Pancreatic Cancer Cell Radiation Survival and Prenyltransferase Inhibition: The Role of K-Ras

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Abstract

Activating K-ras mutations are found in ~90% of pancreatic carcinomas and may contribute to the poor prognosis of these tumors. Because radiotherapy is frequently used in pancreatic cancer treatment, we assessed the contribution of oncogenic K-ras signaling to pancreatic cancer radiosensitivity. Seven human pancreatic carcinoma lines with activated K-ras and two cell lines with wild-type ras were used to examine clonogenic cell survival after Ras inhibition. Ras inhibition was accomplished by small interfering RNA (siRNA) knockdown of K-ras expression and by blocking Ras processing using a panel of prenyltransferase inhibitors of differing specificity for the two prenyltransferases that modify K-Ras. K-ras knockdown by siRNA or inhibition of prenyltransferase activity resulted in radiation sensitization in vitro and in vivo in tumors with oncogenic K-ras mutations. Inhibition of farnesyltransferase alone was sufficient to radiosensitize most K-ras mutant tumors, although K-Ras prenylation was not blocked. These results show that inhibition of activated K-Ras can promote radiation killing of pancreatic carcinoma in a superadditive manner. The finding that farnesyltransferase inhibition alone radiosensitizes tumors with K-ras mutations implies that a farnesyltransferase inhibitor–sensitive protein other than K-Ras may contribute to survival in the context of mutant K-ras. Farnesyltransferase inhibitors could therefore be of use as sensitizers for pancreatic carcinoma radiotherapy.

Introduction

Pancreatic cancer is the fourth most common cause of cancer death in the United States, with a yearly incidence of ~30,700 cases (1). Surgery is the only potentially curative treatment option but only up to 30% of all patients are resectable. Even with complete surgical resection, only 12% to 15% of patients survive 5 years (2). Radiation plays an important role in the treatment of patients with nonmetastatic disease; combined with chemotherapy, radiation is the most effective treatment of locally advanced disease (reviewed in ref. 3). Chemoradiation is also given in adjuvant situations and is currently being tested in a neoadjuvant setting with promising preliminary results (reviewed in ref. 4).

Even with combined modality therapy, survival rates for pancreatic cancer patients are low compared with other malignancies and recurrence and metastasis after treatment remain high. The cause of resistance to standard therapies is unknown but may relate to the multiple molecular lesions found in these tumors. Pancreatic cancer develops through sequential progression from intraductal hyperplasia to invasive carcinoma (5). K-ras mutations occur very early (6) and are the most frequent mutations in pancreatic cancer followed by mutation or silencing of p53 (7), p16 (8), and DPC4/smad4 (9). Additional changes include abnormal sonic hedgehog gene expression, which was recently reported to be an early contributor to pancreatic cancer progression (10). Addressing the contribution of these lesions to tumor cell survival after treatment may point to new ways to improve therapy for this disease.

Ras activation is known to contribute to cancer cell survival in a number of different tumor types and experimental systems (11–15). For pancreatic cancer, K-ras mutations have been reported as a negative prognostic factor after surgery and adjuvant chemoradiation (16) or surgery alone (17). In addition to oncogenic mutations in K-ras, signaling through Ras proteins can be stimulated by epidermal growth factor receptor (EGFR) ligand overexpression and EGFR activation (18, 19), and HER-2/neu overexpression (20). Survival signaling by Ras after irradiation is transmitted by phosphatidylinositol 3-kinase (PI3K) and Akt in several tumor models (21–23). Therefore, the frequent activation of Akt family members in pancreatic cancer by upstream signals or through amplification of Akt itself (24, 25) could also promote survival after irradiation. Because signaling through the Ras pathways may be related to treatment resistance in general (11, 12, 25–28) and radiation resistance in particular (reviewed in ref. 29), inhibiting Ras activation is a potential strategy for tumor-specific radiosensitization in a large majority of pancreatic cancer patients.

Pharmacologic inhibition of Ras posttranslational processing using prenyltransferase inhibitors (FTI) is a well studied means to inhibit Ras signaling (reviewed in ref. 30). Ras protein function is dependent upon localization to the inner aspect of the plasma membrane. A critical step in Ras protein localization to the membrane is the addition of a 15-carbon farnesyl moiety by the enzyme farnesyltransferase (31). Compared with H-Ras or N-Ras, K-Ras is more resistant to inhibition by farnesyltransferase inhibitors (FTI) both because of its higher affinity for farnesyltransferase (32) and due to the potential for alternate prenylation by geranylgeranyltransferase-I (32, 33). Complete inhibition of the prenylation of K-Ras therefore requires a combination of FTIs and geranylgeranyltransferase inhibitors (GGTI; ref. 34). Nevertheless, FTI inhibition of tumor growth was obtained in pancreatic cancer cell lines with K-ras mutations (35, 36), but in many cases required higher doses of inhibitor
than cells with H-Ras activation (37). Thus, other targets for farnesyltransferase inhibitors may be important in the activity of these compounds when used as single agents against tumors (38, 39).

FTIs induce cell cycle arrest, which has been implicated in the antiproliferative effect of this class of compounds on tumor cells (40–42). However, the interaction between this effect and cytotoxic chemotherapeutic agents has been largely overlooked in clinical trials. Cell cycle arrest induced by FTIs could explain the lack of benefit observed in a phase III trial for pancreatic cancer patients that compared combined treatment with R115777 and gemcitabine to treatment with gemcitabine alone (43) because gemcitabine requires cell cycle progression to be effective (44). Superadditive effects of FTIs combined with other treatment modalities could however be expected when FTIs are combined with radiation, cisplatin, taxol, or epithelones to the extent that these are less dependent on cell cycle progression for activity (reviewed in ref. 30). In light of this possibility, we set out to test if combining radiation, which can kill cells in all phases of the cell cycle, with PTIs is effective in enhancing pancreatic carcinoma cell killing.

To examine this question, a panel of nine human pancreatic carcinoma cell lines including seven with K-ras mutations and two expressing wild-type ras were evaluated for response to radiation combined with PTIs. A panel of inhibitors with varying spectra of activity against K-Ras prenylation was assessed. All of the K-ras mutant cell lines were sensitized to radiation by combination inhibition of farnesyltransferase and geranylgeranyltransferase. Surprisingly, majority of K-ras mutant cell lines but not the two ras wild-type lines were sensitized by FTI treatment alone. Thus, survival signaling via Ras is a likely contributor to radiation survival in a majority of pancreatic tumors and a valid target for therapeutic intervention.

Materials and Methods

Cells. PSN-1 cells were obtained through a material transfer agreement with Merck & Co., Inc. (West Point, PA). PancM cells were derived from a patient with ductal adenocarcinoma on protocol for a phase I study of L-778,123 and radiotherapy (45) and have been described previously (46). All other cell lines were obtained from the American Type Culture Collection (Manassas, VA). Hs 766T, MiaPaCa-2, Panc-1, and PSN-1 cells were cultured in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), penicillin (100 units/mL), and streptomycin (100 mg/mL; Life Technologies). All other cell lines were cultured in high glucose (4.5 g/L) RPMI supplemented with 1 mL/mL sodium pyruvate (Life Technologies) and also with fetal bovine serum, penicillin, and streptomycin as above. All cultures were maintained at 37°C in water saturated 5% CO2/95% air. Cells were regularly tested by PCR to insure absence of Mycoplasma containing an equal concentration of DMSO.

Inhibitors. FTI-2, GGTI-2, and L-778,123 were obtained from Merck Pharmaceuticals (West Point, PA). R115777 was obtained from Janssen Research (Johnson & Johnson, Springhouse, PA). All inhibitors were dissolved as concentrated stock solutions in DMSO and diluted at the time of treatment with medium. Control cells were treated with medium containing an equal concentration of DMSO.

Treatment of cells before clonogenic cell survival assays. For experiments with small interfering RNA (siRNA), cells were seeded at 25% confluence and cultured overnight before transfection with 0.6 pmol of the indicated siRNA duplex (Dharmacon RNA Technologies, Lafayette, CO) using oligofectamine (Life Technologies). Cultures were replated as single cells 24 hours after transfection to assure log-phase growth and to facilitate harvest of single cells for survival plating. The mutant K-ras target sequence 5′-CUUGUGUAUGUGAGCUUG-3′ was used for experiments with Panc-1 and ASPC-1, which are homozygous for the mutation encoded on this siRNA. The wild-type K-ras target sequence 5′-AAGCAAGUGUAUAUGUGUG-3′ that targets both wild-type and oncogenic K-ras alleles was used for experiments with BxPC-3, Hs766T, Capan-2, MiaPaCa-2, and PSN-1. Nonspecific siRNA oligo from Dharmacon RNA Technologies (nonspecific II) was used in experiments with BxPC-3, Hs766T, PancM, and ASPC-1 using the MixA family (47) as a control for transfection and nonspecific effects of siRNA.

For experiments with PTIs, the indicated inhibitor was added to mid-log phase cell cultures at the indicated concentrations 24 hours before plating for survival/irradiation and continued for 24 hours after irradiation. At that time, the medium was replaced with drug-free medium. Control cultures were also refed to control for this manipulation.

Clonogenic cell survival following irradiation. Cells were treated as described above and plated as single cells before irradiation. Conditioned medium (20%) was used in clonogenic survival measurements of AsPC-1, Capan-1, Capan-2, Hs 766T, and PancM. Cells were irradiated with a Mark 1 cesium irradiator (J.L. Shepherd, San Fernando, CA) at a dose rate of 1.7 Gy/min. Colonies were stained and counted 10 to 30 days after irradiation. The surviving fraction was calculated as follows: number of colonies formed / number of cells plated (irradiated) × plating efficiency (unirradiated). Each point on the survival curve represents the mean surviving fraction from at least three dishes.

Tumor generation and clonogenic cell survival in nude mice. Pathogen-free female Ncr nu/nu mice were obtained from Charles River Labs (Wilmington, MA) and were housed aseptically in the animal facilities of the University Laboratory Animal Resources and the Institute for Human Gene Therapy of the University of Pennsylvania. All experiments were carried out in accordance with University Institutional Animal Care and Use Committee guidelines. At 5 to 7 weeks of age, mice were inoculated by s.c. injection into the hind flank with 2.5 × 105 MiaPaCa-2 or PSN-1 cells suspended in 50 μL of matrigel (BD Collaborative Research, Franklin Lakes, NJ). Animals were assigned randomly to treatment groups when tumors attained a volume of 300 to 400 mm3. For L-778,123 treatment, drug was given by continuous micro-osmotic infusion (Alza Corp., Palo Alto, CA) at a dose rate of 80 mg/kg/d × 72 hours before irradiation. Animals then underwent a secondary randomization to receive either 6 Gy using a Mark 1 cesium irradiator (J.L. Shepherd) at a dose rate of 1.7 Gy/min or sham irradiation under anesthesia. Within 1 hour after irradiation, animals were sacrificed by cervical dislocation and tumors were excised, minced, and dissociated for 30 minutes at 37°C in HBSS containing 166 units/mL collagenase XI, 0.25 mg/mL protease, and 255 units/mL DNase. Cells were recovered by staining through an 80-mm mesh and centrifuging at 500 × g and were resuspended in culture medium. Cells were counted by hemocytometer using trypan blue to determine viability, and the counts were verified by Coulter counter analysis. A known number of tumor cells were then plated in 100-mm dishes and cultured for 14 to 21 days, after which colonies were stained and tumor colonies counted. Results are expressed as the plating efficiency determined from replicate dishes plated at multiple initial cell densities.

Whole cell lysate preparation. Cells were lysed on culture dishes with reducing Laemmli sample buffer after rinsing once with PBS. Samples were boiled, sheared, clarified by centrifugation at 14,000 rpm and stored at −20°C. For preparation of total cell tumor lysates, a sample of tumor was flash frozen using dry ice/ethanol and pulverized using a dry ice–cooled mortar and pestle. Pulverized tumor fragments were then resuspended in Laemmli sample buffer and solubilized by sonication and boiling. Protein concentration in lysates was determined by amido black staining (47).

Ras activation assays. Cells were washed once in ice-cold HBS [25 mMm/L HEPES (pH 7.5) and 150 mM/L NaCl] and lysed in ice-cold Mg-containing lysis buffer [MLB: 25 mMm/L HEPES (pH 7.5), 150 mM/L NaCl, 1% NP40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM/L NaF, 10 mM/L MgCl2, 1 mM/L EDTA, 1 mM/L sodium vanadate, 10 μg/mL leupeptin, and 10 μg/mL apropin] for 20 minutes at 4°C. Debris was removed by centrifugation at 15,000 × g for 20 minutes. Protein...
concentration of the supernatants was measured using the Bio-Rad Protein Assay Kit. Equal amounts of protein were used as indicated. Lysates were rocked with 15 μL of packed GST-RBD-agarose beads (Upstate Biotechnology, Lake Placid, NY) at 4°C for 30 minutes. Beads were washed thrice in 0.6 mL MLB and resuspended in SDS-PAGE sample buffer for Western blot analysis.

**SDS-PAGE and Western blot analysis.** Equal amounts of protein were separated on Criterion prestac gel and blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked in PBS containing 0.1% Tween 20 and 5% powdered milk before the addition of primary antibody. H-Ras antibody (Oncogene Research Products, La Jolla, CA) was used at a dilution of 1:5,000; K-Ras antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 1:100 and β-actin clone AC-15 (Sigma, St. Louis, MO) was used at 1:5,000 dilution. Antibody binding was detected using the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). Images were digitized using an AGFA Arcus II scanner, and figures were assembled using Adobe Photoshop and Microsoft PowerPoint.

**Results**

We have previously shown that deletion of the oncogenic K-ras allele from DLD-1 human colon carcinoma cells leads to decreased clonogenic cell survival following irradiation, indicating that mutant K-Ras signaling decreases cellular radiosensitivity (13). This is in agreement with previous studies in NIH3T3 murine fibroblasts and in rat intestinal epithelial cells in which transfection with mutant K-ras resulted in a decrease in cellular radiosensitivity (14, 22). Given that over 90% of human pancreatic carcinomas express mutant K-ras, we hypothesized that mutant K-ras expression might contribute to the low response rate, high local relapse rate, and poor clinical outcome observed in patients with pancreatic carcinoma treated with definitive radiotherapy. To test this hypothesis, the role of K-Ras signaling in determining radiosensitivity was examined in a panel of nine human pancreatic carcinoma cell lines, including seven cell lines that express mutant K-ras mutations and two cell lines that express wild-type ras. The cells in this panel have been extensively characterized and are known to contain abnormalities in genes other than K-ras including p53, p16, Akt, and Smd4 (Table 1).

To determine whether K-Ras signaling contributes to radiation resistance in pancreatic carcinoma cells, siRNA duplexes were designed to specifically inhibit K-ras expression. In these experiments, seven of the nine cells in the panel were successfully transfected with either K-ras-specific siRNA or nonspecific control siRNA duplexes 48 hours before irradiation as described in Materials and Methods. Knockdown of K-Ras by siRNA was maximal at the time of irradiation as shown in PSN-1 cells (Fig. 1A). To examine K-Ras influence on radiosensitivity, an siRNA targeting both oncogenic and wild-type forms of K-Ras was used. MiaPaCa-2 and PSN-1 cell lines are homozygous for an identical oncogenic mutation in K-ras. Knockdown of this mutant K-Ras with an siRNA targeted to a sequence common to wild-type and mutant K-ras led to increased radiosensitivity (Fig. 1B). Similar findings were obtained using siRNA specific for mutant K-ras alleles. AsPC-1 and Panc-1 cells were treated with siRNA specific for the mutations in these cells and knockdown of expression using this siRNA (Fig. 1C) also caused radiosensitization (Fig. 1D). K-Ras expression was also targeted in BxPC-3 and Hs766T cells with low level expression of wild-type K-Ras. Treatment with siRNA that targets all K-Ras had no effect on survival of BxPC-3 (Fig. 1A). The radiation survival of Hs766T was also not affected (Fig. 1D). PancM and Capan-1 cells showed too much toxicity in response to oligofectamine transfection to be evaluated for radiosensitization with siRNA.

Whereas these results indicate that mutant K-Ras signaling promotes increased radiation survival in pancreatic carcinoma cells, siRNA approaches are not currently practical for use in patient therapy. In contrast, PTIs represent a pharmacologic approach to inhibiting Ras and are currently in clinical trials (30). Therefore, we examined the ability of PTI to inhibit the posttranslational processing and activation of K-Ras and modulate radiosensitivity of pancreatic carcinoma cells. Because K-Ras can undergo prenylation by two different enzymes, farnesyltransferase and geranylgeranyltransferase, we began by testing the effect of the dual specificity (FTI + GGTI) L-778,123 on pancreatic carcinoma cell radiosensitivity. MiaPaCa-2, PSN-1, and BxPC-3 cells were pretreated for 24 hours with either DMSO (control) or L-778,123 (5 μmol/L) and clonogenic cell survival following irradiation was determined as described in Materials and Methods. In this report, radiosensitization was considered established only when the combination of inhibitor + radiation resulted in supra-additive effects on clonogenic cell survival. Treatment-mediated radiosensitization was defined as a ratio of control to inhibitor-treated clonogenic survival of >1.1. Radiosensitization was thus established independently of cell growth inhibition. Pretreatment with L-778,123 led to an increase in radiosensitivity in the mutant K-Ras expressing MiaPaCa-2 and PSN-1 cell lines but not the wild-type K-ras expressing BxPC-3 cell line (Fig. 2A). This radiosensitization effect correlated with the inhibition of K-Ras processing (Fig. 2B and C). In addition, affinity chromatography using GST-Raf1max [a fusion protein between glutathione S-transferase (GST) and the Ras binding domain of Raf] showed that inhibition of K-Ras processing correlated with inhibition of K-Ras activation because the amount of K-Ras bound to GST-Raf1max which is an indicator of GTP-bound Ras was reduced (Fig. 2C). We also observed in certain cells that overall K-Ras expression was increased by PTI treatment (Fig. 2). We hypothesize that the increase in Ras expression seen in certain cell lines in this and previous reports (48, 49) after inhibition of prenylation in vitro may be a response by cells dependent upon

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Abbreviations: wt, wild-type; mt, mutant; nd, not determined; ampl, amplified/overexpress.

*Bub-1 mutant.

*BRCA-2 mutant.

*Derived in our lab (46).
Ras signaling to the inhibition of Ras activity. Tests of other pancreatic carcinoma cell lines showed that L-778,123 promotes radiosensitization only in cell lines that express mutant K-ras (Fig. 2D). The specificity of L-778,123 radiosensitization is thus consistent with the siRNA knockdown studies showing that inhibition of oncogenic K-Ras signaling leads to radiosensitization in pancreatic carcinoma cells. It should be noted that treatment of all pancreatic cell lines with PTI resulted in decreased plating efficiency, indicating that these compounds have direct effects on cell growth and survival that are not correlated with mutant K-ras expression.

To delineate more fully the relative contributions of FTI and GGTI activities to radiosensitization of pancreatic carcinoma cells, we first used R115777, a compound that is known to be a highly selective inhibitor of farnesyltransferase over geranylgeranyltransferase. In these experiments, MiaPaCa-2, PSN-1, and BxPC-3 cells were pretreated with an siRNA that recognizes both mutant and wild-type (wt) K-ras. Plating efficiencies (PE) under each treatment condition for unirradiated cells were MiaPaCa-2: Control siRNA (PE = 0.37), K-ras siRNA (PE = 0.25); PSN-1: Control siRNA (PE = 0.55), K-ras siRNA (PE = 0.41); BxPC-3: Control siRNA (PE = 0.19), K-ras siRNA (PE = 0.15). C. Knockdown of mutant K-Ras expression in AsPC-1 and Panc-1 cells using siRNA specific to the mutant sequence. D. Summary of the results from clonogenic cell survival assays comparing survival after transfection of pancreatic carcinoma cells with nonspecific siRNA to survival after transfection with K-ras-specific siRNA. Radiosensitization was scored positive (+) if the survival ratio for control/K-ras siRNA exceeded 1.1 at both 2 Gy and at 10% clonogenic cell survival.

Figure 1. Mutant K-ras knockdown radiosensitizes pancreatic carcinoma cells. Pancreatic carcinoma cell lines were transfected with either nonspecific (control) or K-ras-specific siRNA as described in Materials and Methods. After 48 hours (day 2), cellular sensitivity to ionizing radiation was measured using clonogenic cell survival assays, and knockdown of K-Ras was evaluated using western immunoblotting. A, time course of K-ras expression knockdown by siRNA in PSN-1 cells. B, clonogenic cell survival assays for MiaPaCa-2, PSN-1, and BxPC-3 cells treated with an siRNA that recognizes both mutant and wild-type (wt) K-Ras. Plating efficiencies (PE) under each treatment condition for unirradiated cells were MiaPaCa-2: Control siRNA (PE = 0.37), K-ras siRNA (PE = 0.25); PSN-1: Control siRNA (PE = 0.55), K-ras siRNA (PE = 0.41); BxPC-3: Control siRNA (PE = 0.19), K-ras siRNA (PE = 0.15). C. Knockdown of mutant K-Ras expression in AsPC-1 and Panc-1 cells using siRNA specific to the mutant sequence. D. Summary of the results from clonogenic cell survival assays comparing survival after transfection of pancreatic carcinoma cells with nonspecific siRNA to survival after transfection with K-ras-specific siRNA. Radiosensitization was scored positive (+) if the survival ratio for control/K-ras siRNA exceeded 1.1 at both 2 Gy and at 10% clonogenic cell survival.

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To determine whether PTI treatment might radiosensitize pancreatic carcinoma cells in vivo, MiaPaCa-2 and PSN-1 tumors were established in the flanks of nude mice and tested for L-778,123-mediated radiosensitization of tumor clonogens. Tumor-bearing animals were randomly assigned to receive either L-778,123 (80 mg/kg/d × 3 days) or vehicle treatment by continuous infusion. Animals in each drug treatment group underwent a secondary randomization to either 6 Gy or sham irradiation and tumor viability was assessed by clonogenic cell survival within 1 hour of irradiation. These experiments show that L-778,123 + 6 Gy resulted in a 6- to 8-fold decrease in clonogenic cell survival compared with either L-778,123 or vehicle alone and a 2- to 3-fold decrease in clonogenic cell survival compared with radiation alone (Fig. 4A).

The effect on survival was seen although no change in K-Ras processing could be detected (Fig. 4B). Activity of the inhibitor on farnesylation of H-Ras was however documented in these tumor samples. Taken together, these results suggest that PTI might represent an effective radiosensitizer for pancreatic carcinomas. However, the ability of FTI alone to radiosensitize pancreatic carcinoma cells, combined with in vivo radiosensitization by PTI in the absence of detectable change in K-Ras processing status suggests that mutant K-Ras is not the only important target for PTI-mediated radiosensitization in pancreatic carcinomas.

Discussion

We and others have shown that transfection of rodent fibroblast cell lines with mutant H-Ras, N-Ras, and K-Ras results in decreased radiosensitivity (14, 50–53) and that, conversely, loss of mutant K-ras alleles from DLD-1 human colon carcinoma or N-ras alleles from human HT1080 sarcoma cells leads to increased radiosensitivity (13). However, other studies have shown that transfection of Rat2
cells with oncogenic K-ras leads to increased radiosensitivity (54), indicating that the effect of mutant K-Ras signaling on cellular radiosensitivity might depend on the cell type in which it is expressed.

In this study, we have investigated the role of K-Ras signaling in the radiosensitivity of pancreatic carcinomas. In agreement with our previous work, selective knockdown of K-ras expression in pancreatic carcinoma cells led to radiosensitization only in cell lines that express mutant K-ras. Because >90% of pancreatic carcinomas express mutant K-ras, and the surrounding normal tissues should not express mutant K-ras, these results validate K-Ras as a potential molecular target for increasing therapeutic

Figure 3. FTI radiosensitizes pancreatic carcinoma cell lines that express mutant K-Ras. Pancreatic carcinoma cell lines were treated with either DMSO (control) or the indicated PTI as described in Materials and Methods. After 24 hours, cellular sensitivity to ionizing radiation was measured using clonogenic cell survival assays. Ras processing was evaluated in total cell lysates using Western immunoblotting. A, clonogenic cell survival assays for MiaPaCa-2, PSN-1, and BxPC-3 cells after treatment with 2.5 μmol/L R115777. Plating efficiency (PE) of control and R115777-treated cells. B, Western blot showing that R115777 does not inhibit K-Ras processing in MiaPaCa-2, PSN-1, or BxPC-3 cells. C, summary of the results from clonogenic cell survival assays after control versus 2.5 μmol/L R115777 treatment. Radiosensitization (+) was considered established if survival ratio for control/PTI-treated cells exceeded 1.1 at both 2 Gy and at 10% clonogenic cell survival. D, summary of results from clonogenic cell survival assays after FTI-2 (0.3 μmol/L in PSN-1 and Capan-1; 0.6 μmol/L in BxPC-3, Hs766-T, MiaPaCa-2, and PancM; 1.2 μmol/L in Capan-2; 2.5 μmol/L in Panc-1; 5 μmol/L in ASPC-1) + GGTI-2 (0.06 μmol/L in BxPC-3, Hs766-T, Capan-1, and MiaPaCa-2; 0.12 μmol/L in ASPC-1, Capan-2, PancM, and PSN-1). *nd, not determined due to toxicity of inhibitor treatment.
index in patients receiving radiotherapy for pancreatic carcinoma. We have therefore investigated the specificity and spectrum of activity of PTI as pancreatic carcinoma radiosensitizers. It should be noted that these experiments in no way constitute a head-to-head comparison of these compounds, as dose effect was not an end point, but rather make use of the differing spectra of activity of these inhibitors to probe for the relevant target(s) of prenyltransferase inhibition and in addition rule out the possibility of compound-specific effects on radiosensitivity. Using inhibitors that block both farnesyltransferase and geranylgeranyltransferase (L-778,123), or combining inhibitors that block one (FTI-2, R115777) or the other (GGTI-2) enzyme, we established that inhibition of both farnesyltransferase and geranylgeranyltransferase together led to radiosensitization whereas inhibition of geranylgeranylation alone did not. However, we also found that inhibition of farnesylatation with inhibitors having specificity for farnesyltransferase alone at the doses used here (FTI-2, R115777) was sufficient to radiosensitize a majority of pancreatic tumor cells harboring K-ras mutations. This occurred at inhibitor concentrations where H-Ras farnesylation was blocked but in the absence of detectable inhibition of overall K-Ras processing. Given the unexpected result that FTI treatment was sufficient for radiosensitization of cells expressing mutant K-Ras, this raises the question of the mechanism for the observed effect, because K-Ras is resistant to inhibition by farnesyltransferase inhibitors alone.

Our results contrast with the lack of radiosensitization in a panel of cells including MiaPaCa-2 reported by Mégnin-Chanet et al. using the RPR-130401 farnesyltransferase inhibitor (55). This could result from a difference in the activity of this inhibitor but is more likely to be due to methodologic differences. In that report, verification for inhibition of Ras processing was not carried out; therefore, it is unclear whether significant PTI inhibition was achieved under the conditions tested by these authors. Moreover, the duration of drug exposure before in vitro irradiation was 72 hours. We have observed that the cytotoxic effects of prolonged FTI exposure in vitro can make clonogenic cell survival curves difficult to interpret. Of note, at all radiation doses tested, RPR-130401 induced greater reductions of MiaPaCa-2 clonogenic cell survival than control treatment.

There is an increasing body of evidence that the Ras to PI3K to Akt pathway plays an important role in pancreatic cancer (25, 28, 56–59). High expression of Akt in pancreatic cancer was found to be associated with protection against apoptosis (25, 57). Conversely, inhibition of PI3K by wortmannin and LY294002 reduced phosphorylation of Akt (28), slowed tumor growth in vivo without induction of apoptosis (58), and also slowed growth in mouse models (28). A recent study using adenoviral transduction of dominant-negative Ras, PI3K p85β, or Akt into Panc-1 and HPAF pancreatic cancer lines showed that blockage at these points and at Ras itself suppressed both in vitro and in vivo tumor growth and enhanced apoptosis (59). In separate series of studies, we have defined the contribution of PI3K and Akt to radiation survival in cells with H- or K-Ras activation (60), and this pathway seems to contribute to survival in pancreatic cancer cells as well.3

Although our observations of radiosensitization are somewhat analogous to the findings that PTIs block tumor cell growth irrespective of the ras mutation status of the cells (35, 37), they differ in important ways. First, radiosensitization seems independent of tumor cell proliferation changes induced by these inhibitors. Second, although K-Ras prenylation was not blocked, the radiosensitization observed was nonetheless linked to the presence of a K-ras mutation. One possibility would be that whereas mutant K-Ras signaling leads to increased radioresistance, the target of PTI radiosensitization in pancreatic carcinoma cells is unrelated to this K-Ras effect. In this case, other potential targets could include farnesylated proteins that have been implicated in the inhibition of tumor cell growth by PTIs including RhoB, Pxf, CENP-F, and PRL proteins (42, 61–65). Prior studies have not, however, established a role for these proteins in enhancing Ras-mediated radiation survival, although RhoB may affect radiosensitivity in some settings (66, 67). Another possible

3 Unpublished observations.

Figure 4. MiaPaCa-2 or PSN-1 pancreatic tumor clonogens surviving irradiation in situ after L-778,123 treatment of tumor-bearing mice. MiaPaCa-2 or PSN-1 tumor xenografts were established in the flanks of nude mice. Either 80 mg/kg/d L778,123 or vehicle was given for 72 hours before irradiation. A, clonogenic cell survival assays were used to determine tumor viability after irradiation with 6 Gy. B, K-Ras and H-Ras processing in PSN-1 tumors was determined by Western immunoblots of total tumor cell lysates. Three replicate tumors are shown for each drug treatment condition.
mechanism stems from the observation that pancreatic carcinomas, including the MiaPaCa-2 and Panc-1 cell lines, seem to contain an autocrine transforming growth factor α (TGF-α)/EGFR signaling loop (68, 69) and that transfection of rat intestinal epithelial cells with mutant K-Ras leads to H-Ras activation through autocrine TGF-α/EGFR signaling (70). These observations raise the intriguing possibility that FTI are able to radiosensitize pancreatic carcinoma cells by inhibiting H-Ras farnesylation/activity in the context of TGF-α/EGFR autocrine signaling promoted by mutant K-Ras. This possibility is currently under investigation.

In summary, these data show that mutant K-Ras signaling contributes to radiation resistance in pancreatic carcinoma cells. Moreover, FTI radiosensitize pancreatic carcinoma cells that express mutant K-Ras both in vitro and in vivo. These observations suggest that combination PTI + radiotherapy might prove to be more effective than radiation alone in treating patients with carcinoma of the pancreas.

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