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Generation of a novel, cyclooxygenase-2–targeted, interferon-expressing, conditionally replicative adenovirus for pancreatic cancer therapy

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Krumdiek tissue slicer

Abstract

BACKGROUND: Oncolytic adenoviruses provide a promising alternative for cancer treatment. Recently, adjuvant interferon (IFN)-alfa has shown significant survival benefits for pancreatic cancer, yet was impeded by systemic toxicity. To circumvent these problems adenovirus with high-level targeted IFN-alfa expression can be generated.

METHODS: Conditionally replicative adenoviruses (CRAds) with improved virulence and selectivity for pancreatic cancer were generated. The vectors were tested in vitro, in vivo, and in human pancreatic cancer and normal tissue specimens.

RESULTS: Adenoviral death protein and fiber modifications significantly improved oncolysis. CRAds selectively replicated in vitro, in vivo and showed persistent spread in cancer xenografts. They showed high-level replication in human pancreatic cancer specimens, but not in normal tissues. Improved IFN-CRAd oncolytic efficiency was shown.

CONCLUSIONS: Optimized cyclooxygenase-2 CRAds show highly favorable effects in vitro and in vivo. We report a pancreatic cancer–specific, highly virulent, IFN-expressing CRAd, and we believe that adenovirus-based IFN therapy offers a new treatment opportunity for pancreatic cancer patients.

Pancreatic ductal adenocarcinoma is a highly lethal disease, with an estimated 43,140 new cases and with nearly 37,000 deaths in 2010.[1] Because of the deep retroperitoneal location of the pancreas and because early stage disease typically has no specific symptoms, it is uncommon for this cancer to be diagnosed at a surgically resectable stage. Of the newly diagnosed cases, approximately 85% to 90% of patients will have inoperable disease at presentation because of locally advanced stage or metastases.[2] Chemotherapy with gemcitabine is currently the standard of care in the adjuvant setting; however, overall survival remains poor, with a median survival of approximately 22 to 24 months in selected series.[3]

Oncolytic adenovirus is an extensively studied vector in cancer therapy. In particular, conditionally replicative adenoviruses (CRAds), oncolytic adenoviruses that are de-

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signed specifically for replication restricted to cancer cells, have been developed.[4,5] Clinical trials in various cancers including pancreatic, prostate, ovarian, and glioblastoma multiforme[6,7] have established the tolerability of CRAds and such experience with its use has contributed greatly to its well-understood safety profile. However, clinical trials to date also have highlighted several significant disadvantages of CRAd therapy, as follows: limited infection of cancer cells, limited intratumoral spread, limited specificity for cancer cells with off-target effects including hepatotoxicity, and immunogenicity.[5,8–10] Thus, there is a pressing need to develop Ad vectors for cancer therapy with both increased efficacy and virulence toward pancreatic cancer, while at the same time possessing high selectivity for cancer cells with the intent to avoid liver and systemic toxicity.

Pancreatic cancer cells are resistant to infection with conventional CRAds because of the profound lack of the coxsackie-adenovirus receptor (CAR) on the cell surface.[13] We and others previously reported improved infectivity of adenoviral vectors toward pancreatic cancer cells through genetic modification of the viral capsid proteins to alter tropism by binding to alternative cell surface receptors on pancreatic cancer: integrins (arginine-glycine-aspartic [RGD] fiber) and Ad3 receptor (5/3 fiber).[11–13] In addition, improved killing ability has been achieved with adenoviral death protein (ADP) overexpression, an enhancer of apoptosis and viral spread.[14] A further increase in antitumor effect can be achieved by incorporating an antitumor transgene into the CRAd genome. This local transgene expression, coupled with strong vector spread, will advance oncolytic virotherapy for pancreatic cancer significantly and has the potential to create an entirely new class of therapy.

Recently, interferon (IFN)-alfa, a cytokine with direct and indirect antitumor effects, has shown promising improvements in survival in multimodality adjuvant therapy. This was first reported by the Virginia Mason group study,[15] which found a statistically significant improvement in survival with interferon-based adjuvant chemoradiation over gemcitabine-based adjuvant therapy at 26 months of follow-up evaluation, with 84% survival at 2 years. A subsequent phase II study by Linehan et al,[16] using adjuvant interferon-based chemoradiation with postradiation gemcitabine instead of 5-FU, resulted in a 56% 2-year actuarial survival rate, which was identical to that reported by Picozzi et al[17] in the multicenter phase II trial. However, this regimen suffered from systemic side effects, with an incidence rate as high as 95%, and more than 25% of patients required a dose reduction owing to IFN systemic toxicities.[15–18] Overall, this indicates a pressing need for the development of highly active agents for the treatment of pancreatic cancer. As shown by these previous studies, IFN could be a powerful tool for the generation of such a modality. To use IFN as a targeted therapy, the challenge of limiting systemic IFN toxicities must be overcome. One strategy would be to design a viral vector to selectively infect pancreatic cancer cells, replicate within them, and release therapeutic amounts of IFN locally to avoid systemic effects. However, such modified viruses expressing IFN as a therapeutic transgene for pancreatic cancer have not been reported, except as an early generation nonreplicating construct.[19]

In this work we have designed an adenovirus for optimal infectivity of pancreatic cancer with increased virulence as a result of IFN and ADP expression. This is a conditionally replicative adenovirus controlled by the tumor-specific promoter cyclooxygenase-2 (Cox2), allowing the virus to target pancreatic cancer while sparing toxicity to the liver. We hypothesize that this vector will possess superior attributes of cancer selectivity, cell infectivity, and cell killing compared with adenovirus vectors previously studied. Such a strategy hopefully will lead to a more powerful yet better tolerated means of interferon administration in pancreatic cancer patients.

Materials and Methods

Cell lines and animals

The human pancreatic ductal adenocarcinoma (PDAc) cell lines AsPS1, S2VP10, S2013, MiaPaCa2, Panc1, and HS766T were maintained in Dulbecco's modified Eagle medium (DMEM) (Mediatech, Herndon, VA) with 20% fetal bovine serum (FBS) for ASPC-1 and 5% FBS for all other cell lines. MiaPaCa2, Panc1, AsPC1, HS766T, the Cox2-positive human non–small-cell lung adenocarcinoma cell line A549, the Cox2-negative human breast cancer cell line BT474, and epidermoid carcinoma cell line A431 were obtained from the American Type Culture Collection (Manassas, VA). BT474 was maintained in Roswell Park Memorial Institute medium supplemented with 15% FBS and bovine insulin (.01 mg/mL; Life Technologies, Rockville, MD). The 911 cells (a kind gift from Dr Van Der Eb, Leiden University, The Netherlands[20]) were maintained in DMEM supplemented with 5% FBS. Human umbilical vein endothelial cells (HUVEC) were obtained from ATCC and cultured in EGM-2 medium (Cambrex Biosciences, Walkersville, MD). All media were supplemented with penicillin (100 IU/mL) and streptomycin (100 μg/mL).

Female athymic nude mice (NCr-nu/nu; National Cancer Institute at Frederick, Frederick, MD) at 6 to 8 weeks of age were used for in vivo studies. All animals received humane care based on the guidelines set by the American Veterinary Association. All experimental protocols involving live animals were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Adenoviral vectors

Replication-deficient Ad vectors expressing the human interferon-alfa gene under control of cytomegalovirus immediate early promoter (CMV) were constructed using homologous recombination with the region E1-deleted, fiber-

modified adenoviral backbones designed in our laboratory. The human IFN encoding plasmid kindly was provided by Dr Aoki (Genetics Division, National Cancer Center Research Institute, Tokyo, Japan).[19] The infectivity-enhanced Cox2 promoter-controlled CRAds were generated using homologous recombination in *Escherichia coli* as described previously.[11,13,21,22] The ADP-overexpressing oncolytic viruses with ether luciferase or IFN expression cassette were constructed using pShuttleΔE3ADPKanF2 cloning.[11,23] Replication-incompetent CMV promoter-driven luciferase expression vectors with the genetically modified RGD fiber (RGDCMV-Luc), Ad5/Ad3-chimeric fiber (5/3CMV-Luc), or native Ad5 fiber (Ad5CMV-Luc) were used to analyze infectivity enhancement and have been described previously.[21,22] Wild-type Ad5 (Ad-Wt) and its RGD isogenic versions (RGD-Wt) were used as nonselective replicative control vectors.

All viruses were propagated in the 911 cell line and purified by double CsCl density gradient ultracentrifugation, followed by dialysis against phosphate-buffered saline (PBS) with 10% glycerol. The vectors were titrated by plaque assay, and viral particle (vp) number was measured spectrophotometrically with absorbance at 260 nm. Vectors were stored at −80°C until ready for use. Viral structure was confirmed by polymerase chain reaction for Cox2, a mutant replication competent Ad contamination, and fiber structure, as described previously.[13,21]

In vitro analysis of infectivity with luciferase-expressing Ads

Cells (5×10^4 cells/well) grown in 24-well plates were infected with 100 viral particles (vp) per cell for 48 hours, followed by lysis with 100 μL of cell culture lysis buffer (Promega, Madison, WI) and Luc activity was determined with the Luciferase Assay System (Promega). All experiments were performed in triplicate.

Detection of Cox2 promoter–dependent CRAd replication

Cultured cells (5×10^4 cells/well) were infected with .1 vp/cell in 100 μL DMEM. The infection medium was replaced with 1 mL of medium 2 hours later and cells were incubated for 2 days. At this time, the cells were lysed with cell culture lysis buffer (Promega), and luciferase activity was determined with the Luciferase Assay System (Promega). Experiments were performed in triplicate and standardized with protein concentration quantitated by the DC protein assay (Bio-Rad, Hercules, CA).

In vitro quantitative analysis of cancer cell killing ability

Cells were seeded in 96-well plates at 3,000 cells per well, then infected with Ad vectors at varying multiplicities of infection in 100 μL of appropriate medium. The cells were incubated under standard conditions and the number of living cells was measured colorimetrically at serial time points using the Cell Titer Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. The proportion of living cells at each time point was normalized to the number of living uninfected cells. All experiments were performed in triplicate.

Human tissue samples

After approval by the University of Minnesota Institutional Review Board, resection specimens of biopsy-proven pancreatic ductal adenocarcinoma, adjacent normal pancreas, and normal liver were obtained fresh, and immediately sliced to 200-μm thickness (Krumdieck Tissue Slicer; Alabama Specialty Products, Inc, Munford, AL).

Viral infection of human tissue slices

Tissues (200-μm thick) were plated in 12-well plates containing 1 mL/well of medium consisting of 50% FBS Ham's F12 (Mediatech) and 50% FBS DMEM with 1% penicillin/streptomycin, 1% amphotericin B, 15% FBS, and 10 μg/mL dexamethasone. Infection was performed at a multiplicity of infection of 100 vp/cell estimated by average weight to calculate the approximate cell number per tissue slice. The infection media was replaced 3 hours later with appropriate 50% FBS culture media. At day 4, viral DNA was extracted from the slices using a QIAamp DNA Blood Mini kit (Qiagen, Germantown, MD). Viral copy number was quantitated by SYBR Green reverse-transcription polymerase chain reaction (Applied Biosystems, Foster City, CA) with the adenoviral E4 primers[13] and compensated with β-actin. All experiments were performed in triplicate.

In vivo bioluminescent imaging of CRAd replication in a mouse xenograft model

HS766T and A431 cells (1.0×10^6) were injected into both flanks of nude mice. When tumors reached 6 to 10 mm in diameter, a single virus dose of 10^{10} vp in 50 μL PBS was injected intratumorally. Bioluminescent light imaging was performed under anesthesia with 2% isoflurane and after intraperitoneal injection of 3 mg D-Luciferine (Molecular Imaging Products, Bend, OR) using an in vivo imaging system as we described previously.[11,23]

In a separate experiment, A549 cells (1.0×10^6) were injected into both flanks of nude mice. When tumors reached 6 to 8 mm in diameter, a single virus dose of 10^{10} vp in 50 μL PBS was injected systemically via the tail vein. Bioluminescent light imaging was performed under the same conditions as described earlier.

Statistical methods

Statistical analysis of viral effects in vitro and in vivo was performed with Excel (Microsoft, Redmond, WA). The Student *t* test of means was used with a 2-tailed *P* value of less than .05 considered statistically significant. Data are expressed as mean ± standard deviation of at least 3 results.

Results

Adenoviral vector structure

We generated multiple experimental and control vectors as shown in Fig. 1. RGD-CMV-IFN and 5/3-CMV-IFN are unable to replicate owing to deletion of the critical viral E1 region (ΔE1), which is required for replication, and are designed to express the cytocidal IFN gene under control of the continuously active CMV promoter. These viruses have either the RGD or 5/3 capsid fiber modification for increased infectivity toward target cells. RGDCox2CRAd-F and 5/3Cox2CRAd-F are the replication-competent oncolytic agents in which cellular Cox2 activity of the infected cell is used to trigger E1 gene expression and therefore allow the virus to replicate.[11,22] Nonselective, wild-type replicative 5/3WtΔE3ADP-Luc and 5/3WtΔE3ADP-IFN both have an expression cassette replacing the nonessential viral E3 region, which is known to overexpress ADP, an enhancer of viral spread,[21,23] as well as either the therapeutic IFN gene or the Luc reporter. Both viruses are have enhanced infectivity as suggested by changing their tropism to Ad3 receptor (5/3 fiber modification). Of note, gene expression from the adenovirus E3 region follows a late profile owing to control by the major late promoter and therefore is consistent with the replication cycle. Strong transgene expression from this locale also has been observed previously to take place. The vectors 5/3Cox2CRAdΔE3ADP-Luc and 5/3Cox2CRAdΔE3ADP-IFN have the following characteristics: expression of the E1 region, which is critical for replication, is controlled by the Cox2 promoter, conferring cancer specificity; all E3 genes have been deleted and instead either ADP-Luc or IFN are expressed in a replication-dependent fashion; and the vectors are fiber-modified (5/3) to overcome CAR deficiency of pancreatic cancer cells.

The RGD and 5/3 fiber modifications significantly increase adenoviral infectivity in PDAc

To analyze transduction efficiency of fiber-modified viral vectors in pancreatic cancer, S2VP10, S2013, AsPC1, and MiaPaca2 cells were infected with the following: (1) Ad-CMV-Luc vector with the native Ad5 fiber structure; (2) RGDCMV-Luc vector equipped with the RGD fiber modi-

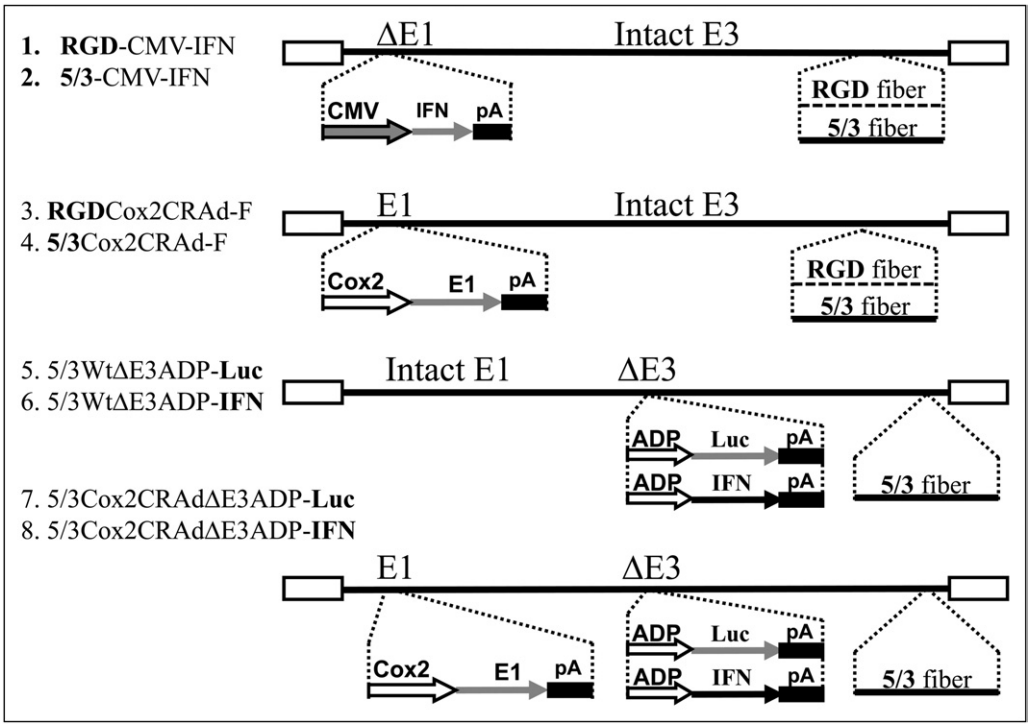

Figure 1 Schematic structure of adenoviral vectors. Several important structural features are reflected including wild-type or Cox2-controlled replication, capsid fiber modification by RGD or 5/3, and insertion of ADP and transgene luciferase or human interferon-alfa into the adenoviral E3 region: (1) **RGD**-CMV-IFN (replication-deficient control); (2) **5/3**-CMV-IFN (replication-deficient control); (3) **RGD**Cox2CRAd-F; (4) **5/3**Cox2CRAd-F; (5) 5/3WtΔE3ADP-**Luc**; (6) 5/3WtΔE3ADP-**IFN**; (7) 5/3Cox2CRAdΔE3ADP-**Luc**; and (8) 5/3Cox2CRAdΔE3ADP-**IFN**.

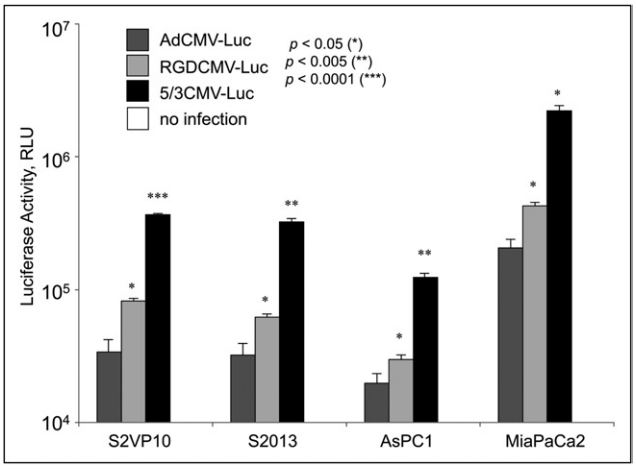

Figure 2 Fiber-modified adenoviral vectors (5/3CMV-Luc and RGDCMV-Luc) significantly increased the infectivity of pancreatic cancer cells compared with the identical control vector with a native Ad5 fiber (AdCMV-Luc). Pancreatic cancer cell lines S2VP10, S2013, AsPC1, and MiaPaca2 were infected with replication-deficient viral vectors encoding luciferase. Both Ad5/Ad3 and RGD fiber-modified viral vectors showed a significant increase in luciferase gene expression compared with the fiber-unmodified counterpart.

fication; and (3) Ad5/3CMV-Luc vector with the 5/3 fiber-knob chimera. Fiber modification was the only structural change made to this identical replication-incompetent viral vectors encoding the CMV promoter-driven luciferase. The adenovirus-induced gene expression was analyzed by Luc assay (Fig. 2). In all 4 pancreatic cancer cell lines, fiber-modified viral vectors showed significant increase in Luc expression, with Ad5/3CMVLuc showing the greatest Luc activity followed by RGDCMV-Luc. Compared with fiber-unmodified AdCMV-Luc, Ad5/3CMV-Luc increased Luc activity by 91% whereas RGDCMV-Luc showed a 59% increase in Luc activity in S2VP10. The Ad5/Ad3 fiber-modified vector significantly outperformed AdCMV-Luc by 90%, 84%, and 91%, and RGDCMV-Luc by 48%, 34%, and 51% in S2013, AsPC1, and MiaPaca2, respectively, and showed the highest level of transduction efficiency in 4 of 4 PDAc cell lines.

The targeted Cox2-controlled virus avoids replication in Cox2-nonexpressing cell populations

To understand the ability of the Cox2-controlled virus to selectively avoid replication (and therefore replication injury) in Cox2-negative cell populations including hepatocytes, the organ of greatest concern, representative cell lines with both low Cox2 activity A431, BT474, HUVEC, and high Cox2 (HS766T), were infected with 5/3WtΔE3ADP-Luc and 5/3Cox2CRAdΔE3ADP-Luc (Fig. 3). These viruses have the most complex structure and are designed for the highest efficacy, incorporating ADP overexpression, massive transgene expression, and fiber modifications to enhance infectious ability. Reporter gene activity in Cox2-positive HS766T pancreatic cancer cells using 5/3Cox2CRAdΔE3ADP-Luc approaches that of 5/3WtΔE3ADP-Luc. Minimal reporter gene activity of 8% or less was detected using 5/3Cox2CRAdΔE3ADP-Luc in cells of low Cox2 activity ($P < .0001$ compared with 5/3WtΔE3ADP-Luc). Importantly, reporter gene activity using 5/3Cox2CRAdΔE3ADP-Luc in HUVEC cells, a normal and untransformed human cell line with low Cox2 activity, was found to be significantly less than activity using 5/3WtΔE3ADP-Luc. These data emphasize the close dependence on Cox2 status of the target cell for replication, and therefore the ability of the Cox2 CRAd to differentially replicate in normal versus cancerous tissue in a highly selective fashion.

The targeted viral construct controlled by the Cox2 promoter shows selectivity and persistence in a cancer xenograft model

Pancreatic cancer HS766T (high Cox2 activity) and Cox2-negative A431 cells were used to form subcutaneous tumor xenografts in the flanks of nude mice (Fig. 4). Mice were injected with a single intratumoral injection of either a tumor-targeted Cox2–replication-controlled (5/3Cox2CRAdΔE3ADP-Luc) or wild-type replication (5/3WtΔE3ADP-Luc) virus expressing luciferase in a replication-dependent manner. During the following 5 weeks, the mice were anesthetized, injected intraperitoneally with the luciferase substrate, D-luciferine, and

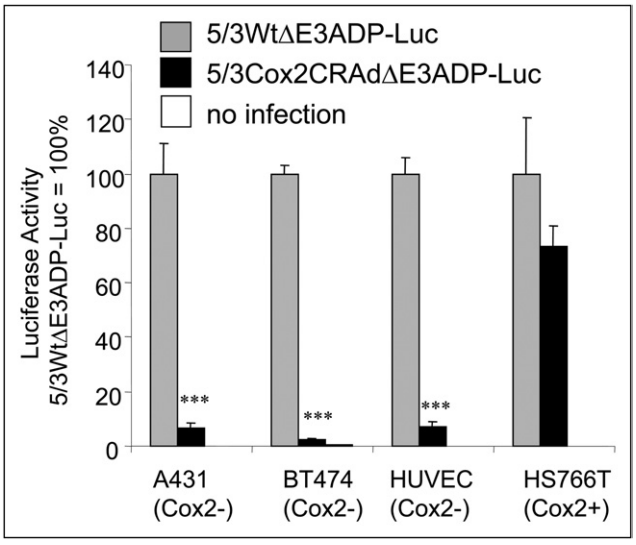

Figure 3 The Cox2-controlled virus selectively avoids replication in Cox2-negative cell populations. The Cox2-negative cell lines A431, BT474, HUVEC cells, and the strongly Cox2-positive pancreatic cancer cell line HS766T were infected with fiber-modified viruses with wild-type replication or Cox2-promoter–dependent replication. Cox2-negative cells fail to support viral replication of Cox2-controlled virus. ***$P < .0001$.

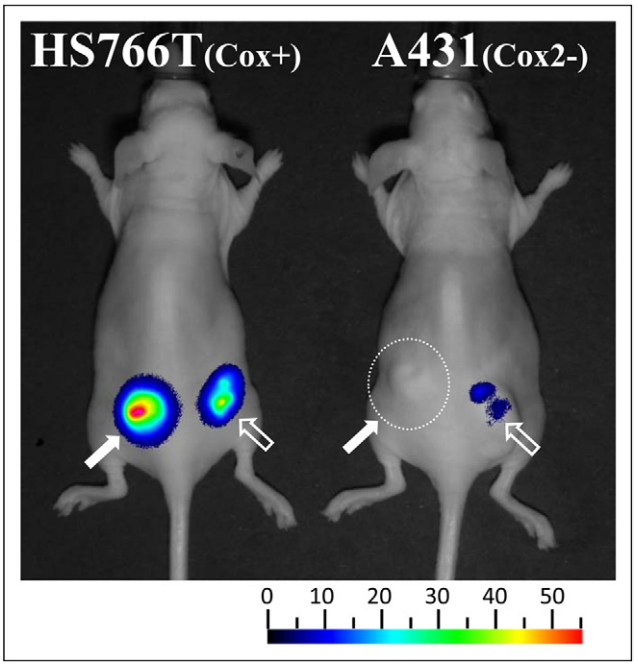

Figure 4 The targeted viral construct controlled by Cox2 shows selectivity and persistence in a cancer xenograft model. Subcutaneous xenografts were established in the flanks of nude mice using the PDAc cell line Hs766T and Cox2-negative control cells A431. 5/3Cox2CRAdΔE3ADP-Luc (left side) and 5/3WtΔE3ADP-Luc (right side) were injected intratumorally. Strong viral replication was seen with both viruses in PDAc xenografts but only with 5/3WtΔE3ADP-Luc in A431 tumors. Representative image at day 4 postinfection.

replication-dependent luciferase activity was measured using noninvasive bioluminescence imaging. Both oncolytic adenoviruses showed tumor-associated bioluminescence at high levels in Cox2-positive HS766T tumors: bioluminescence peaked around day 4 (Fig. 4), and persisted for up to 4 weeks. Importantly, no bioluminescence was found in mice bearing Cox2-negative A431 xenografts that were challenged with the Cox promoter-controlled Ad5/3Cox2ΔE3ADP-Luc.

Furthermore, we performed an experiment to assess whether the same results would be found using intravenous delivery of virus instead of intratumoral delivery. High Cox2-expressing A549 lung cancer cells were used to form subcutaneous flank xenografts in nude mice (Fig. 5). Mice then were injected with 5/3Cox2CRAdΔE3ADP-Luc by intravenous delivery into the tail vein, and repli cation-dependent luciferase expression was imaged over the next several weeks. Not only did our novel 5/3Cox2CRAdΔE3ADP-Luc track subcutaneous Cox2-positive tumors with this delivery strategy, an important proof-of-concept finding for clinical application, but viral activity again was persistent for up to 30 days. Thus, this strategy is capable of in vivo cancer selectivity and sustained replication by both intravenous and direct intratumoral administration.

The targeted Cox2-controlled CRAd shows selective replication ex vivo in human PDAc specimens but not in normal tissues

To study the selectivity of the Cox2 promoter for pancreatic cancer ex vivo, we infected resected specimens of pancreatic cancer, normal pancreas, and normal liver with RGDCox2CRAd-F, an RGD-modified CRAd requiring Cox2 activity of the infected cell for replication, and compared them with RGD-Wt, the control wild-type adenovirus without tumor specificity (Fig. 6). In pancreatic cancer specimens, RGDCox2CRAd-F replicated as avidly as RGD-Wt, indicating the strong activity of this promoter in PDAc. In sharp contrast, normal pancreatic tissue did not support CRAd replication and replication was minimal in normal liver tissue ($P < .005$, $P < .05$ compared with the wild-type replication control RGD-Wt). When the replication of these 2 vectors was compared in each tissue, the percentage of compensated viral copy number RGDCox2CRAd-F in pancreatic adenocarcinoma tissue was significantly higher than

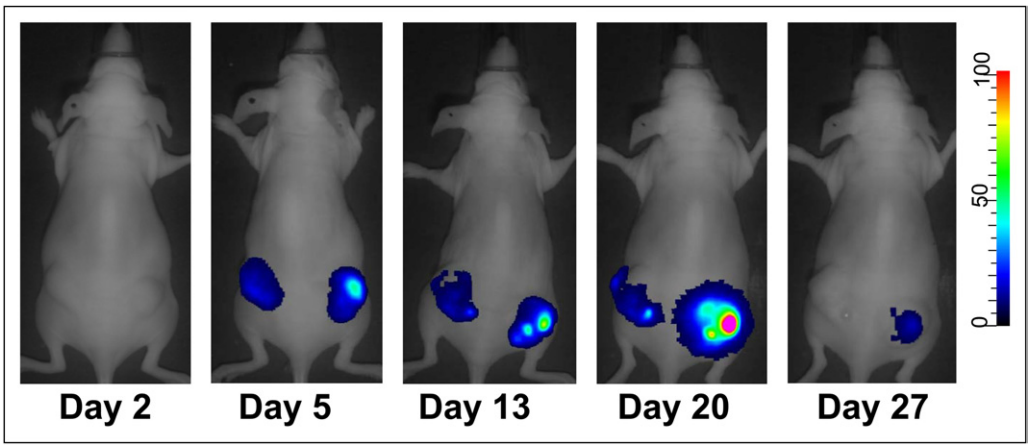

Figure 5 Systemically delivered CRAds replicate and visualize the tumor xenografts. Subcutaneous xenografts of A549 (Cox2-positive) were formed in nude mice followed by a single intravenous injection of 5/3Cox2CRAdΔE3ADP-Luc expressing luciferase in a replication-dependent manner. Tumor-associated bioluminescence was persistent for up to 30 days.

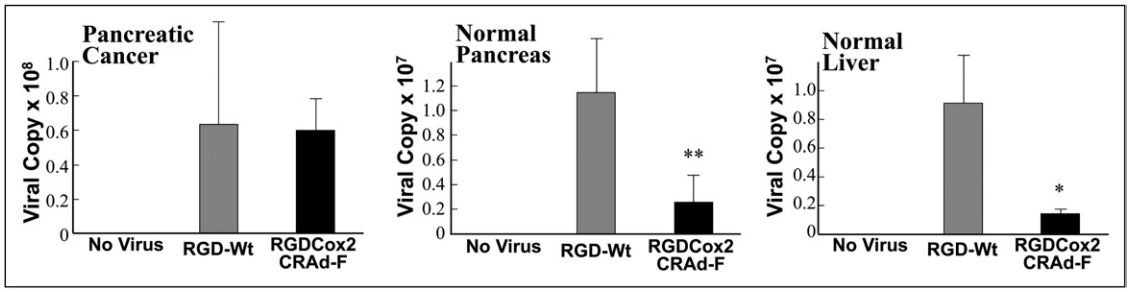

Figure 6 The targeted Cox2-controlled CRAd shows selective replication ex vivo in human PDAc specimens but not in normal tissues. Fresh resection specimens of pancreatic cancer, normal pancreas, and normal liver were thinly sliced and infected with RGDCox2CRAd-F or RGD-Wt. In pancreatic cancer samples, RGDCox2CRAd-F replicates as avidly as RGD-Wt, although in normal pancreas and liver minimal replication of RGDCox2CRAd-F is found, indicating the high level of cancer specificity of the Cox2 promoter. *$P < .05$, **$P < .005$.

in normal pancreas and normal liver. This indicates not only the suitability of Cox2 as a tumor-specific promoter for this virus ex vivo, but also the particular advantage of using Cox2 activity as a driver of viral replication to avoid replication toxicity in liver, the organ of greatest concern with adenovirus.

IFN expressed by Ad has a direct tumoricidal effect independent of viral replication

Next, we have generated RGD and 5/3 fiber infectivity-enhanced replication-deficient Ads expressing the human IFN protein (RGD-CMV-IFN and 5/3-CMV-IFN) and analyzed the cytotoxic effect of adenoviral-induced IFN expression in pancreatic cancer cell lines (Fig. 7). MiaPaca2 and HS766T cell lines were infected with IFN-expressing Ads at different titers (100, 1,000, and 10,000 vp/cell). As a control adenovirus, the identical replication-incompetent adenovirus expressing luciferase (RGD-CMV-Luc) has been used. In MiaPaCa2, increasing the viral titer from 100 to 10,000 resulted in a reduction in the number of living cells from 82% to 49% using RGD-CMV-IFN and from 75% to 27% using 5/3-CMV-IFN ($P < .05$ for both), and in HS766T cells, the same increase in titer resulted in a trend toward reduction of living cells from 87% to 68% using RGD-CMV-IFN and 66% to 35% using 5/3-CMV-IFN ($P < .05$). Results indicate a statistically significant increase in cell killing ability with increasing titers of virus and thus increasing amounts of virally derived IFN.

Increased oncolytic efficiency of IFN-expressing Cox2-controlled CRAds in vitro

Based on these results, to further increase the oncolytic potency of our infectivity-enhanced Cox2 CRAds, we generated oncolytic adenovirus which can massively express IFN from the viral E3 region, identical to Luc-expressing Ads tested earlier (Fig. 8). This adenovirus (5/3Cox2CRAdΔE3ADP-IFN) represents our most advanced CRAds and possesses multiple, integrated antitumor effects through fiber modification, ADP, and IFN overexpression. To analyze its improved cytocidal activity, we infected 5 different PDAc cell lines with a new IFN-expressing CRAd (5/3Cox2CRAdΔE3ADP-IFN) at low titers. We compared them with a nonreplicating IFN-producing virus (5/3-CMV-IFN) an IFN-producing virus without restriction on its replication (5/3WtΔE3ADP-IFN), a CRAd without IFN and ADP (5/3Cox2CRAd-F), and the gold standard adenovirus without cancer specificity and fiber modification, Ad-Wt. It is evident in all cell lines that 5/3Cox2CRAdΔE3ADP-IFN significantly outperforms Ad-Wt in cell killing ability ($P < .05$ for all comparisons at

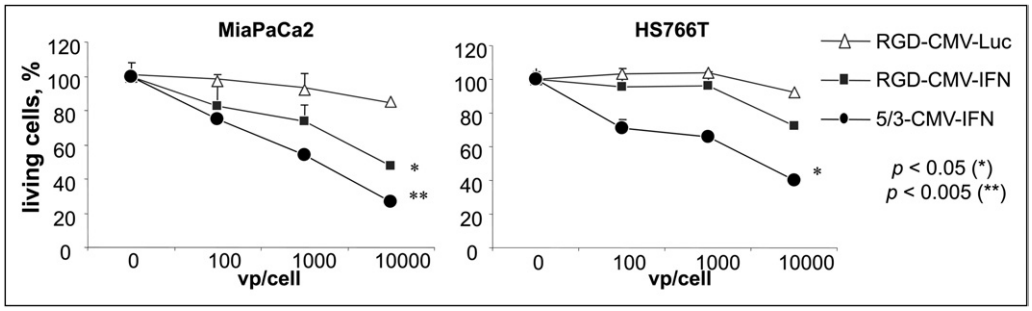

Figure 7 IFN expressed by adenovirus has a direct tumoricidal effect independent of viral replication. Cancer cell lines were infected with replication-incompetent RGD-CMV-IFN, 5/3-CMV-IFN, and an identical luciferase-expressing vector as control. Increasing IFN-alfa production from increasing viral titers leads to a dose-dependent cell killing effect.

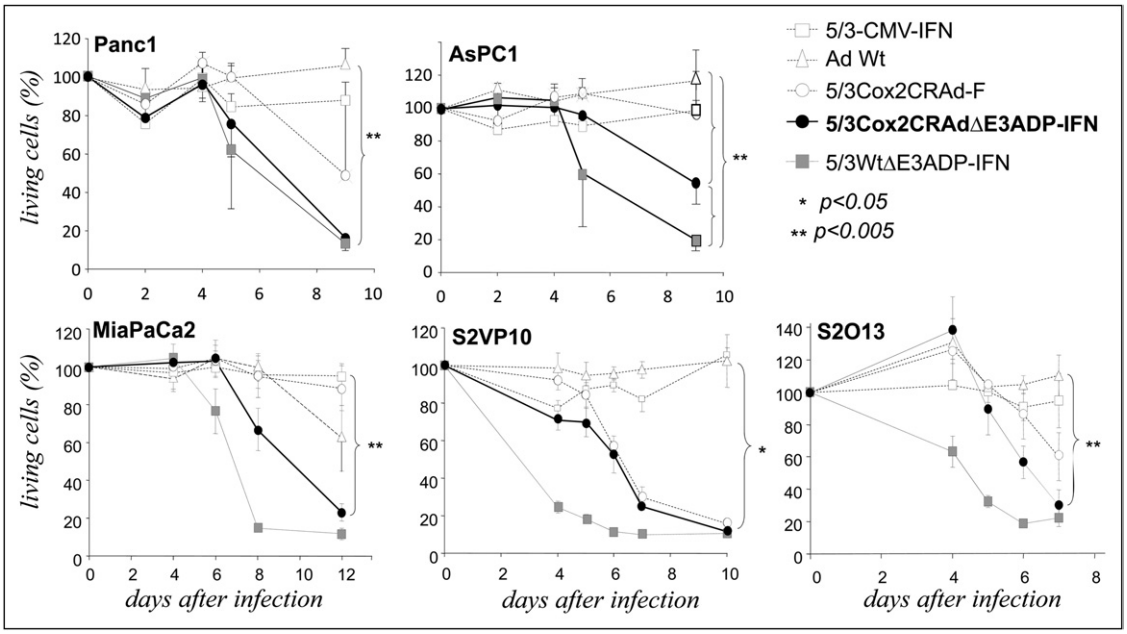

Figure 8 Increased oncolytic efficiency of IFN-expressing Cox2-controlled CRAds in vitro. Human PDAc cell lines Panc1, ASPC1, MiaPaCa2, S2VP10, and S2O13 were infected at day 0. Cell viability was determined with a colorimetric cell proliferation assay. The results are shown as the proportion of living cells remaining relative to uninfected cells. 5/3Cox2CRAdΔE3ADP-IFN shows significantly higher cell killing ability compared with Ad-Wt, the gold standard control virus, and is comparable in effect with 5/3WtΔE3ADP-IFN virus lacking cancer specificity.

the final time point). In addition, the tumor-selective IFN-expressing CRAd (5/3Cox2CRAdΔE3ADP-IFN) achieves the same cell killing ability as its powerful nonselective counterpart, 5/3WtΔE3ADP-IFN, in 3 cell lines (S2O13, S2VP10, and Panc1) and trends toward equivalence in the remaining 2 cell lines. When compared with 5/3Cox2CRAd-F, the effect of the E3 region modifications for ADP and IFN expression is evident because 5/3Cox2CRAdΔE3ADP-IFN lyses pancreatic cancer cells more rapidly and completely in 4 of 5 cell lines tested. Thus, 5/3Cox2CRAdΔE3ADP-IFN broadly displays an increased pancreatic cancer cell killing ability through fiber modification and ADP and IFN overexpression.

Comments

Pancreatic cancer remains a highly lethal disease, which is highlighted by the closely similar numbers of new diagnoses and annual deaths from this malignancy. In the minority of patients in whom resectability is possible,[2] patients still die after a median time of 2 years or less.[3] Thus, a new, highly active, nonsurgical means of treatment is needed, both to improve rates of resectability as well as to improve overall survival when used in the adjuvant setting.

Oncolytic adenovirus has been studied in the setting of pancreatic cancer. Several strategies have been used including CRAds based on frequent mutations or differences in the genetic profile of pancreatic cancer from normal cells, viruses expressing a suicide gene such as a metabolic enzyme to activate a chemotherapeutic prodrug, and cytokine-expressing adenoviruses.[24] A conditionally replicative adenovirus requiring p53-deficient target cells for replication has been described.[25,26] This virus was moved quickly to phase I and II studies where it showed a good safety profile, but disappointingly had short-lived replication and showed responses in the minority of patients.[5,27] Suicide gene therapy, as with an oncolytic Ad-expressing thymidine kinase to activate ganciclovir,[28] is a promising strategy; however, evidence has been conflicting regarding its efficacy. Recently, a different gene, the sodium-iodide symporter, which can be used to deliver radioactive iodine, has been reported as a therapeutic transgene expressed by a CRAd system against prostate cancer,[29] and this and similar strategies hold promise for different tumor types as well. Cytokine-expressing Ads, including interleukin-2, interleukin-12, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), tumor necrosis factor-α, as well as interferons, also have been reported.[30–33] However, although exciting in terms of efficacy in experimental systems and proof-of-concept demonstrations, these strategies generally suffer from problems such as poor adenoviral infectivity and intratumoral spread and lack of adenoviral selectivity for cancer, which would make human clinical use more feasible.[9] Clearly, advanced adenoviral vectors designed for tumor-selective replication to allow for systemic delivery as well as optimized for tumor killing ability with enhanced infectivity and virulence all must be satisfied simultaneously.

We report here an IFN-expressing CRAd that has been designed with these two critical requirements in mind: not only must it display strict selectivity for pancreatic cancer and avoid liver replication, but it also must incorporate advanced design features for enhanced potency. The most advanced virus described here, 5/3Cox2CRAdΔE3ADP-IFN, has been designed for superior cancer selectivity by Cox2-based promoter control, as well as being significantly more lethal to cancer with improved infectivity by fiber modification, ADP overexpression, and massive local IFN expression.

Because infection of cancer cells requires highly efficient binding and internalization, we and others[12,34,35] have modified viral tropism with genetic modification of the viral capsid. We used the 5/3 and RGD fiber modification to overcome profound CAR deficiency of pancreatic cancer cells, and confirmed that across multiple pancreatic cancer cell lines, these do in fact significantly improve infectivity.

Selectivity also is achieved in our design at the transcriptional level. Most adenoviruses ultimately will home to the liver and exert replication toxicity there. Therefore, to be clinically usable, liver replication must be avoided. Pancreatic cancer is known to be highly Cox2-positive,[35] whereas the normal tissues and liver, the organ of most concern, are not.[36] We tested the selectivity of our advanced virus 5/3Cox2CRAdΔE3ADP-IFN in several ways. In vitro, we showed a clear differentiation between the levels of replication in Cox2-positive cells compared with Cox2-negative cell types. Significantly, this also includes the normal human endothelial cell line HUVEC, in which replication of 5/3Cox2CRAdΔE3ADP-IFN was virtually absent. Next, we showed that Cox2 promoter control is feasible in vivo using subcutaneous xenografts of Cox2-positive pancreatic cancer cells as well as Cox2-negative tumors in nude mice. We observed both selectivity and persistence of infection, which was borne out both in direct intratumoral injection as well as with the most clinically relevant route, intravenous delivery. These observations of the selectivity for pancreatic cancer by Cox2 status were highlighted by the finding that our Cox2 CRAds also perform in a highly cancer-selective fashion when tested on actual clinical specimens of pancreatic cancer. We observed excellent replication of Cox2 CRAd in cancer samples along with minimal activity in both normal pancreas and liver. These findings underscore the promise of our strategy of selectivity and its clinical potential.

Even with high selectivity, a viral vector with low potency will not achieve its desired effects. We therefore sought to improve on the second critical issue in vector design: low efficacy. Our virus 5/3Cox2CRAdΔE3ADP-IFN uses 2 strategies here: ADP overexpression for enhanced apoptosis and spread, as well as massive IFN expression. This cytokine has both direct and indirect antitumor effects. Clinically, IFN-based chemoradiotherapy for pancreatic cancer is known to greatly improve outcomes.[15–17] Therefore, this is the cytokine of choice in our approach.

There have been a few attempts to study adenovirus-induced IFN expression. Aoki et al have made several important contributions in this area. It has been shown that IFN, expressed by a replication-incompetent Ad, can cause growth inhibition and regression of pancreatic tumors, and also can exert systemic effects in immunocompetent models.[19,37] Doronin et al[38] generated a CRAd with similar ADP-IFN expression and enhanced antitumor effect in a hepatocellular cancer xenograft model. We confirmed earlier observations of the therapeutic effect of IFN by using replication-deficient fiber-modified Ads to infect several pancreatic cancer cell lines, and found a dose-dependent increase in killing ability, which indicates the direct toxic effects of IFN expression. Taking this strategy further, we incorporated IFN expression into our most advanced conditionally replicating virus 5/3Cox2CRAdΔE3ADP-IFN, which also was fiber modified for increased infectivity, further enhanced by ADP overexpression, and cancer selective by Cox2 status. We also determined that this virus possesses excellent virulence toward pancreatic cancer, greatly outperforms the IFN-lacking counterparts and a wild-type control adenovirus, and achieves comparable effects in vitro to the IFN-expressing virus with unrestricted replication. This increased complexity of our most advanced virus allows it to satisfy the fundamental requirements of both high selectivity as well as optimal virulence.

We have shown a virus that builds on multiple previous achievements of both ourselves and others in the field and report a pancreatic cancer–specific, highly virulent, replication-competent, IFN-expressing CRAd. We are actively working to characterize this virus in vivo in immunocompetent animal models, and ultimately hope to see it deployed for the treatment of human patients. We believe this strategy to have great promise to expand the usability of IFN-alfa–based chemoradiotherapy for this grim disease.

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