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Oncolytic Reovirus against Ovarian and Colon Cancer[1]

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ABSTRACT

Reovirus selectively replicates in and destroys cancer cells with an activated Ras signaling pathway. In this study, we evaluated the feasibility of using reovirus (serotype 3, strain Dearing) as an antihuman colon and ovarian cancer agent. In *in vitro* studies, reovirus infection in human colon and ovarian cell lines was assessed by cytopathic effect as detected by light microscopy, [^{35}S]Methionine labeling of infected cells for viral protein synthesis and progeny virus production by plaque assay. We observed that reovirus efficiently infected all five human colon cancer cell lines (Caco-2, DLD-1, HCT-116, HT-29, and SW48) and four human ovarian cancer cell lines (MDAH2774, PA-1, SKOV3, and SW626) which were tested, but not a normal colon cell line (CCD-18Co) or a normal ovarian cell line (NOV-31). We also observed that the Ras activity in the human colon and ovarian cancer cell lines was elevated compared with that in normal colon and ovarian cell lines. In animal models, intraneoplastic as well as i.v. inoculation of reovirus resulted in significant regression of established s.c. human colon and ovarian tumors implanted at the hind flank. Histological studies revealed that reovirus infection *in vivo* was restricted to tumor cells, whereas the surrounding normal tissue remained uninfected. Additionally, in an i.p. human ovarian cancer xenograft model, inhibition of ascites tumor formation and the survival of animals treated with live reovirus was significantly greater than of control mice treated with UV-inactivated reovirus. Reovirus infection in *ex vivo* primary human ovarian tumor surgical samples was also confirmed, further demonstrating the potential of reovirus therapy. These results suggest that reovirus holds promise as a novel agent for human colon and ovarian cancer therapy.

INTRODUCTION

Reoviruses are common isolates of the respiratory and gastrointestinal tract of humans; however, they are not associated with any known human diseases and thus are considered to be benign (1). Despite their lack of clinical pathogenesis, reoviruses have been studied extensively because they represent the prototype of a large group of viruses known as the double-stranded RNA viruses, some of which are important human pathogens (*e.g.*, rotaviruses). Recent studies on the molecular basis of host cell permissiveness to reovirus have revealed that transformation of NIH-3T3 cells with EGFR, v-*erbB*, or *ras* (all activators of the Ras signaling pathway) result in drastic enhancement of reovirus infection of these cells (2, 3). Although only ~30% of all human tumors have mutations in the *ras* gene, 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 (perhaps as high as 80%) could be susceptible to reovirus oncolysis. Subsequent animal studies demonstrated tumor regression by intratumoral injection of reovirus into immune-deficient (SCID) mice bearing human glioblastoma U87 xenografts and immune-competent mice bearing *ras*-transformed tumor allografts (4).

The fact that *ras* mutations are also common in colorectal and ovarian cancers has led us to probe the possibility of these cancers being susceptible to reovirus treatment. Colorectal cancer is the third most common cancer diagnosed in Americans (5). Approximately 130,000 cases of colon and rectal cancer were diagnosed in the year 2000. Ovarian cancer is a major gynecological malignancy that ranks fifth as the cause of cancer deaths in women. In the United States, ~23,000 new cases were predicted in the year 2000, and ~14,000 women will die of the disease (5). The K-*ras* proto-oncogene is mutated in ~50% of human colorectal cancers (6, 7). In other studies of ovarian carcinomas and borderline malignant tumors, the incidence of mutations in *ras* shows a wide variation, between 4 and 48% (8–10). A recent study has shown that 47% of samples from ovarian adenocarcinoma peritoneal fluids carry a point mutation at codon 12 of the K-*ras* gene (11).

In the present study, we tested a number of human colon and ovarian cancer cell lines for their susceptibility to reovirus infection *in vitro* and examined the correlation between this susceptibility and activation of the Ras pathway. We also implanted tumors in immune-compromised animals and assessed the ability of reovirus to cause tumor regression and promote survival of these animals. Finally, we tested *ex vivo* primary human ovarian cancer surgical specimens for their susceptibility to reovirus infection *in vitro*. Our studies indicate that reovirus is an effective therapeutic against both colon and ovarian cancer.

MATERIALS AND METHODS

Cells and Virus. Human colon cancer cell lines (Caco-2, DLD-1, HCT-116, HT-29, and SW-48), a normal human colon fibroblast cell line (CCD-18Co), human ovarian cancer cell lines (MDAH 2774, PA-1, SKOV3, and SW626), and a normal ovarian fibroblast cell line (NOV-31) were obtained from the ATCC (Rockville, MD). The cells were maintained according to ATCC protocols. The Dearing strain of reovirus serotype 3 was propagated in L929 cells (from ATCC) grown in suspension in Joklik's modified Eagle's medium (Life Technologies, Inc.) containing 5% FBS. Virus was purified according to the protocol of Smith *et al.* (12) with the exception that β-mercaptoethanol was omitted from the extraction buffer.

Reovirus Infection *in Vitro*. The cell lines described above were prepared in six-well plates and infected with reovirus at a MOI of 20 pfu/cell. For metabolic labeling with [^{35}S]methionine, 50 μCi/ml of [^{35}S]methionine (Amersham) were added to the culture medium for 12 h. After the incubation, the medium was removed, and cells were washed with PBS and lysed in lysis buffer [50 mM Tris (pH 7.6), 1% NP40, 150 mM NaCl, 50 mM NaF 1 mM Na_3VO_4, 5 mM EDTA, 1 mM phenylmethlsulfonyl fluoride, and 10 μg of aprotinin/ml]. Lysates were cleared of debris by centrifugation, and supernatants were stored at −70°C until use. Polyclonal rabbit antireovirus serotype 3 serum was used for immunoprecipitation of [^{35}S]methionine-labeled reovirus proteins from cell lysates, as described previously (13). [^{35}S]Methionine-labeled protein and immunoprecipitated reovirus proteins were subjected to 10% SDS-PAGE, followed by autoradiography.

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; SCID, severe combined immunodeficient; ATCC, American Type Culture Collection; MOI, multiplicity of infection; pfu, plaque-forming unit(s); MEK, mitogen-activated protein kinase kinase.

Virus Titration. Cells grown in six-well plates were infected with reovirus at an MOI of 20. After 48 h of incubation, cells and supernatants were frozen and thawed three times. Viral titer was determined by plaque assays using L929 cells.

Ras Activation Assay and Western Blot Analysis. Seventy to 80% confluent cells grown in 10-cm dishes were lysed with 1 × Mg^{2+} lysis buffer (Ras activation assay kit; Upstate Biotechnology). To determine the level of activated Ras (Ras-GTP) in these cells, 1 mg of cell lysate was incubated with 10 μl of Raf-1 Ras binding domain agarose conjugate at 4°C for 30 min. The beads were then collected, washed, resuspended in 2× Laemmli buffer, and boiled for 5 min. This was then followed by SDS-PAGE and Western blotting with an anti-Ras antibody (clone RAS 10) according to the manufacturer's instructions. To determine the level of total Ras, cell lysates were directly subjected to SDS-PAGE and Western blotting with anti-Ras antibody. For Western blot analysis of MEK1/2, the membrane was first blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 20 min and then incubated with an anti-MEK1/2 phospho-specific antibody or anti-MEK1/2 antibody (New England Biolabs) overnight at 4°C. After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse antibody, and specific bands were detected with an ECL system (Amersham).

Animal Studies. Six to 8-week-old female SCID/NOD mice and CD-1 nude mice were obtained from the Cross Cancer Institute (Edmonton, Alberta, Canada) and from Charles River Laboratory (Montreal, Quebec, Canada), respectively. Animals were maintained under specific pathogen-free conditions and treated according to a protocol approved by the University of Calgary Animal Care Committee. For intraneoplastic treatment, when palpable tumors (20–25 mm^2) were established, live reovirus or UV-inactivated virus was administered intratumorally at a dose of 1.0 × 10^7 pfu at day 0 (SCID mice) or 5 × 10^8 pfu at days 0 and 14 (nude mice) in 50 μl of PBS. Two-dimensional tumor measurements were performed with calipers every other day until control animals showed severe morbidity because of excess tumor burden. For systemic treatment with reovirus, nude mice bearing established human colon HCT-116 tumors at the hind flank were injected i.v. into the tail vein with 5 × 10^8 pfu of either live or UV-inactivated reovirus in 50 μl of PBS. Tumor size was measured every other day for 28 days. For the ascites model of human ovarian cancer, nude mice were injected i.p. with 2 × 10^6 MDAH 2774 cells in 200 μl of PBS. Two groups of 10 animals were treated with live- or with UV-inactivated-reovirus at 5 and 19 days after cell injection. The animals' body weight was measured every week, and mice were euthanized if their tumors exceeded 20% of body weight or if the animal appeared to be experiencing distress. For this model, the weights of live and UV-irradiated, virus-treated animals were compared with untreated healthy mice bearing no tumors.

Histology. For histological analysis, tumors (or remaining masses) were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. Sections were then immersed in xylene, followed by rehydration in decreasing

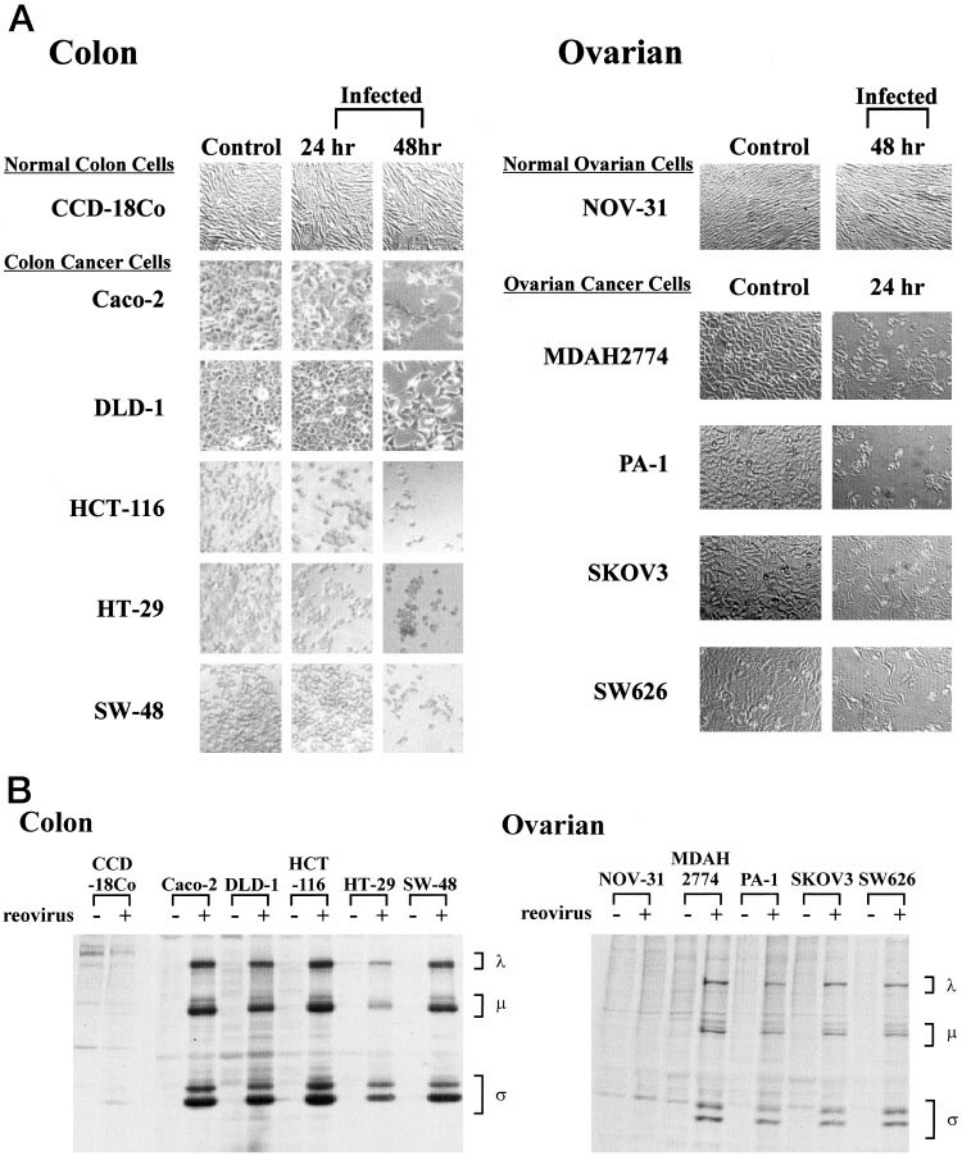

Fig. 1. Cytopathic effects (*A*) and reovirus protein synthesis (*B*) in human colon and ovarian cancer cell lines and normal cell lines infected with reovirus. Cells were exposed to reovirus at a MOI of 20 pfu per cell. *A*, cells were photographed at the indicated time points after infection. *B*, reovirus protein synthesis in uninfected (−) and reovirus-infected (+) colon and ovarian cell lines. Cells were labeled with [^{35}S]methionine for 12 h at 24 h after infection (colon cell lines) and at 12 h after infection (ovarian cell lines). Cell lysates were prepared, and reovirus proteins contained therein were immunoprecipitated with a polyclonal antireovirus antibody and analyzed by SDS-PAGE. *Right*, the three size classes of reovirus proteins, λ, μ, and σ.

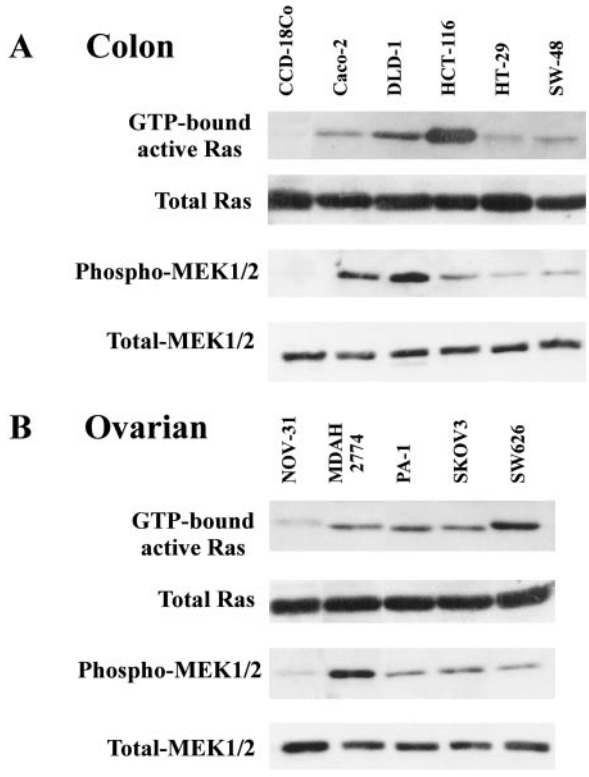

Fig. 2. Ras activities and phospho-MEK1/2 levels in colon cell lines (*A*) and ovarian cell lines (*B*). The GTP-bound form of Ras was pulled down with a Raf-1 Ras binding domain-agarose conjugate. Ras-GTP proteins bound to the conjugate were subjected to SDS-PAGE, followed by Western blotting with an anti-Ras antibody. Activation of MEK1/2 was assessed by Western blotting of whole cell lysates with a phospho-MEK1/2-specific antibody. Immunoblot analysis of total cell lysates with anti-Ras antibody or anti-total MEK1/2 antibody identified the level of total protein.

concentrations of ethanol. Endogenous peroxidase was inactivated in 3% hydrogen peroxide in methanol for 15 min. Sections were then incubated in primary rabbit antireovirus polyclonal antibody (1:1000 in PBS with 10% goat serum and 0.1% Triton X-100) partially purified by ammonium sulfate precipitation. Slides were washed in PBS and then subjected to avidin-biotin-horseradish peroxidase staining as recommended by the manufacturer (Vector, Burlington, Ontario, Canada) and counterstained in hematoxylin.

Reovirus Infection of *ex Vivo* Biopsy Samples. Surgical tumor biopsy samples were removed from patients and kept in DMEM containing 20% FCS and 2× antibiotics on ice. Within 4 h after resection, the specimens were washed in sterile PBS and mechanically disrupted with needles and filtered through a 100-μm pore size nylon cell strainer to make single cell suspensions. Cells were resuspended in medium, infected with reovirus at a MOI of 20, and radiolabeled with [^{35}S]methionine. At various times after infection, cells were harvested and lysed. The lysates were analyzed for viral proteins as described above.

RESULTS

***In Vitro* Reovirus Infection.** To determine the susceptibility of human colon and ovarian cancer cell lines to reovirus, tumor-derived colon cell lines (Caco-2, DLD-1, HCT-116, HT-29, and SW-48) and ovarian cell lines (MDAH 2774, PA-1, and SW626), as well as a normal colon cell line (CCD-18Co) and a normal ovarian cell line (NOV-31), were infected with reovirus at a MOI of 20 pfu/cell. As shown in Fig. 1*A*, little morphological changes were observed in noncancer cell lines, CCD-18Co and NOV-31, at 48 h after infection. In contrast, all of the tumor cell lines infected with reovirus exhibited cytopathic effects (rounding and clumping of cells). At 72 h after infection, >95% of all cancer cell lines examined were destroyed (data not shown). To confirm that cell killing was induced by reovirus replication, the cells were pulse-labeled with [^{35}S]methionine at 24 h after infection (for colon cancer cells) or at 12 h after infection (for ovarian cancer cells). Cell lysates were then prepared, and reovirus proteins contained therein were immunoprecipitated with a polyclonal antireovirus antibody and analyzed by SDS-PAGE. The results (Fig. 1*B*) show significant viral protein synthesis in all tumor-derived cell lines but little viral protein synthesis in the normal cell lines, CCD-18Co and NOV-31. As expected, the cancer cell lines produced significantly more progeny virus than the normal cells (data not shown).

Ras and MEK1/2 Activities in Colon and Ovarian Cancer Cell Lines. The GTP-bound active form of Ras is able to stimulate the downstream effector, Raf-1, through its binding to the Raf-1-Ras binding domain. To examine the activation of Ras in cancer cell lines, the amount of Ras-GTP was measured using Raf 1-Ras binding domain conjugated to agarose beads (Upstate Biotechnology) to pull down active Ras. Lysates were prepared from cells at 80% confluence under normal growth conditions. As shown in Fig. 2, elevated levels of Ras activity were observed in all colon and ovarian cancer cell lines compared with their normal counterparts. We also examined the phosphorylation status of MEK1/2, a downstream effector that is phosphorylated and activated by Raf, by Western blotting with a phospho-MEK1/2-specific antibody. The level of phospho-MEK1/2 was found to be higher overall in the cancer cell lines than in the normal cell lines.

Effect of Reovirus on s.c. Tumors. To assess whether the *in vitro* susceptibility of colon and ovarian cancer cell lines is predictive of the effectiveness of reovirus as an anticancer agent *in vivo*, we evaluated

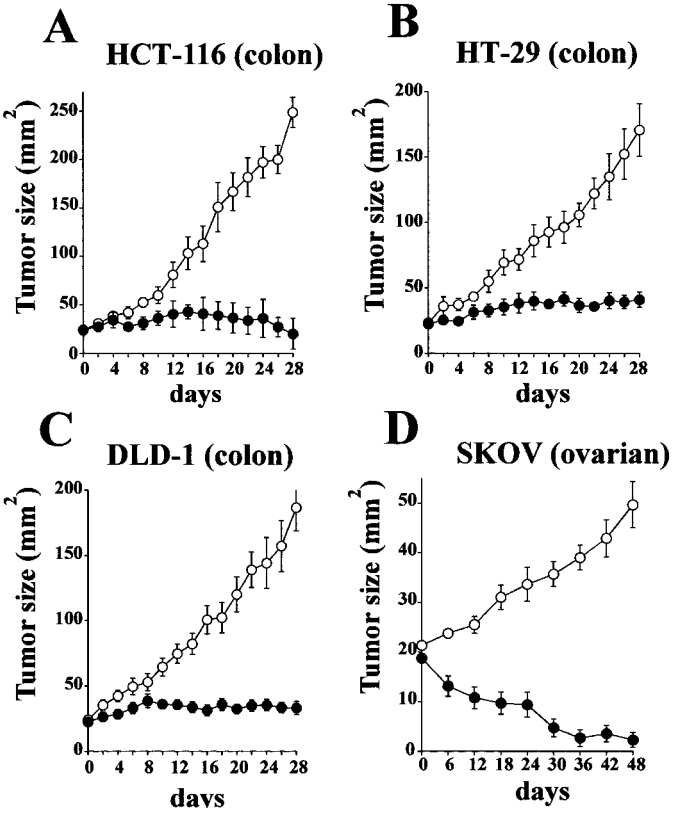

Fig. 3. Effects of intraneoplastic reovirus treatment on s.c. human colon cancer cell line xenografts, HCT-116 (*A*), HT-29 (*B*), and DLD-1 (*C*) in SCID mice and a human ovarian cancer cell line xenograft, SKOV3 (*D*), in nude mice. s.c. tumors received live reovirus (●) or an equivalent dose of UV-inactivated reovirus (○; see "Materials and Methods" for details). *Bars*, SE (n = 6–7).

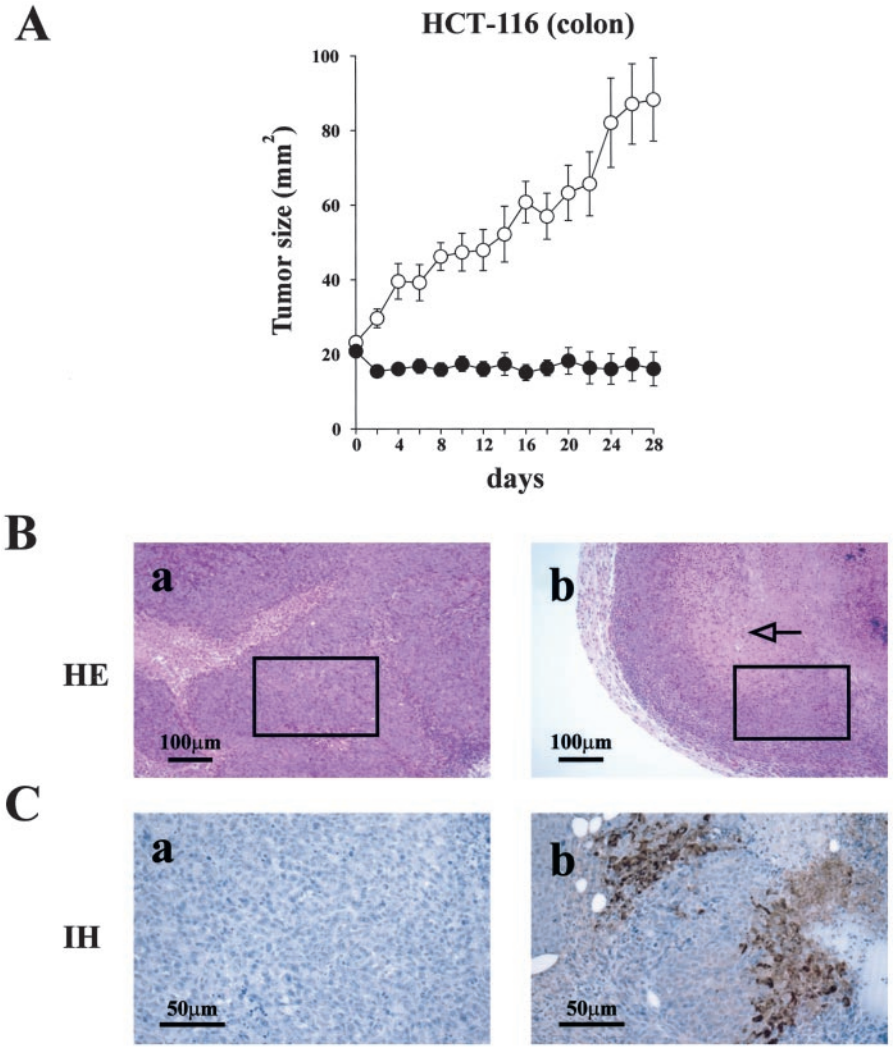

Fig. 4. Effects of i.v. reovirus treatment on s.c. human colon tumors implanted in nude mice. *A*, mice bearing HCT-116 tumors received a single i.v. injection of live reovirus (●) or UV-inactivated reovirus (○). *Bars*, SE (n = 6). H&E staining (*B*) and immunohistochemical staining with rabbit anti-reovirus antibody (*C*) were also carried out on HCT-116 tumors treated with UV-inactivated reovirus (*a*) or live reovirus (*b*). *Arrow*, necrotic area (pictures shown in *C* represent higher magnification of boxed areas in *B*).

the therapeutic effects of intratumoral reovirus injection on s.c. colon and ovarian tumor xenografts. We first examined the efficacy of reovirus treatment on the colon tumor xenografts established by implantation of HCT-116, HT-29, and DLD-1 cells in SCID mice (Fig. 3, *A–C*). Established tumors (20–25 mm^2) received a single injection of reovirus (1×10^7 pfu) or UV-inactivated reovirus, and tumor size was followed for 4 weeks. The mean tumor volume in all three human colon tumor types was found to be significantly reduced in animals treated with live virus compared with those that received UV-inactivated virus. A similar study was carried out using the human ovarian SKOV3 cell line implanted in the hind flanks of CD-1 nude mice (Fig. 3*D*). The mice were treated intratumorally with 5×10^8 pfu of reovirus at days 0 and 14 upon establishment of the tumors. Again, tumor volume was significantly reduced in live virus-treated mice compared with control mice treated with UV-inactivated virus.

Systemic Therapy of Reovirus on Human Colon Tumors. To test the possible use of i.v. delivery of reovirus for the treatment of colon tumors at remote sites, we introduced reovirus into the tail veins of nude mice bearing established HCT-116 tumors at the hind flank (Fig. 4*A*). We found that a single i.v. injection of reovirus (5×10^8 pfu) resulted in the inhibition of tumor growth. Tumors receiving live reovirus showed lysis of tumor cells, as indicated by lesions of necrosis and fibrosis in the remaining tumor mass compared with control animals (Fig. 4*B*). Immunohistochemical staining of the tumors with antireovirus antibody revealed active viral replication within these tumors (Fig. 4*C*). Therefore, at least in immune-compromised animals, systemic delivery of reovirus is effective in causing regression of tumors at a remote site.

Effect of Reovirus on an Ascites Model of Human Ovarian Cancer. In the advanced diagnosed stages of ovarian cancer (International Federation of Gynecology and Obstetrics stages III and IV), the tumor spreads throughout the peritoneal cavity or to distant sites, such as the liver and the pleural surface. To determine whether reovirus therapy is effective at this stage of ovarian cancer, we used a mouse ascites model of human ovarian cancer. CD-1 nude mice were injected i.p. with 2×10^6 MDAH2774 cells 5 days before the initiation of reovirus treatment (day 0). The therapeutic regimen consisted of one i.p. injection of 5×10^8 pfu of live reovirus or UV-inactivated virus (mock-treated control) every 2 weeks. No ascites burden was visible in reovirus-treated mice at day 28, whereas ascites formation was evident in the control mice (Fig. 5*A*). Ascites tumor formation in the control mice correlated with increased body weight of these animals (Fig. 5*B*). In contrast, little weight gain was observed with the reovirus-treated mice when compared with animals without tumor implantation. Furthermore, all of the control mice treated with inactive virus died by day 42 (median survival, 34.6 days), whereas 9 of the 10 live virus-treated mice were still alive after 60 days (Fig. 5*C*; these animals appeared healthy even after 100 days, when they were eventually sacrificed).

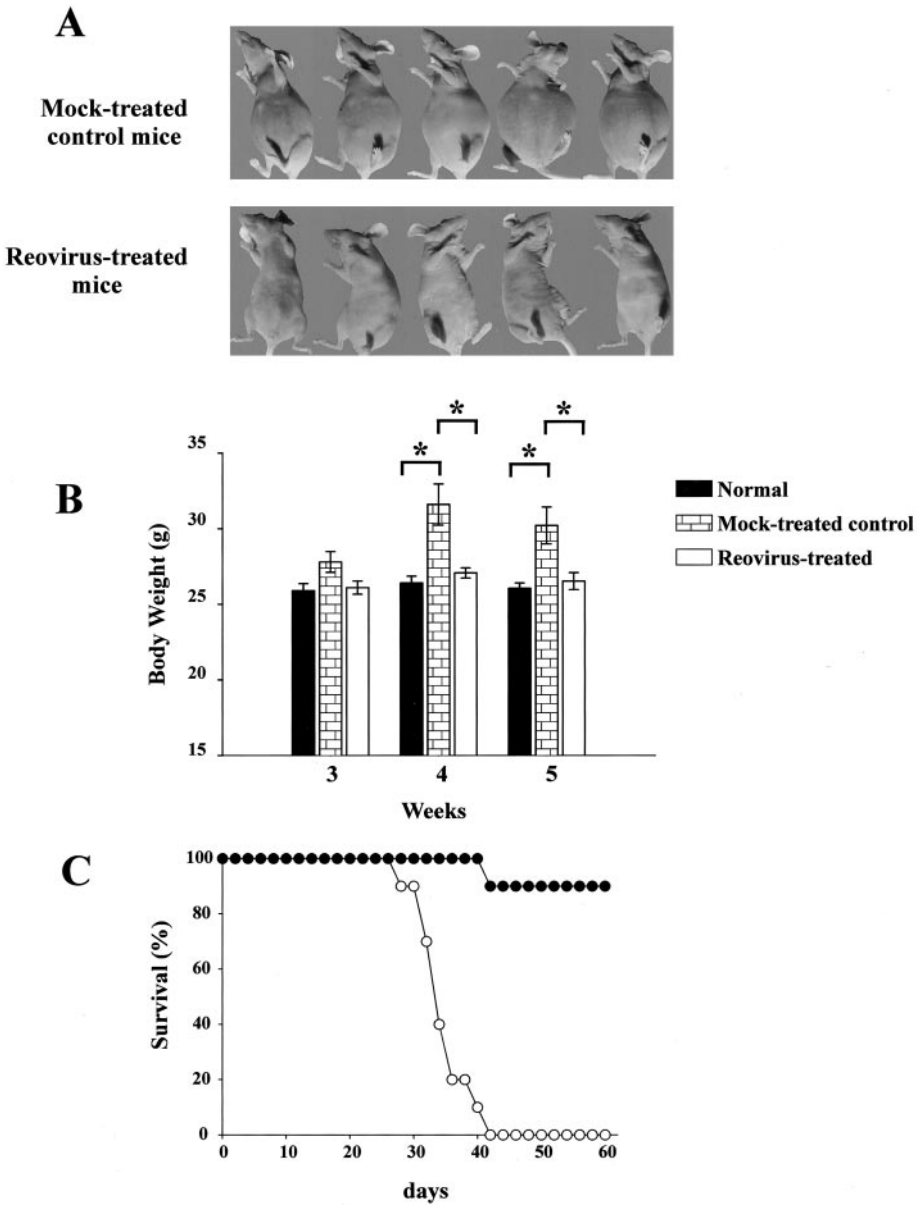

Fig. 5. Effects of reovirus therapy on ascites ovarian tumor formation in nude mice. CD-1 nude mice were injected i.p. with MDAH2774 cells at day 0, followed by i.p. injection with live-reovirus or UV-inactivated reovirus at days 5 and 19. *A*, photos of representative UV-inactivated virus-treated and live-virus treated mice at day 28. *B*, body weight of the mice at 3, 4, and 5 weeks after cell injection. *Bars*, SE (*, $P < 0.01$ by students' *t* test). *C*, Kaplan-Meier survival analysis of tumor-bearing mice treated with live reovirus (●; $n = 10$) or with UV-inactivated reovirus (○; $n = 10$).

Reovirus Infection in Primary Human Ovarian Tumor Biopsy Samples. To see if reovirus also infects tumor biopsy samples, three independent primary human ovarian tumor surgical specimens were prepared for infection *in vitro* (Fig. 6). The clinical backgrounds of the samples were endometrioid adenocarcinoma (Fig. 6, *A* and *B*) and papillary serous adenocarcinoma (Fig. 6*C*). Susceptibility to reovirus infection was assessed by either Western blot with antireovirus antibody (Fig. 6*A*) or [^{35}S]methionine labeling of infected cells (Fig. 6, *B* and *C*). Although there appeared to be some variability in susceptibility between the samples (with sample B being the least susceptible), there was evidence of reovirus protein synthesis in all cases, demonstrating the potential use of reovirus as an antiovarian cancer agent in humans.

DISCUSSION

To use replication-competent viruses for human cancer therapy, safety and efficacy considerations demand that a virus exhibits efficient infection in the target with low pathogenicity in normal tissues. We showed previously that Ras signaling plays a critical role in dictating host cell permissiveness to reovirus. In the present study, we demonstrate that all of the human colon and ovarian cancer cell lines tested were susceptible to reovirus infection, whereas normal colon and ovarian fibroblast cells were resistant. Activating mutations of K-*ras* are present in some human colon cancer cells, such as HCT-116 and DLD-1 (14), whereas Caco-2 and HT-29 cells are known to harbor a normal *ras* proto-oncogene (15). The status of *ras* mutations in the human ovarian cell lines used in this study is unknown at present. However, overexpression of EGFR in colon and ovarian cancer has been well documented (16–18). Moreover, increased expression of ErbB2, a transmembrane receptor with homology to EGFR, has also been observed in 83% of colon tumors (19) and 30% of ovarian tumors (20). Additionally, Src, a nonreceptor tyrosine kinase the activity of which also results in the stimulation of Ras signaling, is commonly activated in colon and ovarian cancers (21–23). It is not surprising, therefore, that the present study shows that the activity of Ras or a downstream element, MEK1/2, was elevated in all cancer cells compared with normal cell line counterparts. Thus, the susceptibility of human colon and ovarian cancer cell lines to reovirus

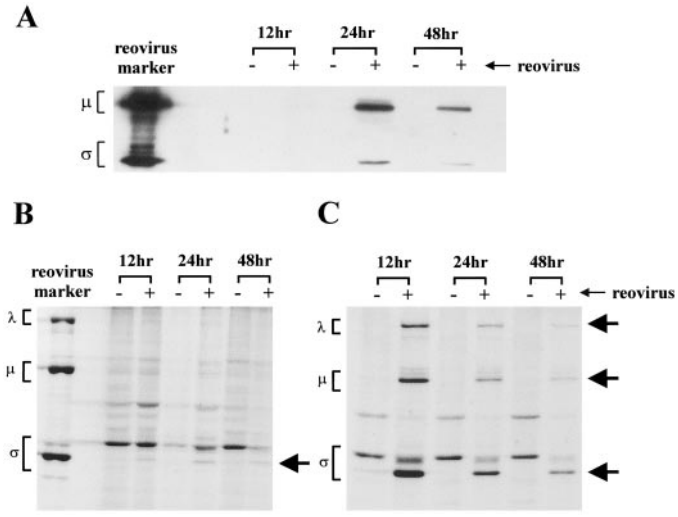

Fig. 6. Reovirus infection of primary human ovarian cancer cells. Single cell suspensions prepared from three separate surgical specimens of human ovarian cancer were either uninfected (−) or infected with reovirus (+). Reovirus protein synthesis was assessed by Western blotting (*A*) or by immunoprecipitation of [^{35}S]methionine-labeled viral proteins with polyclonal antireovirus antibody (*B* and *C*). *Arrows*, reovirus proteins.

is not only attributable to activating mutations in *ras* itself but can also be a result of activation or overexpression of regulatory elements in Ras signaling pathways. This also echoes our previous observation that cells resistant to reovirus infection became susceptible upon transfection with the genes encoding EGFR, v-ErbB, or Sos, all of which are capable of activating Ras signaling (1, 2, 24).

The present study shows that reovirus is effective against all five colon and all four ovarian cancer cell lines tested *in vitro*. Direct inoculation of reovirus into s.c. tumors *in vivo* resulted in marked inhibition of tumor growth. Significantly, i.v. administration of the virus into immune-compromised animals was also found to be very effective, with evidence of active virus replication in tumors remote from the virus injection site. This has raised the possibility of combining reovirus with immune suppressants for the treatment of metastatic cancers in humans in the future. In our preliminary studies, we found that although remote site injections of reovirus failed to effectively inhibit tumor growth in immune-competent mice preimmunized with reovirus before treatment, combination therapy with immunesuppressive drugs fully restored the oncolytic efficacy of reovirus to inhibit remote tumor growth (data not shown). Reovirus treatment was also effective against widespread ovarian tumor xenografts in the peritoneal cavity in the mouse ascites model of human ovarian cancer, with complete cure of 90% of the animals. The high survival rate of these animals is particularly encouraging in view of the common spread of ovarian cancer in the peritoneum at later stages of cancer development.

The use of *ex vivo* biopsy ovarian cancer tissues for *in vitro* studies has also yielded interesting results in that, given the diversity in sample origin, all three specimens were found to be susceptible to reovirus infection. These results are consistent with our demonstration that all four ovarian cancer cells lines tested are infectible by reovirus, suggesting that the susceptibility of these cell lines is not an artifact of extended propagation in culture. However, larger samplings of tumor specimens would be required to provide a more definitive conclusion of the extent of human tumor susceptibility to reovirus. Also, the correlation between *in vitro* susceptibility and therapeutic efficacy in a clinical setting remains to be established.

In conclusion, reovirus therapy is effective *in vitro*, *in vivo*, and *ex vivo* for the treatment of many colon and ovarian cancers, including localized and metastatic tumors. Currently, the main mode of treatment used for colon and ovarian cancer therapy is surgical resection, which is often followed by combination chemotherapy in patients with advanced stages of cancer. Unfortunately, chemotherapy for the treatment of these cancers is less than ideal because drug resistance is a common problem. Therefore, new approaches with different mechanisms of action must be explored. Reovirus specifically targets cancers with an activated Ras signaling pathway and may therefore hold promise as a novel therapeutic in the treatment of a variety of cancers, including colon and ovarian cancer.

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