Effective Treatment of Pancreatic Cancer Xenografts with a Conditionally Replicating Virus Derived from Type 2 Herpes Simplex Virus

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Abstract

Purpose: Pancreatic cancer is a devastating disease that is almost universally fatal because of the lack of effective treatments. We recently constructed a novel oncolytic virus (FusOn-H2) from the type 2 herpes simplex virus. Because the replication potential of FusOn-H2 depends on the activation of the Ras signaling pathway, we evaluated its antitumor effect against pancreatic cancer, which often harbors K-ras gene mutations.

Experimental Design: Human pancreatic cancer xenografts were established in nude mice either s.c. or orthotopically (n = 8/group). FusOn-H2 was injected either directly (s.c. tumors) or by the i.v. or i.p. route (orthotopic tumors). Tumor volume, weight, and survival time were recorded for each animal. Statistical analyses were done by Student’s t test.

Results: A single intratumor injection of FusOn-H2 completely eradicated s.c. pancreatic cancers in all animals. Systemic injection of the oncolytic virus produced clear antitumor effects but did not abolish tumors in any animal. The most striking antitumor effect was seen when the virus was given i.p. Delivery of FusOn-H2 by this route completely eradicated established orthotopic tumors in 75% of the animals and completely prevented local metastases.

Conclusions: FusOn-H2 has potent activity against human pancreatic cancer xenografts and may be a promising candidate for investigative virotherapy of this malignancy.

Pancreatic cancer, which is usually diagnosed at an advanced stage, carries the highest fatality rate among all human cancers and accounts for the fourth highest number of cancer deaths in the U.S. (1). The dismal prognosis associated with this tumor has not improved over the past three decades, largely because effective treatments for metastatic disease have not been developed. This outlook may be changing due to the recently acquired ability to isolate or to genetically engineer viruses that could act as oncolytic agents in the treatment of solid tumors such as pancreatic cancer.

Oncolytic viruses infect, replicate in, and destroy cancer cells without harming normal cells. Nonengineered oncolytic viruses are naturally occurring viruses that preferentially target tumor cells (2), but they are limited in number and are not available for all types of cancers. This disadvantage can be overcome with “engineered” oncolytic viruses that have been genetically modified to target cancer cells. In general, there are three ways to engineer viruses to target malignant cells. One approach is to alter surface viral proteins involved in the entry of viruses into cells, so that the virus is specifically targeted to tumor cells (3, 4). Another approach is to modify the virus to replicate only in the absence of a functional tumor suppressor gene, such as p53, which is mutated in many cancer cells (5). In the third approach, the virus is altered so that it will replicate only in dividing cells (6). Several viruses modified for oncolytic purposes are currently in clinical trials for a variety of solid tumors of different tissue origins. Oncolytic herpes simplex viruses (HSV), for example, were originally designed for the treatment of brain tumors (7), but have since been used effectively to treat other solid tumors (8). Oncolytic HVS have a major advantage over other oncolytic viruses: they can be effectively counteracted with an antiviral drug (acyclovir or gancyclovir) in the event of undesired infection or HSV-induced toxicity.

There are two serotypes of HSV: HSV-1 and HSV-2. Current oncolytic HSVs are derived exclusively from HSV-1, and are most commonly constructed by deleting one of the essential genes required for viral replication. These include r34.5 or ICP6 gene, or both (6, 7, 9–11). Inactivation of either or both of these genes enables the virus to selectively replicate in dividing cells, whereas sparing normal nondividing cells (12–14). However, HSV-2 has several unique features that may be explored for its conversion to an oncolytic agent. For example, we recently constructed an oncolytic virus from HSV-2 by exploiting a unique feature of the virus’ ICP10 gene, which contains a well-defined region in its NH2 terminus that encodes serine/threonine protein kinase (PK) activity (15). This PK domain can bind and phosphorylate the...
GT(Pase-activating protein Ras-GAP, leading to activation of the Ras/MEK/MAPK mitogenic pathway, and c-Fos induction and stabilization, and is required for efficient HSV-2 replication (16, 17). A mutant HSV-2 virus (FusOn-H2), deleted for its PK domain, replicated selectively in, and thus, lysed human tumor cells with an activated Ras signaling pathway (18).

Here, we report a therapeutic evaluation of FusOn-H2 against pancreatic cancer xenographs growing in nude mice. Our results show that this mutant virus is a potent oncolytic agent against both orthotopic and metastatic pancreatic tumors. Indeed, two i.p. injections of the virus at a moderate dose completely eradicated the orthotopic and metastatic tumors in most animals, suggesting a potential clinical role for FusOn-H2 in the treatment of pancreatic cancer.

Materials and Methods

Cell lines and viruses. African green monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Rockville, MD). MPanc-96 cells were a kind gift from Dr. Craig Logsdon, University of Texas, M.D. Anderson Cancer Center (19). This human pancreatic cell line has been found to harbor a mutation in exon 1 of \textit{K-ras} and a frameshift in exons 5 to 8 of \textit{p53} (20). The cells are poorly differentiated and readily form disseminated tumors after i.p. implantation (20). Both Vero and MPanc-96 cells were cultured in DMEM containing 10% fetal bovine serum. Female Hsd athymic (nu/nu) mice (obtained from Harlan, Indianapolis, IN) were kept under specific pathogen-free conditions and used in experiments when they attained the age of 5 to 6 weeks.

To construct FusOn-H2, we used PCR to amplify the \textit{ICP10} left-flanking region of the wild-type HSV-2 strain 186 (wt186) genome (equivalent to nucleotide span 85994-86999 in the HSV-2 genome), the ribonucleotide reductase domain, and the right-flanking region (equivalent to nucleotide span 88228-89347). These PCR products were cloned together into pNeb193, generating pNeb-ICP10-PK, hence the new plasmid contains a mutated \textit{ICP10} gene, in which the left-flanking region of the \textit{HSV-2} genome, the ribonucleotide reductase domain, and the right-flanking region (equivalent to nucleotide span 88228-89347) were deleted.

Further details on the construction of FusOn-H2 are reported elsewhere (18).

Phenotypic characterization and oncolytic activity of FusOn-H2 against a human pancreatic cancer cell line. To phenotypically characterize the new virus, we seeded MPanc-96 cells into six-well plates, and then infected them the following day with either wt186 or FusOn-H2 at a dose of 0.01 pfu/cell. Cells were cultured in a maintenance medium (containing 1% fetal bovine serum) and were incubated for up to 2 days to allow the fusion pattern and plaques to develop. To measure the in vivo killing effect of the viruses, we seeded MPanc-96 tumor cells into 24-well plates and infected them with FusOn-H2 or wt186 at 1, 0.1, or 0.01 pfu/cell, or left them uninfected. Cells were harvested 24 or 48 hours later by trypsinization, and the number of viable cells determined with a hemocytometer after trypan blue staining. The percentage of viable cells was calculated by dividing the number of cells excluding trypan blue in the infected well by the number excluding the stain in the well that was left uninfected. The experiments were done in triplicate, with mean cell numbers used for the final calculation.

Animal studies. MPanc-96 cells were harvested from subconfluent cultures by a brief exposure to 0.25% trypsin and 0.05% EDTA. After trypsinization was stopped with medium containing 10% fetal bovine serum, the cells were washed once in serum-free medium and resuspended in PBS. Only single cell suspensions with >95% viability were used for in vivo injections. On day 0, 2 \times 10^6 MPanc-96 cells were inoculated either into the right flank (for establishing s.c. tumor) or into the pancreas of 5- to 6-week-old female Hsd athymic (nu/nu) mice (obtained from Harlan). For intrapancreatic injection, mice were anesthetized with 2.5% Avertin and the injection site cleaned with 70% ethanol. A 1-cm incision was made in the left subcostal region, and the pancreas was exposed. A suspension of 2 \times 10^6 tumor cells in a volume of 50 \mu L was injected into the pancreas. The peritoneum and skin were closed with a 4.0 surgical suture. With the s.c. tumor model, treatment was initiated 2 weeks after tumor cell implantation, when the tumors reached \(5 \pm 5\) mm in diameter. Mice received a single intratumor injection of either 2 \times 10^5 pfu of FusOn-H2 in a volume of 100 \mu L or the same volume of PBS. The tumors were then measured weekly and their volumes determined by the formula: tumor volume (mm\(^3\)) = \[\text{length (mm)} \times \text{width (mm)}\]^2 \times 0.52. With the orthotopic tumor model, treatment was started 1 week after tumor cell implantation. On days 8 and 15 postimplantation, the mice were injected i.p. at a site distant from the area of tumor cell inoculation or systemically with 2 \times 10^5 pfu of FusOn-H2 in a volume of 100 \mu L or the same volume of PBS (i.p. only). On day 36 postinoculation, all surviving mice were euthanized by CO\(_2\) exposure and evaluated macroscopically for the presence of orthotopic tumor and metastases in the abdominal cavity. The orthotopic and metastatic tumor nodules were then explanted, counted, and weighed. In both s.c. and orthotopic experiments, the animals were euthanized when their tumors reached 2 cm in diameter or when they became moribund during the observation period (the time of euthanization was recorded as the time of death).

Fig. 1. Phenotypic characterization of FusOn-H2 in MPanc-96 cells. MPanc-96 cell monolayers were infected with the indicated viruses at 0.01 pfu/cell. Micrographs were taken at 48 hours after infection (original magnification, \(>200\)).
Statistical analysis. Quantitative data are reported as the mean ± SD. Statistical analyses were done by Student’s t test, with the exception of the survival data that were analyzed by the Kaplan-Meier plot and the log-rank (Mantel-Cox) test using MedCalc software (Mariakerke, Belgium). P < 0.05 was considered significant.

Results

Phenotypic characterization of FusOn-H2 in human pancreatic cancer cells. We previously showed that FusOn-H2 could induce syncytia formation in human tumor cells of different tissue origins, including breast and ovary, in addition to its ability to selectively replicate in these cells (18). To determine if it could also induce cell membrane fusion in human pancreatic cancer cells, we infected MPanc-96 cells in a six-well plate with either the parental wt186 or FusOn-H2 virus, or left the cells uninfected. We then monitored the cultures for a cytopathic effect and syncytia formation. As shown in Fig. 1, syncytia formation was clearly present in cultures infected with FusOn-H2, but not in cells infected with the parental wt186 virus. Instead, infection with wt186 induced a typical cytopathic effect, characterized by cell rounding and swelling. These results indicate that FusOn-H2 is also fusogenic in human pancreatic cancer cells. As the cells in the syncytia usually die within 2 to 3 days (21, 22), the ability to induce syncytia formation thus represents an additional tumor killing mechanism of FusOn-H2.

Efficiency of in vitro killing of pancreatic cancer cells by FusOn-H2. To assess the oncolytic activity of FusOn-H2 in vitro, we infected MPanc-96 cells seeded in 24-well plates with this virus or with wt186 at different multiplicities of infection. The cells were harvested at either 24 or 48 hours postinfection, and cell viability was determined by trypan blue staining. As early as 1 day postinfection (Fig. 2A), FusOn-H2 killed a substantial number of tumor cells even in the wells infected with virus at a multiplicity of infection as low as 0.01 pfu/cell. By 48 hours (Fig. 2B), FusOn-H2 had almost completely eradicated the tumor cells exposed to virus doses of 1 or 0.1 pfu/cell. Even at the lowest virus dose, 0.01 pfu/cell, more than half of the tumor cells were killed. Overall, the oncolytic activity of FusOn-H2 was equivalent or even superior to that of its parental wild-type HSV, wt186.

Evaluation of FusOn-H2 oncolytic activity against s.c. pancreatic cancer. We initially chose a s.c. tumor model to evaluate
Together, these data show the efficacy of FusOn-H2 with 2\(^{106}\) pfu of FusOn-H2 in an orthotopic pancreatic tumor model. Freshly harvested MPanc-96 cells (2\(^{106}\)) were injected into the right flanks of nude mice at a dose of 2 \times 10^6 per mouse. Two weeks later, tumors \(\sim 5\) mm in diameter were palpable in all animals. The mice were then randomly divided into three groups (\(n = 8\) each) and their s.c. tumors injected with 2 \times 10^6 pfu of FusOn-H2 in a 100 \(\mu\)L volume or the same volume of PBS. Tumor sizes were measured weekly for 4 weeks. FusOn-H2 treatment led to tumor shrinkage as early as 1 week postinjection; by 2 weeks, half of the mice treated with the virus were tumor-free; and by 4 weeks, all mice in the FusOn-H2-treated group were tumor-free (Fig. 3A). By contrast, mice treated with PBS began to die of progressive tumor by 26 days after tumor cell implantation or had to be euthanized due to rapid tumor growth and cachexia (Fig. 3B). By the end of the experiment, fewer than half of these mice were still alive and all of them bore large tumors (Fig. 3A). These results show the ability of FusOn-H2 to eradicate established pancreatic tumors when given intra-tumorally.

**Evaluation of FusOn-H2 oncolytic activity against orthotopic pancreatic cancer and its local metastases.** We next evaluated FusOn-H2 in an orthotopic pancreatic tumor model. Freshly harvested MPanc-96 cells (2 \times 10^6) were injected into the pancreas of nude mice to establish pancreatic tumors in the orthotopic site. One week after tumor cell implantation, mice were randomly divided into three groups and were injected with 2 \times 10^6 pfu of FusOn-H2 either systemically (through the tail vein, \(n = 7\)) or i.p. (\(n = 8\)), or with PBS as a control (i.p. only, \(n = 6\)). A repeated injection with the same dose of virus was given 1 week later. Four weeks after the first therapeutic injection, all mice were euthanized and examined for orthotopic tumors and local metastases. A typical pathologic specimen from each treatment group is shown in Fig. 4A. The PBS control specimen contained a clear example of local metastatic disease (hatched arrow) in addition to the orthotopic tumor (solid arrow). Overall, five mice from the PBS control group had local metastases, indicating successful establishment of this cancer model.

Systemically delivered FusOn-H2 had a significant therapeutic effect on the growth of established pancreatic cancers at the primary site. By the time the animals were sacrificed, the orthotopic tumors in PBS-treated mice had attained relatively large sizes (mean volume, 683.75 mm\(^3\); Fig. 4; Table 1), in contrast to the orthotopic tumors in mice treated systemically with FusOn-H2 (mean volume, 175 mm\(^3\), \(P < 0.01\)). Systemic delivery of FusOn-H2 also inhibited local metastases by the orthotopic tumor (Table 1). The most striking result came from i.p. administration of FusOn-H2, which led to complete eradication of orthotopic tumors in 75% of the animals (Table 1). The remaining animals had significantly smaller tumors than those in either the PBS control or the systemically treated group (\(P < 0.01\); Fig. 4B). FusOn-H2 given by this route also completely prevented local tumor metastases. The effectiveness of FusOn-H2 therapy was reinforced by the animal survival rate (Fig. 4C; Table 1). All mice in the group treated i.p. with FusOn-H2 survived, whereas i.v. administration of the virus, although less effective than i.p. delivery, significantly prolonged survival by comparison with the PBS control (\(P < 0.01\)). Together, these data show the efficacy of FusOn-H2 in vivo therapy for experimental orthotopic and metastatic pancreatic tumors, especially when the virus is given i.p. Additionally, the fact that there was no animal death during the early stage of virus infusion either i.p. or systemically, suggests that FusOn-H2 could be safely given through these routes of administration.

**Discussion**

Although better cancer detection methods and more effective surgery and chemotherapy have improved clinical outcome for many solid tumor patients in recent years, there has been little progress in the management of patients with pancreatic cancer.

![Fig. 4. Therapeutic effect of FusOn-H2 against orthotopic human pancreatic cancer xenografts and local metastases established in the peritoneal cavity of nude mice. MPanc-96 cells (2 \times 10^6) were implanted into the pancreas of nude mice. At 8 and 15 days after tumor cell inoculation, the mice received either i.p. or systemic (i.v.) injections of FusOn-H2 at a dose of 2 \times 10^6 pfu, or i.p. injections of PBS (control). Four weeks after the initial injection (i.e., 5 weeks after tumor cell implantation), the mice were euthanized. The orthotopic tumor and local metastases in the abdominal cavity were examined, counted and excised. A. Gross appearance of a typical specimen from each group: solid arrows, orthotopic tumors; hatched arrow, metastatic disease. B. Comparison of orthotopic tumor volumes after i.v. or i.p. treatment with FusOn-H2. The data are reported as mean ± SD. C. Kaplan-Meier plots of survival data collected over the 4-week observation period. *: \(P < 0.01\) compared with i.v. administration; **: \(P < 0.01\) compared with PBS.]
In particular, the standard treatment options leave much to be desired. The highest cure rates are attained when the tumor is truly localized to the pancreas but this stage of the disease accounts for <20% of cases overall. The majority of patients have locally advanced disease at the time of diagnosis and typically are treated with chemotherapy and radiation, with subsequent survival times rarely exceeding 9 months. Thus, patients with any stage of pancreatic cancer would benefit from novel and effective therapeutic strategies.

Unlike traditional forms of cancer gene therapy in which viruses are used in a defective form to deliver therapeutic genes, oncolytic viruses directly kill tumor cells through their selective replication and consequent cytolytic effects. The current group of oncolytic HSVs are exclusively derived from HSV-1. Originally designed and constructed for the therapy of brain tumors, they have since been shown to be effective against other solid tumors such as breast, prostate, and ovarian cancers. However, oncolytic HSVs seem to show only limited activity against pancreatic cancer and are often required to be used in combination with other therapeutic modalities, such as prodrug therapy and chemotherapy (23, 24). Derived from HSV-2, the mutant FusOn-H2 virus was constructed by deletion of the PK domain of the viral ICP10 gene, a modification totally different from that used to construct HSV-1-based oncolytic viruses. In the present study, FusOn-H2 showed potent oncolytic activity against pancreatic cancer xenografts without being combined with other therapeutic modalities. Two i.p. treatments with a moderate dose of this HSV-2-derived virus led to complete eradication of established pancreatic tumors in the orthotopic site in 75% of the animals. Even systemic (i.v.) delivery of this virus produced a measurable antitumor effect on the orthotopic tumor and its local metastases. One factor that might have contributed to the potent antitumor effect of FusOn-H2 is its ability to induce syncytia formation in tumor cells. We and others recently showed that incorporation of cell membrane fusion activity into an oncolytic HSV-1 can significantly enhance the antitumor effect of the virus (25, 26). Hence, the apparent fusogenic property of FusOn-H2 likely potentiated its oncolytic effect in pancreatic cancer cells.

Besides activating the Ras signaling pathway to facilitate HSV-2 replication, the PK domain of the ICP10 gene has been shown to have antiapoptotic activity during HSV-2 infection (27). Deletion of this domain from the viral genome would therefore be expected to “trick” tumor cells into an apoptotic state after virus infection, thereby enhancing the oncolytic activity of the virus. Indeed, we have observed rapid induction of apoptosis in several human tumor cell lines after infection with FusOn-H2, but not with an oncolytic virus derived from HSV-1. Thus, we suggest that unscheduled apoptosis of pancreatic cells, due to FusOn-H2 infection, also contributed to the antitumor efficacy of this virus. It seems likely that the potent antitumor activity of FusOn-H2 might have been derived from the combined effect of more than one killing mechanism of the virus, e.g., the direct cytolytic effect of virus replication, the ability of the virus to induce syncytia formation, and apoptosis. An added benefit of having multiple killing mechanisms by a single oncolytic agent might be a reduction in the emergence of therapy-resistant tumors. The history of cancer therapy is filled with examples of single agents that were initially effective but soon lost therapeutic activity because their restricted mechanisms of action allowed the generation and overgrowth of resistant tumor cells. We believe the multiple, diverse cytolytic mechanisms used by FusOn-H2 would reduce the likelihood of treatment resistance by pancreatic tumor cells, enabling prolonged therapy with the virus.

Unlike the HSV-1-based oncolytic HSVs that target any dividing cell, the replication potential of FusOn-H2 requires activation of the Ras signaling pathway. Activating K-ras gene mutations are present in >90% of pancreatic adenocarcinomas (28). Such mutated K-ras genes encode constitutively active forms of K-ras that are resistant to degradation and seem to be important in the initiation and maintenance of pancreatic adenocarcinoma. This cancer therefore represents an ideal therapeutic target for FusOn-H2. Eighty percent of all patients with pancreatic cancer present with local metastatic disease, usually to i.p. lymph nodes and the liver. The results of this and a companion study (18) suggest that the FusOn-H2 oncolytic virus could be safely and effectively administered into the primary tumor and/or peritoneal cavity at the time of laparotomy or perhaps percutaneously by computed tomography–guided injection. Regardless of the delivery strategy, FusOn-H2 warrants evaluation in patients with pancreatic cancer.

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**References**


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