Systemic Reovirus Therapy of Metastatic Cancer in Immune-competent Mice

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ABSTRACT

The human reovirus is an oncolytic virus that specifically targets cancer cells with an activated Ras pathway. Because it is replication competent and highly specific for cancer cells, this virus has the potential to be an effective antitumor cancer agent through remote site delivery. In this study, we exploited the ability of reovirus to replicate in murine cells to test the efficacy of this virus in eliminating distal and/or metastatic tumors in immune-competent mice. We found that i.v. therapy with reovirus not only inhibited metastatic tumor growth but also led to a significant improvement in animal survival. Combining i.v. reovirus treatment with immune suppression (cyclosporine A or anti-CD4/anti-CD8 antibodies) resulted in further reduction in tumor size and a considerable prolongation in survival, compared with viral therapy alone. Combined therapy was also effective in overcoming a preexisting immunity to reovirus (a common occurrence in humans and thus a potential impediment to oncolytic effectiveness) to induce metastatic tumor regression. This is the first study to use systemic delivery of an oncolytic agent in conjunction with immune-suppressive drugs to effect a prolonged animal survival outcome. Together, our results suggest that i.v. reovirus therapy may present a feasible, novel alternative in the treatment of metastatic cancer in humans.

INTRODUCTION

Reoviruses are common isolates of the respiratory and gastrointestinal tract of humans; however, they are not associated with any known human diseases and are therefore considered to be benign (1). Despite their lack of clinical significance, reoviruses have been extensively studied as the prototype of a large group of viruses known as the double-stranded RNA viruses and as a model for viral pathogenesis in neonatal mice (2). Recent studies on the molecular basis of host cell permissiveness to reovirus have shown that NIH-3T3 cells (or their derivatives), which are normally resistant to reovirus infection, became highly susceptible on transfection with genes encoding the epidermal growth factor receptor, v-erbB, sos, or ras, all of which are activators of Ras signaling (3, 4). Reovirus therefore exploits an activated Ras pathway in transformed cells for infection. We have further found that in untransformed (reovirus-resistant) cells, viral transcripts are generated but are not translated (5). A function of the activated oncogene products in transformed cells is therefore to release this translation block. Typically, in resistant, untransformed cells a $M_r$ 65,000 protein identified as PKR is phosphorylated (activated) by viral transcripts. PKR in turn phosphorylates the α subunit of the translation initiation factor 2 (eukaryotic initiation factor 2α), leading to the inhibition of translation of viral genes (i.e., a suicidal loop). In cells transformed by activated Ras or by an element in the Ras signaling pathway, PKR phosphorylation is either inhibited or reversed, allowing viral protein synthesis to ensue, ultimately leading to a lytic infection.

Although only about 30% of all human tumors have mutations in the ras gene (6), the fact that the Ras pathway can be activated by other elements in the absence of mutations in ras itself suggests that a significantly higher percentage of human cancers could be susceptible to reovirus oncolysis. Indeed, we have found that reovirus infects a variety of established human cancer cell lines derived from many cancer types, including breast, brain, colon, ovarian, pancreatic, and prostate cancer (7–9).

Metastatic cancer is the major cause of death in most cancers. In many patients, microscopic or clinically evident metastasis has already occurred by the time the primary tumor is diagnosed (10). Complete treatment of widely spread metastases in distant organs by surgery, radiation, or chemotherapy is nearly impossible. Effective chemotherapeutic treatment, for example, is hindered by the fact that metastatic tumors are generally less sensitive to chemotherapy when compared with their corresponding primary tumors (11, 12). In this regard, replication-competent oncolytic viruses are valuable because as infectious agents they are able to replicate and amplify in remote tumor sites. Furthermore, transfected ras oncogenes have increased the metastatic ability of cancer cells in several rodent models, and activating mutations in ras are common in human metastatic cancers (13, 14).

The objective of this study is to determine whether reovirus introduced i.v. may be able to target inaccessible tumors and initiate oncolysis to effectively treat metastatic cancer in immune-competent animals. We have also examined the efficacy of i.v. reovirus treatment in combination with immune-suppressive therapy in animal models of metastatic cancer. Our results suggest that i.v. reovirus therapy may be a feasible alternative in the management of metastatic cancer in the future.

MATERIALS AND METHODS

Cells and Virus. Ras-transformed C3H-10T1/2 fibroblasts [C3-ras cells (provided by D. Edwards; Ref. 15),] LLC cells (obtained from ATCC), and C-3-L5 cells (provided by P. Lala; Ref. 16) were cultured in DMEM containing 10% fetal bovine serum. The Dearing strain of reovirus serotype 3 was propagated in L929 cells (ATCC) grown in suspension in Joklik’s modified Eagle’s medium containing 5% fetal bovine serum. Virus was purified according to the protocol of Smith et al. (17), with the exception that β-mercaptoethanol was omitted from the extraction buffer.

Animal Studies. Female 6–8-week-old C3H and C57BL mice were obtained from Charles River Laboratory (Montreal, Quebec, Canada). Animals were maintained under specific pathogen-free conditions and treated according to a protocol approved by the University of Calgary Animal Care Committee. Experimental designs are indicated in Figs. 1–5. For tumor cell injection, actively growing cells were harvested, washed, and resuspended in PBS.

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3 The abbreviations used are: PKR, double-stranded RNA-activated protein kinase; LLC, Lewis lung carcinoma; pfu, plaque-forming unit(s); CyA, cyclosporine A; CyP, cyclophosphamide; ATCC, American Type Culture Collection; mAb, monoclonal antibody.
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Reovirus was diluted in saline (5 × 10^6 pfu/100 μl) and injected i.v. for treatment or i.p. for immunization. CyA (50 mg/kg; Sandimmune) and CyP (3 mg/mouse; Sigma) were administered i.p. For depletion of T-cell subsets in vivo, mice received i.p. injection with 500 μg each of ammonium sulfate-purified anti-CD4 mAb (clone GK1.5; ATCC) and anti-CD8 mAb (clone 2.43; ATCC) as indicated in the figure legends. The depletion of CD4 and CD8 T cells was >95%.

Virus Titration and Neutralizing Antibody Assay. For virus titration, mice were sacrificed at various time points after infection. Tumors were weighed, frozen, and stored at −70°C until use. Virus titers present in tumor homogenates were determined by plaque assay using L929 cells. For the neutralizing antibody assay, serum was collected from the tail vein. Antireovirus antibodies were measured as described previously (18, 19).

Histology. To count visible metastases, mouse lungs were removed and fixed in Bouin’s solution. For histological analysis, tumors and other organs were fixed in Bouin’s solution or 10% neutral buffered formalin, embedded in paraffin, and sectioned. Sections were subjected to either H&E staining or immunohistochemical staining as described previously (8).

Statistical Analysis. Two-tailed P values were determined using a nonparametric Wilcoxon test and Student’s t test to determine the significance of the difference between the experimental groups.

RESULTS

Tumor Growth Inhibition in Immune-Competent Mice Treated with i.v. Injections of Reovirus. It was first necessary to determine the maximum tolerated i.v. dose of reovirus in immune-competent (C3H) mice. To this end, several different therapeutic regimens were evaluated, including the combined administration of reovirus with the immune-suppressive drug CyA (Table 1). The highest i.v. dose of reovirus that resulted in 100% animal survival was determined to be 4 injections of 5 × 10^8 pfu at 6-day intervals (total dose, 2 × 10^9 pfu), regardless of cotreatment with CyA. Histological examination of major organs (brain, heart, intestine, kidney, liver, lung, pancreas, and spleen) performed on those animals killed by reovirus overdose revealed pathological changes in both liver and heart tissues (data not shown). This is not unexpected because previous studies have found pathogenesis in the liver and heart of neonatal mice infected with reovirus (20, 21). The maximum tolerated dose of reovirus determined above was used for all our subsequent studies.

To determine whether i.v. reovirus treatment could induce tumor regression in immune-competent mice, we administered tail vein injections of 5 × 10^8 pfu of reovirus to C3H mice bearing established s.c. ras-transformed C3H-10T1/2 fibroblasts (C3-ras cells) implanted in immune-competent C3H mice, and mice were given the maximum tolerated i.v. dose of reovirus. A, tumor regression after i.v. reovirus treatment (5 × 10^8 pfu; injections at days 0, 6, 12, and 18; ○) or saline treatment (●). Points represent the mean tumor size ± SE (n = 6/group). B, kinetics of virus replication in tumors (●) and viral titers in blood (○) after reovirus treatment. ND, not detected (<10 pfu/g).

gradually over time (Fig. 1B). Immunohistochemical staining with an antireovirus antibody revealed active replication of the virus within the tumors (data not shown). These data suggest that systemic delivery of reovirus results in specific targeting of tumors at a remote site, leading to significant inhibition of tumor growth in immune-competent mice.

Application of i.v. Reovirus Therapy to Experimental Models of Metastasis. A single tail vein injection of C3-L5 cells, derived from primary transplantable murine mammary tumor T58, leads to the rapid formation of lung metastases in syngeneic C3H mice (16). To determine the efficacy of i.v. reovirus treatment in this metastatic cancer model, C3H mice first received i.v. injection with 5 × 10^8 C3-L5 cells (day 0; Fig. 2A). The i.v. reovirus therapeutic regimen established above was initiated either at day 5 (early stage; microscopic metastases; Fig. 2A, a) or at day 14 (late stage; visible lung surface metastases, 0.5–1.0 mm in diameter; Fig. 2A, b). In both cases, i.v. reovirus therapy resulted in significantly improved survival of the reovirus-treated mice compared with mock-treated mice (60% versus 10% for those treated early, and 40% versus 10% for those treated late, P < 0.01 by Wilcoxon test).

To further demonstrate the efficacy of i.v. reovirus therapy in metastatic cancer, we used a tumor removal model using LLC cells (Fig. 2B). In this model, removal of the primary tumor leads to rapid growth of lung metastases, which are normally suppressed by circulating angiogenesis inhibitors secreted by the primary tumor (22). For this study, s.c. LLC tumors were allowed to grow for 2 weeks after cell implantation to a size of ~1500 mm^3. The tumors were then removed surgically (day 0), and i.v. reovirus was administered on day 1 and day 7 after tumor removal. Fourteen days after tumor removal, all mice were sacrificed, and lung surface metastases, lung weight, and histology were examined. The formation of surface lung metastases after tumor removal was significantly inhibited by i.v. reovirus treatment compared with mock treatment (Fig. 2B, a). Moreover, the average lung weight of reovirus-treated mice remained comparable.
We first evaluated the effect of the immune-suppressive CyA on the efficacy of i.v. reovirus therapy of murine C3-ras tumors implanted in the hind flank of immune-competent C3H mice (Fig. 3B). Although i.v. reovirus treatment alone was effective in suppressing tumor growth at earlier times, eventual tumor regrowth was evident. Combining i.v. reovirus therapy with CyA treatment significantly inhibited this tumor regrowth, suggesting that reovirus therapy is more effective when the host’s antireovirus response is suppressed.

In addition to the localized tumor model above, we also examined the efficacy of combined therapy in a disseminated LLC metastatic lung cancer model (Fig. 3C), where C57BL mice received i.v. injection with $3 \times 10^7$ LLC cells to establish tumors. The mice were randomly divided into six groups, with each group receiving one of the following treatments: (a) saline/saline; (b) saline/CyA; (c) saline/T-cell depletion; (d) reovirus/saline; (e) reovirus/CyA; and (f) reovirus/T-cell depletion. The mice in the three groups treated i.v. with reovirus survived significantly longer than each mock-treated counterpart: reovirus/saline versus saline/saline, $P < 0.05$; reovirus/CyA versus saline/CyA, $P < 0.01$; and reovirus/T-cell depletion versus saline/T-cell depletion, $P < 0.01$, by Wilcoxon test. Among the three

with that of normal control animals ($P < 0.01$ by Student’s $t$ test; Fig. 2R, b). Histological examination further revealed that mice treated with reovirus had only microscopic metastases in the lung, whereas mock-treated mice showed marked growth of lung metastases (Fig. 2B, c).

Enhancement of the Efficacy of i.v. Reovirus Therapy by Immune Suppression. Because of an induction of immunity against reovirus in treated animals, it is possible that viral infection and oncolysis within the tumor are inefficient at late stages of treatment, allowing for tumor regrowth. Examination of neutralizing antibody titers against reovirus (a major indicator of an antireovirus immune response; Ref. 23) in treated animals revealed that antireovirus antibodies in the mouse serum were significantly elevated after reovirus treatment (Fig. 3A). To determine whether suppression of the immune response can improve the efficacy of reovirus therapy in our models, we examined the effects of combined reovirus therapy with immune-suppressive drugs or T-cell depletion using mAbs against CD4 or CD8.

Fig. 2. Effects of i.v. reovirus therapy in metastatic cancer models. A, prolonged survival in C3-L5-metastatic cancer model after i.v. reovirus treatment. C3H mice received an i.v. injection of $4 \times 10^5$ C3-L5 cells/mouse. Reovirus ($5 \times 10^8$ pfu, 4 times; red) or saline (blue) treatment was initiated at 5 days (a) or 14 days (b) after the C3-L5 injection ($n = 7–9$ mice/group). B, reovirus-mediated inhibition of lung metastasis growth after primary tumor removal. LLC cells were implanted in C57BL mice, and the primary tumors were surgically removed 14 days later (day 0). Mice were then treated with reovirus ($5 \times 10^7$ pfu, days 1 and 7) or saline i.v. a, number of surface lung metastases; b, lung weight; c, H&E staining of lung sections. Error bars indicate the SE ($n = 9–10$ mice/group).

Fig. 3. Effects of combined i.v. reovirus therapy with immune suppression. A, increased levels of neutralizing antibodies against reovirus in the serum of mice treated with reovirus. B, effects of immune suppression on i.v. reovirus therapy in localized s.c. tumor model. C3H mice bearing established C3-ras cell tumors were treated i.v. with reovirus ($5 \times 10^7$ pfu; $\triangle$ and $\blacktriangle$) or saline (○ and □) at day 0, 6, 12, and 18 followed by i.p. injection of CyA (50 mg/kg; $\triangle$ and $\blacktriangle$) or saline (○ and □) every other day from day 5. Error bars indicate the SE ($n = 7–8$ mice/group). * $P < 0.05$ (day 18–24) by Student’s $t$ test. C, effects of immune suppression on survival in the LLC metastatic model. C57BL mice received i.v. injection with LLC ($3 \times 10^5$ cells) at day 0, followed by i.v. injection with reovirus ($5 \times 10^7$ pfu) or saline at days 7, 13, 19, and 25, in conjunction with or without immune suppression. CyA (50 mg/kg) was injected i.p. every other day from day 12, and purified anti-CD4 or -CD8 mAbs (0.5 mg/mouse) were injected i.p. at days 11, 12, 15, 18, and 23 ($n = 8–9$ mice/group).

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In the serum were observed in all reovirus-injected mice (Fig. 4A). In the C3-ras tumor model. C3H mice were immunized i.p. with live reovirus (5 × 10⁸ pfu). After 2 weeks, elevated levels of antireovirus antibody titers in C3-ras mice were observed in all reovirus-injected mice (Fig. 4A, left). C3-ras cells were then implanted over the hind flank of both these immunized mice and naive controls. After palpable tumors were established, the mice received i.v. reovirus treatment in combination with CyA or another immune-suppressive drug, CyP. The production of antireovirus antibodies in immunized mice treated with CyA or CyP was found to be significantly inhibited, and antibody titers were comparable with that of mice without prior exposure to the virus (Fig. 4A, right). We reported previously that preexisting immunity to reovirus did not abrogate the oncolytic effects of reovirus injected directly into C3-ras tumors (5). In the present study, in which reovirus was introduced systemically, however, we observed that reovirus was ineffective in inhibiting tumor growth in immunized mice (Fig. 4B). However, combination therapy with immune-suppressive drugs (CyA or CyP) fully restored the ability of reovirus to effectively inhibit remote tumor growth.

The efficacy of combined reovirus therapy was further examined in the LLC metastatic lung tumor model using preimmunized mice (Fig. 5). C57BL mice received i.p. injection with reovirus (5 × 10⁸ pfu), and 2 weeks later, these immunized mice as well as naive mice were given i.v. LLC (3 × 10⁵ cells; day 0). Seven days after cell injection, i.v. reovirus treatment with or without immune suppression was initiated, and mice were sacrificed on day 21. The results (Fig. 5) show that although reovirus treatment alone was very effective in reducing the number of lung metastases and in maintaining normal lung weight in the naive mice, its effectiveness was drastically compromised in the preimmunized mice. In contrast, combined therapy of reovirus with CyA or mAb T-cell depletion significantly enhanced its effectiveness in controlling lung metastasis in this latter group of mice.

Collectively, these observations suggest that combining reovirus treatment with immune suppression can effectively counteract the reovirus-treated groups, the survival of mice with immune suppression by CyA or mAb T-cell depletion was significantly improved compared with that of mice treated with reovirus alone: reovirus/saline versus reovirus/CyA, P < 0.01; and reovirus/saline versus reovirus/T-cell depletion, P < 0.01, by Wilcoxon test. Treatment with immune-suppressive drugs alone did not improve survival.

From these data, it is evident that complementing i.v. reovirus treatment with immune suppression not only enhances tumor regression in localized cancer models but also prolongs survival in metastatic cancer models.

Reovirus Oncolytic Effects in Mice with Previous Exposure to the Virus. Although reovirus infections have not been known to elicit significant clinical symptoms in humans, a high prevalence of anti-reovirus antibodies has been observed in healthy volunteers (70–100%; Refs. 18 and 19). This preexisting immunity to reovirus could potentially compromise the efficacy of reovirus treatment in cancer patients. Therefore, to further evaluate the clinical feasibility of using reovirus as an antitumor agent in humans, we examined the efficacy of i.v. reovirus therapy in mice already immunized against the virus (Fig. 4). First, we challenged C3H mice i.p. with reovirus (5 × 10⁸ pfu). After 2 weeks, elevated levels of antireovirus antibodies in the serum were observed in all reovirus-injected mice (Fig. 4A, left).
therapeutic-inactivating capabilities of an immune system previously primed by viral exposure.

**DISCUSSION**

The idea of using viruses as anticancer agents has been around for decades; however, serious attempts to explore this possibility have only been initiated relatively recently. Renewed interest in this approach has in large part been because of recent major advances in both cancer biology and virology that have brought new insights into the mechanistic aspects of oncolytic virus function (24). For example, the human reovirus and herpes simplex virus-1 have both been shown to target cells with an activated Ras signaling pathway (4, 35). G207, an attenuated version of herpes simplex virus-1 with deletions of the \( \gamma 34.5 \) and \( UI39 \) genes, has been tested on animal models of many cancer types (25, 26). The oncolytic mutant adenovirus ONYX-015 infects cells lacking a functional p53 tumor suppressor pathway (27). Finally, oncolytic vesicular stomatitis virus can target tumors with defects in the interferon pathway (28) and tumors with aberrant p53, Ras, or myc function (29).

The oncolytic potential of a virus is commonly first tested in vitro using cancer cell lines grown in culture, followed by animal studies in vivo in immune-deficient mice bearing human tumor xenografts. Although this approach often yields important information on the safety and efficacy of the virus in the treatment of cancer, it fails to address key issues pertaining to the involvement of the host immune response, which would clearly add a new dimension of complexity to the system. Consideration of the host immune system is particularly important in cases where the virus is introduced by systemic delivery for the targeting of cancers that have metastasized. Experimentally, the influence of the host immune system on oncolytic virus therapy can best be assessed through the use of a syngeneic mouse model (mouse tumors grown in immune-competent mice). Reovirus has a major advantage in this regard because of its ability to grow in rodents, thereby allowing for the assessment of its safety and efficacy in the presence of a competent immune system.

We have reported previously (5) that direct injection of reovirus into tumors was effective in both naive and immunized immune-competent mice. However, the question remained whether reovirus delivered systemically could encounter complications in terms of toxic side effects and/or neutralization by the host immune system. In the present study, we have determined the maximum tolerated i.v. dose of reovirus (not to be confused with the minimum effective dose) and have in fact found this to be effective in targeting remote tumors and eliciting significant tumor regression, without detrimental side effects. Therefore, at least in the mouse model, i.v. reovirus treatment has a certain window of safety and efficacy, even in the face of a competent immune system. This understanding may have broad applicability toward a more effective use of oncolytic viruses in general.

Furthermore, in this study, we have demonstrated that immune suppression enhanced the efficacy of reovirus treatment in immune-competent animal models. Combined reovirus/immune-suppressive drug therapy successfully inhibited the regrowth of localized C3H tumors at late stages of treatment and resulted in longer survival times in metastatic LLC cancer models. In mice previously immunized by the virus, we have shown previously that intratumoral injection of reovirus can induce tumor regression, yet in this study, systemic reovirus therapy of immunized mice failed to effect tumor regression. Reovirus therapy, however, was successful in immunized mice when administered in conjunction with immune-suppressive drugs. Moreover, therapeutic safety was demonstrated in immune-suppressed animals because we did not observe acceleration of tumor growth or infection by other pathogens, which are potential risks for cancer patients.

Although established immunity against the virus may neutralize progeny virions and severely compromise oncolytic efficacy, there is also evidence that viral infection in the tumors may also activate the immune system to generate a specific antitumor response (30, 31). Immune activation induced by viral infection, however, does not appear to play a significant role in eliciting the regression of tumors in our model system because we found that general suppression of the immune response significantly enhanced the efficacy of reovirus treatment. This suggests that the host immune system generally neutralizes the virus and hinders its oncolytic activity rather than contributing to tumor regression. The benefit of combining viral therapy with immune suppression might depend on the type of oncolytic virus, as well as the immunogenicity of the cancer cells used. C3-ras and LLC were used for the experiments of the combined therapy presented in this article because they are poorly immunogenic cell lines (Ref. 32; data not shown). Animal models based on nonimmunogenic cancer cell lines more accurately reflect the human clinical situation, where nonimmunogenic tumors represent the majority of metastatic cancers. In fact, the evasion of tumor cells from immune killing through down-regulation of immunogenic surface molecules or via the secretion of immune suppressive effectors is a common feature of metastatic cancers (10). The results of the present study, however, do not rule out the possible evocation of an anticancer immune response because of viral infection in the tumors. It remains to be determined what immune components are involved in antiviral immunity versus anticancer immunity to establish a more targeted method of immune modulation to enhance reovirus efficacy, without suppressing anticancer responses.

At present, Phase I clinical trials using reovirus are currently underway at the Tom Baker Cancer Centre (Calgary, Alberta, Canada). Although direct intratumoral injection is the preferred method of delivery for localized tumors, remote site administration of reovirus may be advantageous in the clinical treatment of metastatic cancer. In the latter case, reovirus might be used to perfuse locally inaccessible and/or diffuse tumors or even to reduce the risk of tumor recurrence after surgical resection of a tumor. In fact, i.v. administration of replication-selective adenosviruses has been shown to be effective in Phase I clinical trials (33, 34). On the basis of the results from the present study, it is likely that combining reovirus therapy with immune-suppressive drugs could improve the efficacy of reovirus treatment of metastatic cancers.

**REFERENCES**

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