 0.0001$; experiment ongoing). C, histological analysis of LV-treated long-term survivors showed no evidence of residual tumor. Left column: scar-like lesion, without residual tumor, was found in the cerebellum (arrow, ×2.5); this lesion replaced the normal parenchyma and also involved the subarachnoid space (×40); the lesion shows sparse blood vessels and cells (but not tumor cells) lying in a cellular background (×400). Right column: all three LV animals that were cured of their tumor had mild hydrocephalus. Slightly enlarged ventricles were found (×2.5, ×40) with a subependymal cellular reaction that was most prominent in the corpus callosum (×40, box). This was composed of prominent microglia, mononuclear cells, and macrophages but no tumor cells (×400). The aqueduct in the midbrain was patent and not obstructed (data not shown). Pictures were taken from H&E-stained sections.
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Fig. 7. Labeling of Daoy MB cells with GFP facilitates monitoring tumor growth in vivo. A, Daoy-GFP cells grown in vitro (∗×400). Cells were transfected with a plasmid-expressing GFP and a neomycin-resistant gene. B, Daoy-GFP tumor grown i.c. in a nude mouse and visualized under a WBFIS.

(zero of three), even those who died from recurrent tumors, had spinal cord or leptomeningeal metastases (Fig. 8F). All DV-treated mice examined (four of four) had spinal cord metastases but none of the LV-treated mice (zero of three) did (Fisher’s exact test, P ≤ 0.003; the other four LV mice are still alive). Because these brains and spinal cords were only saved as frozen tissue (necessary for GFP imaging), detailed examination of the tissue for signs of hydrocephalus or periventricular inflammation could not be made (although none was apparent). As expected, the fluorescing areas viewed by WBFIS were proven to be MB tumors.

DISCUSSION

This study shows the potential usefulness of a replication-competent virus that kills MB cells with activated Ras pathways or through other elements of Ras-signaling pathways. The efficacy of reovirus was demonstrated in established human MB lines, cell lines derived from Ptc1+/− mice that spontaneously form MB, an orthotopic MB model, and in several tumor specimens from MB patients. We found that long-term survivorship occurred, and the incidence of metastases to the spine and leptomeninges was dramatically reduced when reovirus was administered repeatedly. This is potentially important because clinically MB disseminate throughout the craniospinal axis, necessitating radiotherapy to the whole brain and entire spinal cord. Although this provides superior tumor control and prolongs survival, radiotherapy also causes disabling cognitive, growth and neuroendocrine deficits in these young patients. Reovirus might reduce the incidence of spinal and leptomeningeal metastases in MB patients and allow radiotherapy to be more focused (i.e., administered only to the tumor bed) or reduced in some patients.

Little is understood regarding the regulation of MB metastases, and we do not know the precise mechanism by which reovirus reduces MB dissemination. Three observations suggest that it does so by infecting MB cells that have high levels of activated Ras and are more likely to disseminate. First, we have previously shown that reovirus infects and kills tumor cells that have high levels of activated Ras or elements in its downstream pathways (7, 10). Second, we found that MB cell lines with high levels of activated Ras (H-Ras/K-Ras or N-Ras) were highly susceptible to reovirus infection and cell killing, whereas those with low levels of activated Ras were resistant. Third, a recent study (41) has also found that up-regulation of Ras/MAPK pathways might predispose MB to dissemination. Using gene expression profiling to compare metastatic and nonmetastatic MB, 85 candidate predictor genes associated with MB dissemination were identified. A number of these was involved in downstream Ras/MAPK signaling, suggesting these might be appropriate targets for novel MB treatments directed against MB metastases. Although highly suggestive, conclusive proof that the mechanism through which reovirus reduces MB dissemination is by infecting MB cells with activated Ras awaits the demonstration (e.g., by using an inducible or adenoviral expression system of activated or dominant negative Ras in vivo) that manipulating Ras activation directly affects both reovirus oncolyis and MB dissemination. As well, our data suggests that the role of Ras in reovirus susceptibility may be independent of the Ras isoform activated because Daoy, D283, and U222 had H-Ras/K-Ras activation, but ONS76 had high levels of N-Ras activation. The low incidence of a single-base mutation in codon 61 of the N-Ras gene has been detected in MB (50). The previous observation that NIH3T3 cells transfected with Sos and H-Ras (20) conferred susceptibility to reovirus suggests Ras is important for viral oncolysis. Data presented here support this idea and extends this to include activated N-Ras. Finally, in the absence of detailed time course studies and given the limitations of available imaging technologies to identify the dissemination of isolated tumor cells in vivo, we could not tell if repeated reovirus administration prevented MB dissemination or treated it once it occurred.

Our study has several limitations that are common to many preclinical cancer models. First, although we selected Daoy for in vivo studies because it was very susceptible and had a high level of activated Ras, as with any established cell line, it is unlikely to be completely representative of MB in patients. For example, Daoy has no wild-type p53 (51), and p53 mutations are commonly believed to be rare in MBs. However, mutations or alterations in the p53 pathway occur in 10–14% of MB patients (52–57). To address this limitation, we examined MB surgical specimens and a MB cell line derived from Ptc1+/−/p53+/+ mice and found them to be susceptible to reovirus killing. Experiments to test this approach in MBs in Ptc1+/−/p53+/+ mice in vivo are planned. Similarly, on the basis of models derived from gene expression profiles (41), we may have unwittingly biased our results by studying the most metastatic MB line. On the other hand, using Daoy has allowed us to focus on more aggressive MBs that need better therapies and to assess the effects of reovirus therapy on dissemination of the tumor, a very clinically relevant feature of MB. MB dissemination is likely the result of a complex interaction between tumor cells and the extracellular environment. Therefore, reovirus might only augment the barriers to migration/dissemination and, as a result dissemination, may be much harder to block in patients. A second limitation is that the mice we studied were immunocompromised, and our model largely ignores the contribution of the
Fig. 8. Multiple i.t. reovirus administrations prolonged survival *in vivo*, produced long-term survivors, and reduced the frequency of spinal and leptomeningeal metastases. A, survival was significantly prolonged with multiple i.t. reovirus injections (*P* = 0.001), and four of seven (57.1%) animals were long-term survivors. B, average fluorescent tumor size (as measured by WBFIS), of the LV group compared with the DV group (*P* < 0.0001). C and D, graphs showing tumor size of a tumor that responded (C), and a tumor that did not respond (D) to multiple reovirus administrations (*arrows* indicate the day of virus administration). E, spinal or leptomeningeal metastases (leptomeningeal metastases not shown here) were found in all of the DV but none of the LV-treated group (Fisher's exact test, *P* = 0.003; spinal cord metastasis indicated by *red arrows*).
host immune response. Observations that manipulation of the immune response can improve the efficacy of oncolytic viruses in targeting remote tumor cells (58, 59) suggest this could be an important consideration in patients. Finally, we cannot make conclusions regarding the short- and long-term side effects of administering a live, replication competent virus into the brains of children with brain tumors. We found that mice treated with reovirus gained weight and appeared to thrive, but several LV-treated mice that were cured of their tumors had mild hydrocephalus and subependymal inflammation. Whether this was attributable to a reovirus infection of the ependymal or subependymal cells (60), residua from a treated MB tumor deposit, or even an unknown contaminant of the virus we prepare in our laboratory (this is not prepared under GMP conditions) is unknown. It is possible that some patients may require a cerebrospinal fluid shunt after viral inoculation.

Reovirus appears to be benign when administered to adults (12, 14, 61, 62), even intracerebrally (14), and young immunocompetent animals but is lethal in newborns (62) and severely immunocompromised animals (e.g., severe combined immunodeficiency mice; Refs. 7, 10, 12). There is a clear age dependency to fatal reovirus infection in the early newborn period. For example, all newborn mice inoculated at 8 days of age die from reovirus infection, whereas all mice survive if they are inoculated with reovirus when they are 2 days older (62). The basis underlying the dramatic switch in susceptibility of the newborn brain to reovirus infection is unknown. If this were understood, it might be possible to define a precise period of risk of significant neurotoxicity and exclude very young patients from reovirus treatment. A comprehensive evaluation of the potential toxicity of intracerebral administration of reovirus in younger and older primates, using virus prepared under GMP conditions, would be required before this is considered for a clinical trial in MB patients. A Phase I trial of intralesional administration of reovirus in patients with cutaneous metastases from systemic cancer was recently completed (63), and no significant toxicity was found (serial magnetic resonance imaging and lumbar punctures were performed on all patients), although these patients were all adults, and the virus was not delivered into the brain. A Phase II/III study in reovirus in adults (P. A. F. is the principal investigator) with recurrent malignant gliomas is currently underway. This will provide some information regarding the intracerebral administration of reovirus in patients with brain tumors.

We have investigated the use of reovirus as an oncolytic virus in MB. Our study suggested that reovirus had activity against experimental models of MB. If a comprehensive study of its short- and long-term side effects in the brain of young primates is favorable, it may be considered for a clinical trial in MB patients.

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Note Added in Proof

To further address the issue that Daoy has mutated p53 and p53 mutations are uncommon in MB patients we evaluated this approach in vivo with ONS76, which has wt p53 (64). Similar results were obtained and survival was prolonged in LV-treated mice. The median survival for the DV group was 73 days versus 127 days (Logrank test P = 0.0411) in the LV group treated with a single i.t. administration of reovirus.

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