Oncolytic Viral Therapy for Human Pancreatic Cancer Cells by Reovirus

Tsuyoshi Etoh,1 Yoshihisa Himeno, Toshifumi Matsumoto, Masanori Aramaki, Katsunori Kawano, Akira Nishizono, and Seigo Kitano

Departments of Surgery I [T. E., Y. H., T. M., M. A., K. K., S. K.] and Microbiology [A. N.], Faculty of Medicine, Oita Medical University, Oita, Japan

ABSTRACT

Purpose: Pancreatic cancer has a poor prognosis and few effective therapies are available. The oncolytic effect of reovirus has been observed in cancer cells with an activated Ras signaling pathway, and pancreatic cancer may be a candidate target for reovirus because K-ras mutation is frequently found in pancreatic cancer.

Experimental Design: In this study, we examined the feasibility of using reovirus (serotype 3) as an antihuman pancreatic cancer agent.

Results: Reovirus was able to infect five human pancreatic cancer cell lines (Panc1, MIApaca-2, PK1, PK9, and BxPC3) in vitro. We also confirmed that the Ras activity in these cancer cell lines was elevated compared with that in the normal cell line and that susceptibility to reovirus was associated with the Ras activity of these cells. In a unilateral murine xenograft model using Panc1 and BxPC3 cell lines, each tumor growth was suppressed by intratumoral injection of reovirus. Furthermore, local injection of reovirus also had systemic antitumor effects in a bilateral xenograft model using Panc1 cell line. Immunohistochemical examination revealed that reovirus replication was observed within the tumor but not in surrounding normal tissue.

Conclusions: These results suggest that reovirus can be considered for a novel therapy against pancreatic cancer.

INTRODUCTION

Human reovirus is a unique oncolytic, nonenveloped virus containing 10 segments of double-stranded RNA as its genome. Reoviruses are common isolates of the respiratory and gastrointestinal tract of humans, but in general, reoviral infections are asymptomatic. Recent molecular studies have demonstrated that reovirus requires an activated Ras signaling pathway via direct Ras mutation or independent pathways downstream of Ras such as epidermal growth factor receptor or Her-2 (Neu/ErbB-2) for infection of cultured cells (1). Aberrant ras function (mostly constitutive activation) contributes to the development of many types of neoplastic human diseases (2), ~80% of pancreatic (3), 50% of colorectal (4), and 40% of lung carcinomas (5). In addition, reovirus treatment of immune-competent C3H mice bearing tumors established from ras-transformed C3H-10T1/2 cells also resulted in tumor regression (6). These results suggest that reovirus may have applicability in the treatment of several cancers with an activated Ras signaling pathway. Recently, it has been reported that reovirus may be used as an antitumor agent against human colon, ovarian, and breast cancers (7, 8).

Pancreatic cancer is the fourth and fifth leading cause of cancer death in men and women, respectively, with a dismal 5-year survival rate of ~<5% (9, 10). The principal reasons for the poor prognosis include a lack of symptoms until tumor has progressed beyond a resectable stage and the tumor’s propensity for early metastasis to regional lymph nodes and the liver. At present, although surgical resection is the most effective treatment against pancreatic cancer, the outcome has been unfavorable. In addition, both adjuvant chemotherapy and radiation therapy do not contribute a significant impact on improving disease survival (11, 12). Therefore, the development of novel approaches is important for the treatment of this disease.

The idea of using viruses as oncolytic agents might be an attractive tool for pancreatic cancer therapy. Clinical applications of oncolytic cancer therapy require tumor-specific delivery and minimal side effects (13). The aim of this study is to investigate the oncolytic effect of reovirus in vitro and to examine the relationship between this susceptibility and an activated Ras signaling pathway. In addition, we assess whether reovirus is able to effect regression of xenograft tumors in immune-incompetent animal models.

MATERIALS AND METHODS

Cell Lines and Virus. Five cancer cell lines, Panc1, MIApaca-2, PK1, PK9, and BxPC3, provided from the Japanese Cancer Research Bank (Tokyo, Japan) were used in this study. Four cell lines (Panc1, MIApaca-2, PK1, and PK9) were cultured in RPMI 1640 containing 10% FBS2 and antibiotics, and MIApaca-2 was cultured in DMEM containing 10% FBS and antibiotics. NIH3T3 was cultured in DMEM containing 10% FCS and antibiotics as the normal cell line. Reovirus serotype 3 was kindly provided by Dr. Kensuke Hirasawa (University of California). Reovirus serotype 3 was purified according to the protocol of Smith et al. (14) with exception that 2-mercaptoeth-
andol was omitted from the extraction buffer. Cells grown in 6-well plates were infected with reovirus at a 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, and virus was lysed in the viral buffer and stored at 4°C as described previously (15).

**Cytotoxicity in Cell Lines.** We evaluated the effect of reovirus on pancreatic cancer cells in culture. To detect reovirus protein synthesis, pancreatic cancer cells were infected with reovirus at an MOI of 10 PFUs/cell and, at the indicated times after infection, pulse-labeled with [35S]methionine for 3 h. The labeled proteins were electrophoresed on a 10% SDS-PAGE as described previously (15). For evaluation of susceptibility to reovirus in vitro, each cell line was plated onto 6-well dishes at 1 × 10^5 cells/well. Twenty-four h later, each cell was infected with reovirus at an MOI of 0.1 viral particle/cell and maintained in RPMI 1640 with 10% FBS at 37°C. The control was UV-inactivated reovirus. On days 1–7 after infection, we observed morphological changes and counted the number of viable cells by a hemocytometer using the trypan blue exclusion method.

**Measuring Ras Activity.** Ras activity in pancreatic cancer cells was examined using a ras activation assay kit (Upstate Biotechnology). In brief, each cell lysate was incubated with 5 μl of a 50% slurry of Raf-1 RBD-agarose for 30 min at 4°C. The labeled proteins were electrophoresed on a 10% SDS-PAGE and blotted with a monoclonal anti-Ras antibody (1 μg/ml). The antibody was then reacted with horseradish peroxidase-conjugated goat antimouse secondary antibody and detected by the Enhanced Chemiluminescence-WB system (Amersham, Tokyo, Japan; Ref. 16).

**Subcutaneous Tumor Xenograft Model in Nude Mice.** Six-week-old male athymic BALB/c nude mice, purchased from Kyushu Animal Co., were kept under pathogen-free conditions according to the Animal Center Guidelines. The tumor cells (1 × 10^6) were s.c. implanted unilaterally or bilaterally in the flanks of the mice under anesthesia. When the tumors reached a diameter of ~5 mm, the mice were randomly divided into two groups (5 mice/group). In unilateral xenograft experiments, multiple intratumoral injections of reovirus (1.0 × 10^6 PFUs) were performed every other day. In bilateral xenograft experiments, the mice were given a series of multiple but only unilateral intratumoral injections of reovirus over 21 days (four injections of 1.0 × 10^6 PFUs each for the first 9 days, followed by four injections of 1.0 × 10^6 PFUs every 24 h for the next 12 days). Control injections were with equivalent amounts of UV-inactivated virus. The tumor size was calculated by external morphological changes and counted the number of viable cells by a hemocytometer using the trypan blue exclusion method.

**Immunodetection of Reovirus Replication.** To identify the localization of reovirus in the xenografts, immunohistochemical analysis was performed using primary rabbit antireovirus polyclonal antibody (1:1000 in PBS with 10% goat serum and 0.1% TritonX-100), which was also kindly provided by Dr. Kensuke Hirasawa, then partially purified by ammonium sulfate precipitation. Reovirus protein was detected using the avidin-biotin peroxidase method (LASB kit; Dako, Kyoto, Japan) as described previously (17).

**RESULTS**

**In Vitro Cytotoxic Efficacy.** To evaluate the susceptibility toward the reovirus, five pancreatic cancer cell lines were infected at a MOI of 0.1 viral particles/cell. Five pancreatic cancer cell lines were efficiently destroyed by reovirus within 1 week. As shown in Fig. 1A, the CPE appeared from day 1 after infection, and 50% or greater cytotoxicity was demonstrated at day 7 after infection. In contrast, the NIH3T3 control cells were resistant to reovirus infection. In addition, severe morphological changes were also seen in infected cancer cell lines (Fig. 1B). To additionally confirm reovirus replication in these cells, reovirus protein synthesis was examined after 48-h exposure to reovirus using a methionine assay. After infection, reovirus protein synthesis was detected in all pancreatic cancer cell lines but not in the control cell line (Fig. 1C).

**Detection of Ras Activities in Pancreatic Cancer Cell Lines.** The amount of Ras-GTP was measured using Raf1-Ras binding domain conjugated to agarose beads to pull down active Ras. The Ras activation assay demonstrated that ras activities in all cancer cell lines were increased compared with that in cell line (Fig. 2).

**Oncolytic Efficacy of Reovirus Treatment in Vivo.** To examine oncolytic effects of reovirus treatment on pancreatic cancer in vivo, we established tumors derived from Panc1 and BxPC3 pancreatic cancer cell lines. Then we performed intratumoral injections of reovirus into these established s.c. tumors. As a result, these tumor growths were significantly decreased, and tumor regression was observed in all five mice by 14 days after treatment (P < 0.05; Fig. 3, A and B).

To investigate the systemic effect of reovirus, we treated bilateral Panc1 xenografts by injection with a high dosage of reovirus into only one tumor as indicated in “Materials and Methods.” Regression of both injected and un.injected Panc1 xenografts was observed in all five mice by 21 days after treatment (P < 0.05; Fig. 4). Local intratumoral injection of reovirus could effect remote tumor regression, indicating that systemic delivery could occur in our experimental system.

**Posttreatment Histology in a Bilateral Xenograft Mouse Model.** Specimens stained with H&E demonstrated viral-induced CPEs and necrosis as seen by neutrophil infiltration in injected tumors (Fig. 5A). To evaluate for evidence of selective intratumoral replication of reovirus, we performed immunohistochemistry using antireovirus antibody. Reovirus proteins were detected within injected tumors in a bilateral experiment (Fig. 5B). Areas of viral presence within the tumors were associated with areas of CPEs and necrosis. No inflammation, hemorrhage, necrosis, or other pathological effects were seen in the surrounding normal tissue. Similarly, reovirus proteins were detected within the contralateral tumors in a bilateral experiment (Fig. 5C). In contrast, there was no reovirus protein in UV-inactivated reovirus injected tumor tissue (Fig. 5D).

**DISCUSSION**

Reovirus is a novel oncolytic agent for cancer therapy based on targeting the activated Ras signaling pathway (18). Roughly 50% of all cancers have an activated ras signaling pathway because of activating mutations in the ras gene itself and genes upstream or downstream of ras (1). K-ras mutation is
the most common genetic abnormality identified in pancreatic cancer. Here, we have evaluated whether the oncolytic potency of reovirus in pancreatic cancer cells depends on increased Ras activity in these cells. With regard to the status of ras mutations in the pancreatic cancer cell lines used in this study, Panc1, PK1, PK9, and MIApaca-2 are known to have K-ras mutations (19). In contrast, BxPC3 is known to have a normal ras proto-oncogene (20). In our study, the antitumor effect of reovirus was seen not only in the four pancreatic cancer cell lines with K-ras mutation but also in BxPC3 cancer cells without K-ras mutation in vitro. Furthermore, both Panc1 and BxPC3 xenografts were successfully treated with reovirus injection in vivo. Lytic potency seen in BxPC3 without K-ras mutation may be explained by the presence of signaling leading to activation of pathways downstream of Ras (either through Ras or independent of Ras). Of these signaling molecules, epidermal growth factor receptor is overexpressed in pancreatic cancer tissue compared with normal pancreatic tissue (21, 22) and Src protein, a nonreceptor tyrosine kinase, which leads to ras activation is activated in pancreatic cancer (23). In addition, extracellular signal-regulated kinase phosphorylation has been demonstrated in BxPC3 as well as in the other cancer cell lines with K-ras mutation (24, 25). These results may support that the activity of Ras was elevated in all cancer cell lines examined in this study. It is suggested that increased Ras activity may play an important role in the cytotoxicity of reovirus against pancreatic cancer cells.

**Fig. 1** Effect of reovirus on human pancreatic cancer cell lines Panc1, PK1, PK9, MIApaca-2, and BxPC3 cells and the normal NIH3T3 cell line. A, CPE. Cell lines grown to 80% confluently were exposed to reovirus (MOI = 0.1 PFUs/cell), and the CPE was demonstrated in all cancer cell lines. B, morphological changes after reovirus infection. Severe morphological changes were seen in all cancer cell lines after 48 h of infection. C, reovirus protein synthesis in uninfected (−) and reovirus-infected (+) cell lines. Cells were infected with reovirus at an MOI of 10 PFUs/cell and, at the indicated times after infection, pulse-labeled with [35S]methionine for 3 h. The labeled proteins were electrophoresed on a 10% SDS-PAGE. Right, the three size classes of reovirus protein, λ, μ, and σ.

**Fig. 2** Measuring Ras activity by Ras activation assay. The GTP-bound form of Ras 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. Ras activity was increased in all pancreatic cancer cell lines compared with the control cell.
In this study, we confirmed that all five pancreatic cancer cell lines examined were infectable by reovirus. There is the possibility that reovirus susceptibility in cell lines may be an artifact of extended propagation in culture. To elucidate the change of nature as cancer cells or reovirus susceptibility induced by immortalization of cells, we have to examine the capacity of reovirus to replicate in primary human pancreatic cancer tissue in vivo in the future. Furthermore, we need to evaluate the effect of reovirus against large series of clinical samples for clinical setting.

With regard to systemic effect of reovirus, we investigated whether reovirus locally injected was able to affect remote tumor sites. As a result, reovirus could have significant systemic antitumor activity. It could be considered that there are two mechanisms why reovirus locally injected was able to affect distant tumor sites. First, the virus could be directly cytolytic to the tumor cells thereby contributing to tumor remission. Norman et al. (7) reported that reoviral protein was detected in contralateral tumors by immunohistochemical staining, indicating the tumor regression was attributable to viral replication within tumor xenografts. We have also confirmed that reoviral protein...
was detected in the contralateral tumor by immunohistochemistry. Second, the presence of the virus might induce specific or nonspecific immune responses that lyse tumor cells. Especially, a role of NK cells seems to be important for direct or indirect antitumor responses. Recently, it has been reported that NK cells are highly cytotoxic against ErbB2-expressing cancer cells without affecting normal human cells (26). Furthermore, it has been reported that replication-deficient adenovirus vectors inhibit regional lymph node metastasis independent of a therapeutic transgene, an effect that is mediated, at least, in part, by IFN-γ and NK cells (27) Of these mechanisms, we consider the former is convincing because the tumor regression induced by reovirus replication has been demonstrated in our bilateral xenograft model.

Our result shows that reovirus has the potential to specifically destroy pancreatic cancer cells. In our xenograft model, there were no side effects in treated mice, and the high dose administration was acceptable and effective. Coffey et al. (6) has reported that the immunodeficiency of the SCID mice accounts for the high mortality rate of these mice upon exposure to reovirus. This indicates the possibility of occurrence of toxicity against normal tissue. The presence of a competent immune system may influence the toxicity (28). For clinical trials, it will be necessary to clarify these issues using immune competent animal models in the near future.

Most patients with pancreatic cancer die from liver metastasis and peritoneal dissemination despite multimodality therapy, including surgery, chemotherapy, and radiation therapy. Therefore, novel therapies that can target these resistant tumors are needed. In this study, we suggest that reovirus has several favorable characteristics as a potential cancer therapy for pancreatic cancer: antitumoral activity and selectivity may depend on an activated Ras signaling pathway. For the purpose of treatment for liver metastasis or peritoneal dissemination of this disease, the route of systemic delivery is important to regress these tumors (29–31). i.v. delivery or i.p. delivery may allow reovirus to reach inaccessible tumors and to treat undetectable micrometastasis. However, it might also affect the therapy’s effectiveness and toxicity. Therefore, additional studies are necessary for before clinical trials. If these approaches are successful, oncolytic viral therapy using reovirus may become a novel therapeutic platform for pancreatic cancer treatment.

ACKNOWLEDGMENTS

We thank Dr. Kensuke Hirasawa (Cancer Biology Research Group and Department of Microbiology and Infection Disease, University of Calgary Health Science Center, Calgary, Alberta, Canada) for supplying reovirus and antireovirus antibody. We also thank Dr. Graham F. Barnard (Division of Digestive Disease and Nutrition, University of Massachusetts Medical School, Worcester, MA) for his support.

REFERENCES


