iNOS Regulates the Therapeutic Response of Pancreatic Cancer Cells to Radiotherapy

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is highly resistant to radiotherapy, chemotherapy, or a combination of these modalities, and surgery remains the only curative intervention for localized disease. Although cancer-associated fibroblasts (CAF) are abundant in PDAC tumors, the effects of radiotherapy on CAFs and the response of PDAC cells to radiotherapy are unknown. Using patient samples and orthotopic PDAC biological models, we showed that radiotherapy increased inducible nitric oxide synthase (iNOS) in the tumor tissues. Mechanistic in vitro studies showed that, although undetectable in radiotherapy-activated tumor cells, iNOS expression and nitric oxide (NO) secretion were significantly increased in CAFs secretome following radiotherapy. Culture of PDAC cells with conditioned media from radiotherapy-activated CAFs increased iNOS/NO signaling in tumor cells through NF-κB, which, in turn, elevated the release of inflammatory cytokines by the tumor cells. Increased NO after radiotherapy in PDAC contributed to an acidic microenvironment that was detectable using the radiolabeled pH (low) insertion peptide (pHLIP). In murine orthotopic PDAC models, pancreatic tumor growth was delayed when iNOS inhibition was combined with radiotherapy. These data show the important role that iNOS/NO signaling plays in the effectiveness of radiotherapy to treat PDAC tumors.

Significance: A radiolabeled pH-targeted peptide can be used as a PET imaging tool to assess therapy response within PDAC and blocking iNOS/NO signaling may improve radiotherapy outcomes.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) accounts for 90% of all the pancreatic cancer cases and it remains one of the most lethal malignancies due to limited treatment options and resistance to therapy (1). Notably, limited improvements in overall survival have been achieved for PDAC in comparison with other tumor types, in part, because more than half of PDAC cases are diagnosed at an advanced and metastatic stage of the disease (1). Early detection of PDAC remains a challenge due to nonspecific presenting symptoms, difficulty of imaging early-stage tumors, and lack of tumor biomarkers with both good specificity and sensitivity. The poor prognosis for PDAC can be attributed to the rapid metastasis and resistance to conventional therapeutic approaches (chemotherapy, radiotherapy, and molecular targeted therapy). Clearly, a need exists to identify new therapeutic strategies, including effective combinations of existing therapies to expand treatment options for PDAC.

Emerging therapeutic approaches for PDAC include immunotherapies, molecular targeted therapies, and strategies targeting the tumor microenvironment (2).

While pancreaticoduodenectomy (Whipple procedure) or distal pancreatectomy is performed in patients with resectable PDAC, fewer than 20% of all patients fall into these categories, and only cures about 10% of this subset (3). Patients with unresectable PDAC are treated with chemotherapy or chemotherapy/radiation combination therapies (4). Technological improvements in radiotherapy (RT) approaches, such as intensity-modulated radiotherapy, and image-guided radiotherapy, have dramatically improved the conformity of the radiation dose deposition in the tumor area while increasingly sparing healthy tissues (5).

Radiotherapy induces alterations in the secretory profile of tumor microenvironment (TME) that results in changes in tumor invasion, tumor growth, inflammatory mediators, and regulators of angiogenesis (6–8). Noncancer cells including immune cells, fibroblasts, pericytes, and endothelial cells contribute to the stromal compartment of the TME, and, in PDAC, outnumbers the cancer cells (2, 9). The TME of PDAC is highly inflammatory (10); radiotherapy alters the inflammatory TME by contributing to a fibroinflammatory desmoplasia (11) and to an increase in the expression of TNFα (12). The proinflammatory cytokines IFNα, IFNβ, and IFNγ are further induced in radiotherapy-treated cells (13, 14). Cancer-associated fibroblasts (CAF) expressing both vimentin and α-smooth muscle actin (αSMA) are the predominant cellular components of the PDAC stroma (2). Previous studies have demonstrated that irradiated pancreatic CAFs...
exhibit enhanced tumor–stroma interactions, which impact tumor growth and invasion (7, 15). Pancreatic CAFs survive severe stress, including damage induced by radiotherapy single-fraction doses up to 20 Gy (16).

The expression of inducible nitric oxide synthase (iNOS, NOS2) in tumor cells is associated with poor survival in several cancers (17–20). The iNOS is transcriptionally regulated and induced by inflammatory cytokines (21, 22), contributing to the production of nitric oxide (NO) through conversion of l-arginine into citrulline in the presence of NADPH and oxygen. An increase in NO results in an intrinsic prooxidant environment (22), immune escape (23), growth and invasion (7, 15). Pancreatic tumors exhibit higher expression of iNOS compared with nontumor pancreatic tissue (28). Pancreatic orthotopic implantation of tumor cells expressing low levels of iNOS result in the formation of pancreatic tumors with metastasis in the liver and formation of ascites—an effect that is not observed in tumor models developed by orthotopic implantation of PDAC cells containing high levels of iNOS (21). In addition to or independent of iNOS expression in tumor cells, upregulation of iNOS has been detected in stromal fibroblasts and immune cells (22). Mechanistic studies have demonstrated that in PDAC, CAFs express high amounts of iNOS contributing to the development of tumor chemoresistance via increased NO secretion (29). Inflammatory cytokines induce NO production by CAFs (29), contributing to PDAC chemoresistance (30) and immunosuppression (31).

Not only does NO contribute to tumor growth and metastasis (21, 26, 32, 33), but mechanistic metabolic studies demonstrated a NO-mediated decrease in mitochondrial respiration, which led cancer cells to undergo higher glycolytic rates to maintain ATP production levels (34). These results have further supported the role of NO in cancer metabolism by demonstrating that NO regulates the Warburg effect and promotes cancer growth by inhibiting mitochondrial respiration.

We hypothesize that radiotherapy induces alterations in the inflammatory TME contributing to an increase in PDAC iNOS expression and NO secretion that results in an increase in tumor growth. In this study, we show that conditioned media (CM) from radiotherapy-activated CAFs (RT-CAF) increase NO secretion/iNOS expression by the tumor cells that contributes to PDAC tumor growth, and acidification of the PDAC microenvironment, which can be detected using the radiolabeled pH (low) insertion peptide (pHLIP). Importantly, we demonstrate that this phenomenon can be mitigated with iNOS inhibition in vitro and in vivo, providing a therapeutic option for overcoming this protumorigenic phenotype.

Materials and Methods

Pancreatic cancer cell lines and patient samples

SUIT-2, CAPAN-2, MIA PaCa-2, and BxPC-3 human PDACs were purchased from ATCC and were grown according to standard procedures. The FC1245 murine PDACs were obtained from the David Tuvenson’s group and were transformed in Michel Sadelain’s laboratory to generate FC1245lac− (35). FC1245 and FC1245lac− cells were cultured in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. Cell lines used in this work were purchased in 2016–2019, and they were used within passage number of 15. All the cell lines were Mycoplasma free and authenticated at Memorial Sloan-Kettering Cancer Center (MSKCC, New York, NY) integrated genomics operation core using short tandem repeat analysis.

Deidentified patient samples (nontumor and tumor pancreatic tissues) were obtained before and after radiotherapy from David M. Rubenstein Center for Pancreatic Cancer Research following Institutional Review Board approval. Radiotherapy doses, radiotherapy time-points, and sample collection time-points are described in Supplementary Table S1.

Murine orthotopic PDAC xenograft model

All experiments involving animals were performed according to the guidelines approved by the Research Animal Resource Center and Institutional Animal Care and Use Committee at MSKCC. PDAC orthotopic models were developed as described previously (36) and additional details are given in Supplementary Information.

Contrast injection and CT for pancreatic tumor delineation

Pancreatic tumors were visualized using an image-guided small-animal micro-irradiator (XRad225Cx, Precision X-Ray) following a protocol previously described by Thorek and colleagues (37). Mice were injected with a 3 mL solution of 75 mg iodine/mL (iohexol; Omnipaque, GE Healthcare) at 5 minutes prior to imaging. Data visualization was performed as described previously (37) and tumor volumes were determined using Amira region-grow tool.

Image-guided X-ray radiotherapy of pancreatic tumors

Immediately after visualization of the pancreatic tumor by X-ray CT using a solution of 15 mg iodine/mL, radiation arc therapy was performed in mice bearing orthotopic pancreatic tumors (37). See Supplementary Information for details.

Isolation and expansion of murine and human pancreatic fibroblasts

Fibroblasts were isolated from orthotopic FC1245 PDAC models or human PDAC using methodology described previously by Müerköster and colleagues (29). Further details are described in Supplementary Information.

Conditioned medium from irradiated and nonirradiated cells

To analyze the effects of radiotherapy on NO secretion from fibroblasts on tumor cells and vice versa, cells were cultured with the respective conditioned medium obtained from fibroblasts for 24 hours. Before use, these media was centrifuged at 10,000 rpm for 10 minutes.

Coculture of irradiated and nonirradiated pancreatic tumor cells with fibroblasts

FC1245 pancreatic tumor cells (1 × 10^5 tumor cells per well) were plated into the bottom compartment of a 6-well culture plate (Corning). The fibroblasts were then seeded into the top transwell compartment (2 × 10^5 fibroblasts per well). Medium was replaced after 24 hours, cocultures were irradiated (see below for details on in vitro radiotherapy treatments), and supernatants or cell extracts were analyzed after an additional 24-hour coculture. The supernatants were centrifuged at 10,000 rpm for 10 minutes before use on the analysis.

Nitrite level determination by colorimetric assays

NO is a gaseous free radical with a short life and no available methods to directly measure NO levels exist. Therefore, the levels of
more stable NO metabolites (nitrite, NO$_2^-$) were measured in cell culture supernatants using the NO colorimetric assay (R&D Systems) or the fluorescent probe 4,5-Diaminofluorescein (DAF-2; Cayman Chemical). See Supplementary Information for details.

**In vitro therapeutic treatments**

Details on the different in vitro therapeutic experiments—radiation treatment, iNOS inhibition with 1400W, iNOS knockdown, iNOS amplification, NO donor treatments, glucose uptake, lactate production, cell counting, viability assessment with Trypan blue exclusion, migration, and invasion assays—are described in Supplementary Information.

**NFκB, cytokine, and RNA analyses**

The activity of the NFκB in FC1245 cells cultured in the presence of 150 mg/kg of potassium D-luciferin salt (Caliper Life Sciences) was determined using a NFκB kit according to the manufacturer’s instructions (Bioscience, 60614). See Supplementary Information for details.

Cell supernatants of FC1245 PDAC cells, radiotherapy fibroblasts, nonradiotherapy fibroblasts, and FC1245 incubated with CM from fibroblasts were collected, centrifuged at 16,000 × g for 10 minutes, and saved at −80 °C until the day of the experiment. Cytokines were quantified using the MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead 32 Plex Panel (Millipore).

For RNA analyses, details are described in Supplementary Information. mRNA expression data were processed by BioMark HD System and data analyzed using the real-time PCR analysis software (Fluidigm). mRNA levels were relative to 18S (ΔCt = Ct gene of interest − Ct 18S) and normalized versus the mean of control.

**In vivo therapy studies**

Bioluminescence was measured for 5 minutes after intraperitoneal administering 150 mg/kg of potassium ω-luciferin salt (Caliper Life Sciences) dissolved in PBS. Mice were deemed to have engrafted tumors (typically 7 days postorthotopic implantation of FC1245 PDAC cells) once total bioluminescence surpassed 10$^5$ photons/second/cm$^2$/sr. Mice were randomly grouped into treatment groups (n = 10 per group): vehicle, 1400W, radiotherapy, or a combination of 1400W and radiotherapy. Radiotherapy protocols received three doses of 6 Gy (administered with a 48-hour interval, see sections above for details on radiotherapy protocols). Intraperitoneal administration of 1400W (1 μg/g) was started at day 0. 1400W was administered once daily for 7 days during the radiotherapy treatment period and every 2 days for 2 weeks after the radiotherapy treatment period.

**Western blotting, immunofluorescence, and IHC**

Western blot, immunofluorescence, and IHC were performed as detailed in Supplementary Information.

**Radiolabeling of pHLIP, PET imaging of 18F-FDG, and acute biodistribution studies**

The 18F-FDG was obtained from the Nuclear Pharmacy at MSKCC on the morning of injection. NO2A-variant 3 pHLIP (>95% chemical purity) was purchased and used as received from CSBio Co. For peptide radiolabeling with Gallium-67 ($^{67}$Ga), $^{67}$Ga (gallium-67 citrate, Nuclear Diagnostic Products) was trapped on a silica cartridge (Sep-Pak Light, Waters), washed with water, and eluted as [$^{67}$Ga]GaCl$_3$ with 0.4 mol/L HCl, followed by pH adjustment to approximately 4.0 with 1.0 mol/L Na$_2$CO$_3$. NO2A-cysVar3 was dissolved in DMSO and added to the [$^{67}$Ga]GaCl$_3$. Following addition of 200 μL of AcN, the mixture was incubated at approximately 75°C for 30 minutes, diluted with water, and loaded onto a C-18 cartridge (Sep-Pak Light, Waters). After washing the cartridge with 10 mL of water to remove unbound $^{67}$Ga, the radiolaabeled peptide was eluted with 100% ethanol and reconstituted in PBS for in vivo studies. The radiochemical purity of all constructs used in animal studies exceeded 95%.

At 14 days posttherapy (see above for details on the radiotherapy protocol and 1400W administrations), mice (n = 5 per group) were administered 20 μCi intravenous injection of $^{67}$Ga-labeled pHLIP or 200 μCi 18F-FDG. At 2 hours postintravenous administration of 18F-FDG, PET images were recorded on an Inveon PET scanner (Siemens). All images were visualized in AIMDE 1.04 software (http://amide.sourceforge.net).

Acute biodistribution studies were performed at 24 hours after injection of 67Ga-labeled pHLIP or 2 hours after intravenous injection of 18F-FDG. Mice were sacrificed and organs were harvested, weighed, and assayed in the gamma counter for biodistribution studies. Radioactivity associated with each organ was expressed as percentage of injected dose per gram of organ (%ID/g).

**Results**

**Radiotherapy increases iNOS expression in murine and human pancreatic tumors**

Given that a higher iNOS expression is associated with poor survival in patients with PDAC (20) and with PDAC therapeutic resistance (29), we sought to determine whether radiotherapy interferes with iNOS protein levels in pancreatic cancer. We used immunofluorescence assays to characterize iNOS protein levels in matched normal versus tumor pancreatic samples from patient specimens obtained before treatment [treatment-naïve, nonradiotherapy (non-RT)] and after radiotherapy (Fig. 1A; Supplementary Fig. S1). The radiotherapy-treated PDAC samples were obtained between 1 and 3 weeks after the last radiotherapy treatment fraction (Supplementary Table S1) and the number of cumulative radiation dose given to those patients decreased in the following order patient #1 > patient #2 > patient #3 > patient #4. The tumor tissues in our four different patient cohorts showed a significantly higher iNOS expression when compared with the nontumor pancreas (Fig. 1A; Supplementary Fig. S1). These observations are consistent with previous IHC staining showing higher iNOS expression in tumor when compared with nontumor samples in PDAC cases (20, 28). The iNOS protein levels were higher in radiotherapy-treated tumors when compared with nontreated PDAC tumors (4.6-fold ± 0.1 patient #1, 1.6-fold ± 0.4 patient #2, 2.4-fold ± 0.9 patient #3, and 1.8-fold ± 0.5 patient #4; Fig. 1A). Immunofluorescence staining of iNOS was similar in nonradiotherapy versus radiotherapy-treated nontumor tissues of patients #2 and #3. Nontumor samples of 2 other patients showed a 2-fold increase in iNOS protein levels after radiotherapy (2.4 ± 0.3 patient #1 and 2.3 ± 0.5 patient #4). Additional immunofluorescence staining of αSMA and cytokeratin in radiotherapy-treated samples obtained from patient #3, demonstrated iNOS/αSMA in 56.3% of total αSMA$^+$ cells and iNOS/cytokeratin$^+$ in 90.1% of total cytokeratin$^+$ cells (Fig. 1B and C; Supplementary Tables S2 and S3).

To further compare iNOS protein levels between nonradiotherapy and radiotherapy-treated pancreatic tumors, FC1245Luc$^+$ murine pancreatic cancer cells were orthotopically transplanted to the pancreas of C57BL/6 (B6) mice. FC1245 pancreatic cancer cells isolated from KPC (Kras$^{LSL-G12D/+}$;Pdx1-Cre) mice in the C57BL/6 (B6) genetic background represent a valuable biological PDAC model (38) as they mimic the pathophysiological features of...
Radiotherapy (RT) increases iNOS expression in PDAC. Figure 1. Quantiﬁcation of immunofluorescence staining of iNOS in PDAC tumors with or without radiotherapy treatment (mean ± SEM, n = 3). Confocal images of iNOS staining and patient clinical history are described in Supplementary Fig. S1 and Supplementary Table S1. *P < 0.05; †P < 0.01, compared with corresponding nonradiotherapy tissue; ‡P < 0.05, compared with corresponding nontumor tissue and based on a Student t test. B and C, Immunofluorescence staining of iNOS, cytokeratin, and αSMA in PDAC samples obtained from patient #5 after radiotherapy. DAPI was used to stain cell nuclei. Quantifications of iNOS/αSMA and iNOS/cytokeratin staining were performed using the Halo software and data are shown in Supplementary Tables S2 and S3. Scale bars, 50 µm. D and E, Immunofluorescence staining of iNOS, cytokeratin, and αSMA in FC1245 orthotopic PDAC tumors. DAPI was used to stain cell nuclei. Quantifications of iNOS/αSMA and iNOS/cytokeratin staining were performed using the Halo software and data are shown in Supplementary Tables S4–S7. Tumors were collected at 2 days after a 12 Gy radiotherapy dose. Scale bars, 2,000 µm (D) and 50 µm (E). H&E, hematoxylin and eosin.

Radiotherapy-activated fibroblasts induce inflammatory cytokines secretion, iNOS expression, and NO release by PDAC

Pancreatic TME largely consists of stromal fibroblasts, which are thought to contribute to PDAC resistance to treatment (40, 41). Therefore, we examined the role of CAFs in iNOS expression and NO secretion of PDAC following radiotherapy. Previous studies have demonstrated increased invasiveness of pancreatic cancer cells after coculture with ﬁbroblasts irradiated at a dose of 5 Gy (7). We performed primary cultures of ﬁbroblasts (29) isolated from surgical resections of treatment naïve (nonradiotherapy) and radiotherapy-treated FC1245 PDAcs. (Supplementary Fig. S4A and S4B). To investigate possible paracrine iNOS/NO signaling, we analyzed CM of radiotherapy versus nonradiotherapy CAFs and in vitro coculture models consisting of FC1245 pancreatic cancer cells and CAFs (Fig. 2A; Supplementary Fig. S3). To mimic the high PDAC desmoplastic stromal environment, we followed previous reports and used a tumor—CAF ratio of 1:2 in our in vitro model system (29). Western blot analysis demonstrated similar iNOS expression in FC1245 cancer cells and CAFs that received an extra in vitro irradiation dose of 5 Gy demonstrated, respectively, a 2.2-fold and 3.3-fold increase in iNOS protein levels in FC1245 in non-RT CAFs and RT-CAFs (Fig. 2B). iNOS expression was similar in nonradiotherapy and radiotherapy-treated FC1245 cells (Fig. 2B). Additional Western blot analysis revealed a 1.4-fold ± 0.1 increase in iNOS expression when FC1245 cancer cells are incubated with CM of CAFs for 24 hours (Fig. 2C). Because an extra in vitro irradiation dose increases iNOS expression in CAFs (Fig. 2B), we performed additional studies in FC1245 cocultured with CAFs or cultured with CM of CAFs that received an extra in vitro 5 Gy. We observed a 1.8-fold ± 0.2 increase in iNOS protein levels in FC1245...
Radiotherapy (RT)-activated CAFs enhance PDAC tumor growth. A, CAFs were isolated from nonradiotherapy-treated FC1245 orthotopic PDACs (non-RT CAFs) or from mice treated with a 12 Gy radiotherapy dose (RT-CAFs). FC1245 were cultured with conditioned medium from CAFs or cocultured with CAFs using a transwell in vitro system. B, Western blot of iNOS in total lysates of FC1245, non-RT CAFs, and RT-CAFs. Density of Western blot bands was quantified by scanning densitometry with ImageJ software. C, Western blot analysis of iNOS in total lysates of FC1245. FC1245 cells were cocultured with CAFs (RT-CAFs or non-RT CAFs) or treated with CM from CAFs (RT-CAFs, CAFs, or non-RT CAFs, CM) for 24 hours. Density of Western blot bands was quantified by scanning densitometry with ImageJ software. D, NO$_2^-$ levels in supernatants of FC1245, RT-CAFs, non-RT CAFs, FC1245 treated with supernatants from CAFs, and FC1245 cocultured with CAFs. * P < 0.05; ** P < 0.01; *** P < 0.001 compared with FC1245 and based on a Student test (mean ± SEM, n = 3) in FC1245 cells cultured with CM non-RT CAFs, CM RT-CAFs, and CM RT-CAFs in the presence of 10 μmol/L 1400W. * P < 0.01, compared with FC1245; ** P < 0.001 compared with FC1245 treated with CM from non-RT CAFs. E, Luciferase NF-κB activity assay (mean ± SEM, n = 3) in FC1245 cells cultured with CM non-RT CAFs, CM RT-CAFs, and CM RT-CAFs in the presence of 10 μmol/L 1400W. * P < 0.01, compared with FC1245; ** P < 0.001 compared with FC1245 treated with CM from RT-CAFs and based on a Student t test. 1400W, N-C-(aminomethyl)benzyl)acetamide. F, IFNα, IL1β, and TNFα concentrations in the supernatants of FC1245 PDAC cells (first bar on the graph), non-RT CAFs (second bar), RT-CAFs (third bar), FC1245 incubated with CM from non-RT CAFs (fourth bar), and FC1245 incubated with CM from RT-CAFs (fifth bar). Cell supernatants were collected at 24 hours after cell culture and cytokine activity was determined using the MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead 32 Plex Panel. Data are presented as mean ± SEM; n = 4–6 independent experiments. * P < 0.05; ** P < 0.01 compared with FC1245 and based on a Student t test. G, Bioluminescence images and tumor growth rate (mean ± SEM, n = 4) of pancreatic orthotopic tumors developed by implantation of FC1245luc alone, FC1245luc mixed with non-RT CAFs, and FC1245luc mixed with RT-CAFs into the pancreas of C57BL/6 (B6) mice. * P < 0.05; ** P < 0.01, compared with FC1245. H, Cell growth of FC1245 cultured in the presence or absence of CM of irradiated and nonirradiated CAFs. To deplete iNOS, FC1245 tumor cells were treated with 10 μmol/L 1400W or transfected with INOS siRNA (INOS KD). Data are presented as mean ± SEM, n = 3. * P < 0.05, compared with FC1245 plus CAFs non-RT or FC1245 plus RT-CAFs; ** P < 0.05, compared with FC1245 and based on a Student t test. 1400W, N-C-(aminomethyl)benzyl)acetamide. FC1245 migration (I) and invasion (J) after treatment with CM of CAFs using the Boyden and Matrigel invasion chambers, respectively. Data are presented as mean ± SEM, n = 3. * P < 0.05; ** P < 0.01; *** P < 0.001 compared with FC1245 and based on a Student t test.

Given that inflammatory cytokines such as IFNα, IL1β, and TNFα induce INOS expression through NF-κB (22, 29, 30), we performed analyses of NF-κB and 32 cytokines using a MILLIPLEX kit. NF-κB activity in FC1245 cells was monitored using a NF-κB luciferase reporter assay. Culture of FC1245 with CM from RT-CAFs increased NF-κB in the tumor cells (Fig. 2E). Cytokine analyses were...
performed using the cell supernatant of FC1245, non-RT CAFs, RT-CAFs, and FC1245 cells cultured either with medium from non-radiotherapy fibroblasts or with medium from radiotherapy fibroblasts. IFNγ, IL1β, leukemia-inhibitory factor (LIF), RANTES (regulated on activation normal T-cell expressed and secreted), and IL13 were similar in FC1245 and CAFs (Fig. 2F). Culture of FC1245 PDAC cells in the presence of CM from radiotherapy fibroblasts increased the production of IFNγ, IL1β, and TNFα (Fig. 2F). In addition, when FC1245 cells were cultured with medium from radiotherapy fibroblasts, we observed an increase in the production of IL6, LIF, and RANTES (Supplementary Fig. S8). On the other hand, IL13 decreases when FC1245 cells were cultured with medium from nonradiotherapy fibroblasts (Supplementary Fig. S8). We next used real-time PCR to understand the major genes in radiotherapy-activated CAFs driving these transcriptional differences in FC1245 cancer cells (Supplementary Fig. S9). We observed upregulation in IFNγ, TNFα, and TNFβ in RT-CAFs when compared with non-RT CAFs. When compared with RT-CAFs, nonradiotherapy CAFs demonstrated higher mRNA levels for RANTES, C-X-C motif chemokine 10 (CXCL10), IL12, IL1α, IL1β, IL1R1, IL6Rα, Interferon regulatory factor 7 (IRF7), and STAT1 (Supplementary Fig. S9). These results suggest that radiotherapy-activated fibroblasts induce increased secretion of NO and iNOS expression in the tumor cells through NF-κB, which in turn leads to an elevated release of inflammatory cytokines by the tumor cells.

**RT-CAFs increase pancreatic tumor growth, an effect that can be abolished by genetic or pharmacologic blockade of iNOS.**

In our study, FC1245 pancreatic orthotopic tumor models were used to determine the role of non-RT CAFs and RT-CAFs in PDAC tumor growth in vivo. FC1245 luc−/− alone, FC1245 luc−/− mixed with non-RT CAFs, and FC1245 luc−/− mixed with RT-CAFs were implanted into the pancreas of C57BL/6 (B6) mice and tumor growth was evaluated using bioluminescence. As shown in Fig. 2G, the tumor growth rate was significantly higher in FC1245 cells mixed with RT-CAFs than in the model developed by mixing FC1245 cells with non-RT CAFs or FC1245 cells alone (Fig. 2G). At 14 days after cells’ implantation in the pancreas, we observed significantly higher mSMA−/− tumors developed by FC1245 luc−/− mixed with CAFs when compared with FC1245 luc−/− cells alone (Supplementary Fig. S10). To explain the protumor effect of RT-CAFs, the influence of these cells on the growth of FC1245 cells was investigated in vitro using the Trypan blue assay. Enhanced FC1245 cell growth was observed after cells being cultured with CM of RT-CAFs (Fig. 2H). Culture of FC1245 with CM of nonradiotherapy CAFs did not interfere with PDAC cancer cell growth (Fig. 2H). Next, we used the Boyden chamber (Fig. 2I) and scratch assays (Supplementary Fig. S11) to determine whether RT-CAFs affected the migratory properties of FC1245 cells. Matrigel invasion chamber assays were also used to measure FC1245 invasion properties in the presence of CAFs (Fig. 2I). FC1245 cells in culture with CM of RT-CAFs demonstrated a significant increase in both migration and invasion properties as compared with FC1245 cells alone, which is consistent with previous studies (7). Culture of FC1245 cells with CM of RT-CAFs showed a significantly larger number of migratory and invading cells compared with culture with CM of nonradiotherapy fibroblasts (Fig. 2I and J; Supplementary Fig. S11). We did not detect metastatic spread at 6 days after cells’ implantation in the pancreas (Supplementary Fig. S12). At 14 days, metastatic spread of tumor was observed in the liver, spleen, and stomach in tumors developed by FC1245 luc−/− mixed with RT-CAFs (Supplementary Fig. S13).

Premised on our findings (Figs. 1 and 2), we expected that the protumor effects of RT-CAFs are mediated by iNOS/NO signaling. Therefore, we sought to investigate FC1245 cells growth after iNOS amplification or exposure to physiologic levels of the NO donor diethylenetriamine NONOate (DETA-NO). DETA-NO has a half-life of 20 hours and in aqueous solution it spontaneously decomposes to produce a long lasting NO release. Previous studies have reported that DETA-NO increases migration of lung carcinoma cells and proliferation of breast cancer cells (26, 42). Others have reported that low concentrations of DETA-NO (20−200 nmol/L) induce cancer cell growth, while concentrations higher than 20 μmol/L inhibit cell proliferation (34). In our studies, NO induction in PDAC cells was performed using 1 μmol/L of DETA-NO or with a clustered regularly interspaced short palindromic repeats (CRISPR) activation plasmid (Supplementary Figs. S14 and S15). Treatment of FC1245 pancreatic cancer cells with 1 μmol/L of DETA-NO or with CRISPR activation plasmid increased NO levels and FC1245 cell growth, an effect that is abolished by the addition of the highly selective iNOS inhibitor 1400 W (Supplementary Figs. S14 and S15; ref. 43). To support the hypothesis that NO release from CAFs contributes to radiotherapy resistance of pancreatic carcinoma cells, NO induction in PDAC cells in the presence of RT-CAFs was blocked using siRNA-mediated knockdown of iNOS or pharmacologic treatments with 1400 W (Fig. 2I; Supplementary Figs. S14–S16). In addition, the iNOS inhibitor 1400 W decreased NF-κB induction in tumor cells cultured with CM RT-CAFs (Fig. 2E). These results indicate that CAFs can promote the growth of PDAC cells, an effect that increases when cells are cultured with CM of RT-CAFs, which can be abolished in vitro by iNOS depletion using genetic and pharmacologic approaches. 

**Pharmacologic blockade of iNOS improves therapeutic response of PDACs to radiotherapy.**

Previous studies demonstrated a role for iNOS in PDAC chemoresistance (20). Our findings suggest iNOS/NO signaling as a cause of PDAC resistance to radiotherapy, with NO secretion from RT-CAFs preventing radiotherapy therapeutic efficacy. To translate our findings into a clinically relevant approach, we performed therapeutic studies combining iNOS pharmacologic inhibition with radiotherapy in orthotopic PDAC models. Luciferase-expressing FC1245 pancreatic cancer cells were implanted into the pancreas of C57BL/6 (B6) mice. To determine the impact of radiotherapy dose on PDAC therapy, a pilot therapeutic study was performed using 12 Gy in a single treatment fraction or 18 Gy administered in three fractions of 6 Gy at 48-hour intervals. These dose schedules were selected because, as per the linear quadratic model of cell kill, they have similar biologically effective dose (BED at an alpha/beta = 10, BED10). The three-fraction course of radiotherapy (BED10 = 28.8 Gy) improved survival when compared with a single fraction (BED10 = 26.4 Gy; Supplementary Fig. S17), supporting previous reports that are suggestive of the clinical benefit of radiotherapy administration in fractions (11). In further therapeutic experiments, radiotherapy was administered in 3 doses of 6 Gy at 48-hour intervals. After an engraftment period, tumor-bearing mice as identified via bioluminescence were randomly assigned to treatment groups (nonradiotherapy, radiotherapy, 1400 W, and 1400W/radiotherapy). At 14 days after the first radiotherapy dose, mice treated with 1400 W or 1400W/radiotherapy demonstrated significantly decreased tumor growth and tumors of small size as measured by luminescence (Fig. 3A–C). The weight of tumors at 14 days after the first radiotherapy dose was 1.65 ± 0.60, 0.92 ± 0.04,
and 0.60 ± 0.09 g in mice treated with radiotherapy, 1400W, and 1400W/radiotherapy (Supplementary Fig. S18).

One of the downstream effects of NO in cancer includes evasion to apoptosis (24, 44) and therefore, tumor sections of the different therapeutic groups were immunostained to assess proliferation and apoptosis. The 1400W/radiotherapy-treated mice displayed diminished proliferation of tumor cells based on the reduced number of Ki67⁺ tumor cells compared with control, 1400W, and radiotherapy groups (Fig. 3D). IHC staining of cleaved caspase-3 indicated significantly increased apoptosis in pancreatic tumors treated with radiotherapy and 1400W/radiotherapy (Fig. 3D). Collectively, these results demonstrate that radiotherapy/iNOS inhibition slows pancreatic tumor growth.

**NO secretion from RT-CAFs changes PDAC TME extracellular pH**

One of the mechanisms by which NO modulates tumor cell proliferation is through upregulation of glycolysis (34). NO release into tumor cells reduces mitochondrial oxidative phosphorylation at complexes III and IV of the electron transport chain and increases activity of the 6-phosphofructokinase 1 (PFK-1), which results in the activation of glycolysis promoting a metabolic switch from an aerobic to a glycolytic phenotype (Warburg effect; refs. 34, 45–50). We attempted to study changes in pancreatic cancer cells glycolysis after FC1245 cells incubation with the NO-donor DETA-NO or culture with CM of RT-CAFs. Our results show that NO production in FC1245 cells is increased through exposure to DETA-NO (Supplementary Fig. S14). Glucose consumption and lactate production (Supplementary Fig. S19) are increased compared with without DETA-NO addition. This effect was further inhibited by the addition of the iNOS inhibitor 1400W (Supplementary Fig. S19).

Because our results showed that DETA-NO increased the glycolytic rate when compared with untreated FC1245 cells, we hypothesized that NO release from CAFs could increase glucose consumption and lactate secretion in cancer cells. Cultures of FC1245 with CM of CAFs demonstrated that RT-CAFs led to a significant increase in glucose uptake (1.3 ± 0.1, mean ± SEM, n = 4; Fig. 4A) and lactate production (2.1 ± 0.6, mean ± SEM, n = 4; Fig. 4A). At 14 days postradiotherapy, we observed an increase in GLUT1 expression of PDAC tumors (1.7 ± 0.2, mean ± SEM, n = 3; Fig. 4B). Additional immunofluorescence staining of αSMA, cytokeratin, and GLUT1 in radiotherapy-treated PDAC samples demonstrated GLUT1⁺/αSMA⁺ in 8.4% of total αSMA⁺ cells and GLUT1⁺/cytokeratin⁺ in 34.3% of total cells.
The percentage of GLUT1^þ/cytokeratin^þ cells (Fig. 4C and D; Supplementary Tables S8–S11). The percentage of GLUT1^þ/cytokeratin^þ cells was 7.6-fold higher in radiotherapy-treated samples when compared with control (Fig. 4C and D; Supplementary Tables S8–S11). We next used fluorodeoxyglucose (18F-FDG) positron emission tomography (PET) to validate in vivo changes in GLUT1 and glucose consumption observed in Fig. 4A–D. Our biodistribution and PET imaging results at 14 days post first radiotherapy dose in mice bearing FC1245 orthotopic PDAC tumors demonstrated a significant increase in 18F-FDG PDAC uptake, an effect that decreases in mice treated with radiotherapy/1400W (Fig. 4E and F; Supplementary Fig. S20). As glycolysis promotes intracellular H^þ generation contributing to the membrane dynamics of Na^÷/H^þ flux, as mediated by the sodium/hydrogen exchanger isoform 1 (NHE1), an increase in glycolysis as mediated by NO release from RT-CAFs could activate NHE1. Our results revealed that radiotherapy increases NHE1 expression in FC1245 PDACs, an effect that...
was inhibited with the iNOS inhibitor 1400W (Fig. 4G). In sum, our findings indicate that an increase in iNOS/NO signaling in RT-fibroblasts upregulates PDAC glycolysis.

**pH-targeted pHLIP allows assessment of radiotherapy/I400W response within PDAC**

Activation of NHE1 causes a reversal of the plasma membrane pH gradient (51), resulting in a more alkaline intracellular pH and a more acidic extracellular pH (Fig. 4H–I). As we observed an increase in GLUT1 and NHE1 in radiotherapy-treated tumors (Fig. 4A–G), we attempted to use a pH-targeted imaging probe to detect changes in the extracellular microenvironment pH, as mediated by NO secretion from RT-CAFs. pH (Low) Insertion Peptide (pHLIP) allows detection of tumor cell surface acidity (Fig. 4H; ref. 52). We performed in vivo biodistribution studies with the radiometallated pHLIP variant [{\textsuperscript{67}Ga}]Ga-NO2A-cysVar3 in mice bearing FC1245 orthotopic PDAC tumors. On the basis of our studies demonstrating that at 14 days posttherapy, the tumor growth was slowed in mice treated with 1400W/radiotherapy when compared with radiotherapy alone (Fig. 3A–C), mice were administered with [{\textsuperscript{67}Ga}]Ga-NO2A-cysVar3 at 14 days post first radiotherapy dose. At 24 hours postinjection of [{\textsuperscript{67}Ga}]Ga-NO2A-cysVar3, PDAC radiotherapy-treated tumors had an uptake of 8.6 ± 0.7 percentage injected dose per gram tissue (%ID/g, mean ± SEM, n = 5), while control tumors had an uptake of 3.6% ± 1.9% ID/g, mean ± SEM, n = 5 (Fig. 4I, Supplementary Fig. S21). The increase in [{\textsuperscript{67}Ga}]Ga-NO2A-cysVar3 PDAC tumor uptake, due to an increase in extracellular acidity, was further decreased in mice treated with radiotherapy plus 1400W (3.1% ID/g ± 1.0, mean ± SEM, n = 5, Fig. 4I). Taken together, these results suggest that NO signaling activation in radiotherapy-treated tumors will result in changes of the PDAC extracellular pH (Fig. 4I) that can be detected using radiolabeled pHLIP, suggesting a clinical potential of pHLIP to assess radiotherapy response within pancreatic cancer, and suggesting ultimately that the benefit of radiotherapy intervention in PDAC can be improved by blocking NO signaling.

**Discussion**

The mechanisms of iNOS/NO signaling and their actions on PDAC response to radiotherapy remain to be completely understood. Here, we demonstrate that radiotherapy enhances iNOS expression and subsequent NO secretion in PDAC. Mechanistic studies demonstrated that the CM of irradiated CAFs drives the production of iNOS in PDACs. Cultures of PDAC cells with CM of RT-CAFs demonstrate that NO secretion and iNOS expression by RT-CAFs are further induced by pancreatic tumor cells through NF-kB, which in turn leads to the release of inflammatory cytokines by the tumor cells. In addition, we found that NO increases after radiotherapy in PDAC, leading to the release of inflammatory cytokines by the tumor cells. In our experiments, an extra in vitro radiotherapy dose was necessary to study the mechanisms by which RT-CAFs induce iNOS/NO secretion in the tumors (Fig. 2). When compared with non-RT-CAFs, RT-CAFs demonstrated upregulation of iNOS mRNA (Supplementary Fig. S9). Co-culture of PDAC cells with RT-CAFs and culture with CM of CAFs increased iNOS expression/NO secretion in the tumors cells (Fig. 2C and D). The increase in iNOS expression and NO secretion by the tumor cells was higher when cells were cultured with CAF-CM when compared with cells cocultured with CAFs (Fig. 2C and D). These results suggest that the secretome of RT-CAFs is the major driving force in the production of iNOS in PDACs. The differences in iNOS/NO between FC1245 cocultures and cultures with CAF-CM can be explained by changes in PDAC cells viability (Supplementary Fig. S6) and future studies are necessary to determine the temporal dynamics of iNOS/NO signaling in these in vitro culture systems.

RT-CAFs increased PDAC cell growth, migration, invasion, and metastatic spread of tumor (Fig. 2G–J, Supplementary Fig. S13). Blocking of NO release, by iNOS inhibition with 1400W or using knockdown via siRNA, abolished the promotor effect of RT-CAFs. Admittedly, while we successfully demonstrate that CAFs play a role in the radiotherapy-mediated increase of iNOS/NO signaling in PDAC in vitro, this system does not fully reflect the complexity and cellular diversity of the PDAC microenvironment in vivo.
Because NO regulates several biochemical pathways, the molecular mechanisms by which NO increases in CAFs after radiotherapy, and how INOS/NO signaling can be further induced by the tumor cells facilitating tumor growth (Fig. 2E) are likely broad. At the transcriptional level, TNFα, IFNγ, IL6, and IL1ß are all known to activate NF-κB, which induces INOS expression (22, 29, 30). Here, we show that the protumor inducing effect of radiotherapy-activated fibroblasts occurs through NF-κB and involves increased secretion of IFNγ, IL1ß, TNFα, IL6, and LIF (an IL6 class cytokine) by pancreatic tumor cells. In addition, our studies demonstrate that the INOS inhibitor 1400W decreases NF-κB induction in PDACs cultured with secretome from RT-CAFs.

Preclinical and clinical studies have shown low toxicity of INOS inhibitors (33, 57–59), suggesting their potential as therapeutic agents. For instance, INOS inhibitors have been shown to block colon adenocarcinoma (60) and glioma tumor stem cell tumor growth (33). Our findings that radiotherapy increases INOS expression, suggests INOS inhibition as a promising strategy that could be applied in the treatment of PDAC. In our studies, the INOS inhibitor 1400W delayed tumor growth, an effect that was enhanced with the 1400W/radiotherapy combination therapy (Fig. 3A–C). Importantly, the 1400W/radiotherapy therapeutic approach shows potential to increase preservation of healthy tissues during surgical PDAC interventions due to the observation of increasingly localized tumors with less diffuse margins (Fig. 3B). The 1400W/radiotherapy combination therapy was unable to completely eliminate tumor growth and further studies are necessary to determine whether other INOS inhibitors or combination of INOS inhibition with other treatment regimens may further augment efficacy when compared with 1400W/ radiotherapy. Previous reports in orthotopic intracranial tumors demonstrated higher therapeutic efficacy for the INOS inhibitor BYK191023 when compared with 1400W (33) plausibly due to its improved pharmacokinetic and bioavailability properties.

In ovarian cancer cells, NO decreases mitochondrial respiration, which results in the activation of glycolysis promoting a metabolic switch from an aerobic to a glycolytic phenotype (34). Glycolysis promotes H+ generation, which is removed from the intracellular to the extracellular compartments in exchange with Na+, by NHE1 (51). In our studies, we observed that NO increases glycolytic rate and NHE1 expression in PDAC (Fig. 4A–G). These membrane transport mechanisms contribute to the acidification of the extracellular space.

Equipped with this information, we used a radiolabeled peptide, [68Ga]Ga-NO2A-cysVar3 pHLIP to probe changes in the TME extracellular pH arising from radiotherapy-mediated NO secretion (Fig. 4H and I). The var3 pHLIP was used in our studies due to its remarkable ability to target acidity in vivo (52, 61–63) and a radiolabeled version of var3 pHLIP will be entering in the clinic in the current year of 2020. The radiolabeled var3 pHLIP demonstrated higher targeting accumulation in radiotherapy PDAC tumors when compared with unirradiated PDAC tumors (Fig. 4I). This increase in acidity, due to NO secretion from radiotherapy-activated PDACs, was further decreased when radiotherapy is combined with the INOS inhibitor 1400W.

In conclusion, our data support the notion that the secretome of RT-CAFs initiates a paracrine activation loop increasing INOS/NO signaling in PDAC and additional in vitro radiation is necessary for robust INOS expression by the tumor cells. Our preclinical results demonstrate the potential of INOS inhibition with fractionated radiotherapy regimen for improved response, and additionally that that response can be assessed via radiolabeled pHLIP, highlighting the mechanisms by which NO in the secretome of RT-CAFs contributes to PDAC resistance. Our study supports previous reports demonstrating that the secretome of therapy-activated CAFs contribute to PDAC pathology and that targeting INOS/NO signaling may be a valid therapeutic approach for improving radiotherapy outcomes in PDAC.

Disclosure of Potential Conflicts of Interest

T. Merghoub is a consultant at Pfizer, reports receiving a commercial research grant from IMVAQ, Bristol-Myers Squibb, Surface Oncology, Kyn Therapeutics, Infinity Pharmaceuticals, Peregrine Pharmaceuticals, Adaptive Biotechnologies, Leap Therapeutics, and Aapya and has ownership interest (including patents) in oncolytic viral therapy, alpha virus–based vaccine, neoantigen modeling, CD40, GITR, OX40, PD-1, and CTLA-4. J.S. Lewis has ownership interest (including patents) in pHLIP, Inc. No potential conflicts of interest were disclosed by the other authors.

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