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Photoimmunotherapy lowers recurrence after pancreatic cancer surgery in orthotopic nude mouse models

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Abstract

Introduction—Photoimmunotherapy (PIT) is based on the use of a monoclonal antibody specific to cancer epitopes conjugated to a photosensitizer near-infrared (NIR) phthalocyanine dye (IR700). In this study, PIT with IR700 conjugated to anti-carcinoembryonic antigen (CEA) was used as an adjunct to surgery in orthotopically-implanted human pancreatic cancer in a nude mouse model in order to eliminate microscopic disease in the tumor bed and prevent local as well as metastatic recurrence.

Materials & Methods—Athymic nude mice were orthotopically implanted with the human pancreatic cancer cell line BxPC3 expressing green fluorescent protein (GFP). After tumor engraftment, the mice were divided into two groups: bright light surgery (BLS) + anti-CEA-IR700 + 690 nm laser (PIT) and BLS only. Anti-CEA-IR700 (100 μg) was administered to the treatment group via tail vein injection 24 hours prior to therapy. Tumors were resected and the surgical bed

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was treated with intraoperative phototherapy at an intensity of 150 mW/cm^2 for 30 minutes. Mice were imaged non-invasively for 8 weeks using an OV-100 small animal fluorescence imager.

Results—BLS+PIT reduced local recurrence to 1/7 mice to 7/7 mice with BLS-only (p=0.001) and metastatic recurrence to 2/7 mice compared to 6/7 mice with BLS-only (p=0.03). Local tumor growth continued at a rapid rate after BLS only compared to BLS+PIT where almost no local growth occurred. There was a significant difference in tumor size between mice in the BLS+PIT (2.14 mm^2, 95% CI [6.34, −2.06] and BLS-only groups (115.2 mm^2, 95% CI [141.6, 88.8]) (p<0.001) at 6 weeks after surgery. There was also a significant difference in tumor weight between the BLS+PIT group (6.65 mg, 95% CI [19.65, −6.35] and BLS-only group (1100 mg, 95% CI [1406, 794] at 8 weeks (p<0.001) after surgery.

Conclusions—PIT holds promise in the treatment of pancreatic cancer and may serve as a useful adjunct to surgery in the eradication of microscopic residual disease that can lead to both local and metastatic recurrence. Further studies are warranted to investigate the potential toxicities of PIT, especially with regard to anastomoses such as those involved in pancreaticoduodenectomy.

Keywords

Pancreatic cancer; orthotopic mouse models; photoimmunotherapy CEA; surgery

Introduction

Photoimmunotherapy (PIT) uses tumor specific monoclonal antibodies that are conjugated to the photosensitizer phthalocyanine dye, IR700, which is cytotoxic upon irradiation with near-infrared (NIR) light (1-3). Several monoclonal antibodies (mAbs) have been used with PIT in mouse models of breast cancer, including trastuzumab, a monoclonal antibody directed against human epidermal growth factor receptor 2 (HER2), and panitumumab, a monoclonal antibody directed against human epidermal growth factor receptor 1 (HER1) (4, 5). Cell death was induced immediately after irradiating mAb-IR700–bound target cells with NIR light. In vivo tumor shrinkage after irradiation with NIR light was demonstrated in target cells expressing the epidermal growth factor receptor. The mAb-IR700 conjugates were effective when bound to the cell membrane and produced no phototoxicity when not bound, suggesting a different mechanism for PIT as compared to conventional photodynamic therapies (1).

Pancreatic cancer is a highly lethal tumor with high rates of local and distant recurrence (6, 7). In the present study, we used a chimeric monoclonal antibody against the carcinoembryonic antigen (CEA), which is often overexpressed in pancreatic cancer and has been previously utilized by our laboratory for fluorescence-guided surgery and fluorescence laparoscopy (8-17). The anti-CEA antibody was conjugated to IR700 and used for PIT treatment of human pancreatic cancer after tumor resection in orthotopic mouse models.

Materials and Methods

Cell Culture

The human pancreatic cancer cell line BxPC-3 was stably transduced to express green fluorescent protein (GFP) as previously described (18, 19). Cells were maintained in RPMI

1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), penicillin/streptomycin (Gibco-BRL, Carlsbad, CA), sodium pyruvate (Gibco-BRL), sodium bicarbonate (Cellgro, Manassas, VA), L-glutamine (Gibco-BRL), and minimal essential medium nonessential amino acids (Gibco-BRL). All cells were cultured at 37° C in a 5% CO_2 incubator.

Animals

Athymic nu/nu nude mice (AntiCancer Inc., San Diego, CA), 4-6 weeks old, were used in this study. Mice were kept in a barrier facility under HEPA filtration. Mice were fed with an autoclaved laboratory rodent diet. All mouse surgical procedures and imaging were performed with the animals anesthetized by intramuscular injection of 50% ketamine, 38% xylazine, and 12% acepromazine maleate (0.02 ml). Animals received buprenorphine (0.10 mg/kg ip) immediately prior to surgery and once a day over the next 3 days to ameliorate pain. CO_2 inhalation was used for euthanasia of all animals at 8 weeks after surgery. To ensure death following CO_2 asphyxiation, cervical dislocation was performed. All animal studies were conducted with an AntiCancer, Inc. Institutional Animal Care and Use Committee (IACUC)-protocol specifically approved for this study and in accordance with the principals and procedures outlined in the National Institute of Health Guide for the Care and Use of Animals under Assurance Number A3873-1.

Antibody-Dye Conjugation

A water-soluble silicon-phthalocyanine derivative, IRDye 700DX NHS ester, was obtained from LI-COR Bioscience (Lincoln, NE). 2 mg (~ 14 nmol) of chimeric anti-CEA antibody (Genara Biosciences LLC, Morgan Hill, CA) at a concentration of 2 mg/ml in 0.1 M Na_2HPO_4 (pH= 8.6) was incubated for 2 hours at room temperature with IR700dye NHS ester (135 ug, 70 nmol) prepared in anhydrous DMSO at 5 mmol/L. After the incubation period, the IR700-conjugate was buffer exchanged and purified with phosphate buffer saline (PBS, pH= 7.1) using Amicon Ultra Centrifugal Filter Units (EMD Millipore Corporation, Billerica, MA). The IR700-mAb conjugate was repeatedly diluted with 10 ml volumes of PBS and then concentrated using the filter units until less than 2 % of the unconjugated IR700 dye species remained, as determined by size exclusion HPLC (SE-HPLC). Analysis of the conjugates by SE-HPLC was performed using an Agilent 1100 HPLC system fitted with a TSKgel G2000SWxl column (Tosoh Biosciences, Tokyo, Japan). The SE-HPLC elution buffer was 1X PBS (pH =7.1) with a flow rate of 1 ml/min. UV/Vis detection at 280 nm and 690 nm was used to determine the average dye-to-antibody ratio (DAR) for each conjugates. With this sample, a purity of 97.6% with 0.5% free dye and a DAR of 4.1 was achieved.

Orthotopic tumor implantation

After confluence, BxPC-3-GFP human pancreatic cancer cells (1×10^6 cells) were injected subcutaneously into the flanks of nude mice and allowed to engraft and grow over a period of 2-3 weeks. Tumors were then harvested and 1 mm^3 tumor fragments from subcutaneous tumors were sutured to the tail of the pancreas using 8-0 nylon surgical sutures (Ethilon; Ethicon Inc., Somerville, NJ). On completion, the tail of the pancreas was returned to the abdomen, and the incision was closed in one layer using 6-0 nylon surgical sutures (Ethilon)

(20, 21). The implanted tumor fragments were allowed to grow over a period of 2 weeks until they were 4-6 mm^3 in volume.

Experimental protocol

A total of 14 mice were used for the experiment (Figure 1). After orthotopic pancreatic tumor engraftment, the mice were divided into 2 groups of 7 mice, comprising a treatment group of bright light surgery (BLS) +PIT and a control group (BLS-only). Thus the experimental design could determine if the addition of PIT to BLS conferred benefit to BLS-only which is the current standard of practice. Anti-CEA-IR700 (100 μg) reconstituted in 100 μl PBS was injected via the tail vein in the treatment group 24 hours prior to surgery, while the control group had 100 μl PBS similarly injected 24 hours prior to surgery. After 24 hours, each of the mice had their pancreatic tumors exposed and resected with bright light surgery. The treatment group was subjected to phototherapy with a 690 nm laser (Aspyrian Therapeutics, San Diego, CA) at 150 mW/cm^2 for 30 minutes for a total of 270 J/cm^2. The surrounding normal tissues were protected with aluminum foil during PIT. Mice were intravitally imaged for GFP expression at the time of therapy and weekly thereafter with the tumor exposed to evaluate response to therapy (please see below). After 8 weeks the mice were sacrificed, at which point they were imaged and had their tumors removed and weighed.

Animal Imaging

Mice were intravitally imaged for GFP expression weekly using the Olympus OV100 small animal imaging system (Olympus Corp. Tokyo, Japan), containing an MT-20 light source (Olympus Biosystems Planegg, Germany) and DP70 CCD camera (Olympus Corp. Tokyo, Japan) (22). Images were used to calculate tumor area with Image-J (National Institute of Health Bethesda, MD) and were processed with the use of Photoshop elements-11 (Adobe Systems Inc. San Jose, CA). Tumor area has previously been shown to correlate with tumor volume (23).

Statistical Analysis

All statistical analysis was done using SPSS software version 21 (IBM, Armonk, NY). For pairwise comparisons, quantitative variables were calculated using the paired-samples Student’s t-test and confirmed with the Wilcoxon rank-sum test. A p-value <0.05 was considered significant. 95% confidence intervals obtained on analysis of the data were configured into the error bars of the appropriate figures and graphs.

Results

Metastatic and local recurrence after BLS-only or BLS+PIT

BLS+PIT reduced local recurrence to 1/7 mice to 7/7 mice with BLS-only (p=0.001) and metastatic recurrence to 2/7 mice compared to 6/7 mice with BLS-only (p=0.03) (Table 1). In mice with metastatic recurrence, metastases were noted in the liver, spleen, abdominal wall, and small bowel.

Local tumor growth before and after BLS-only and BLS+PIT

Tumor sizes were assessed on a weekly basis by quantitative intravital GFP imaging to evaluate response to therapy and overall progression. In the BLS-only group, there was a steady increase in tumor size over the course of the experiment due to the presence of residual disease after surgical resection, clearly demonstrated by GFP imaging, achieving a maximum average value of 115.2 mm^2 by 8 weeks (95% CI [88.76, 141.64]). In the BLS +PIT group however, there was a steady decrease in visible tumor, with tumor disappearance noted in all but one mouse at 3 weeks. In all but one mouse, there was no recurrence of tumor by 8 weeks, resulting in a maximum average value of 2.14 mm^2 (95% CI [−2.08, 6.32]). The difference between the two groups was significant with $p<0.001$ (Figures 2 & 3).

At the termination of the experiment at 8 weeks, the tumors in both groups were excised and weighed. Complete excision was confirmed with the OV-100 in assessing for the GFP fluorescence signal. The average tumor weight of the BLS-only group was 1099.7 mg (95% CI [793, 1406]) and 6.7 mg (95% CI [−6.3, 19.7]) for the treatment group with $p<0.001$ between the two groups (Figure 4). Only one of the 7 mice in the BLS+PIT group had residual tumor.

Discussion

Pancreatic cancer continues to be a highly lethal cancer with surgical resection as the only potential curative treatment. Furthermore, only 15-20% of patients are eligible for resection, with a poor prognosis even after “complete resection”. 5-year survival after pancreaticoduodenectomy after resection for node-negative disease is 25-30% and 10% for node-positive disease (24, 25).

In a previous study, we initially determined efficacy of PIT on BxPC3 human pancreatic cancer cells in vitro and were able to corroborate these findings in-vivo (26). We demonstrated a high level of CEA antigen expression on target tumor cells and demonstrated efficacy in-vitro with PIT using an Anti-CEA-IR700 conjugate. Using an orthotopic mouse model of pancreatic cancer, there was a significant difference in overall tumor burden between the PIT and control groups in favor of the treated groups when assessing for both tumor size and tumor weight 5 weeks after initial therapy. However, despite the high efficacy of PIT, there was however a 100% recurrence rate in both groups.

The present study has demonstrated that PIT as adjunct therapy with BLS reduced both metastatic and local recurrence. It is of particular importance to pancreatic cancer that metastatic recurrence could be reduced by PIT since metastatic recurrence after pancreaticoduodenectomy is a major and frequent cause of lethality in patients with this disease. Although PIT would have much potential for patients with (+) margin CEA pancreatic cancer, our results suggest that PIT can be used for apparent (−) margin CEA pancreatic cancer, since apparent (−) margins may be positive and detectable by fluorescence in the future.

Thus, PIT holds promise in the treatment of pancreatic cancer and may serve as a useful adjunct to surgery in the eradication of potentially metastatic microscopic disease. Further toxicity experiments are also warranted to determine if PIT would be toxic to the anastomoses required in pancreaticoduodenectomy.

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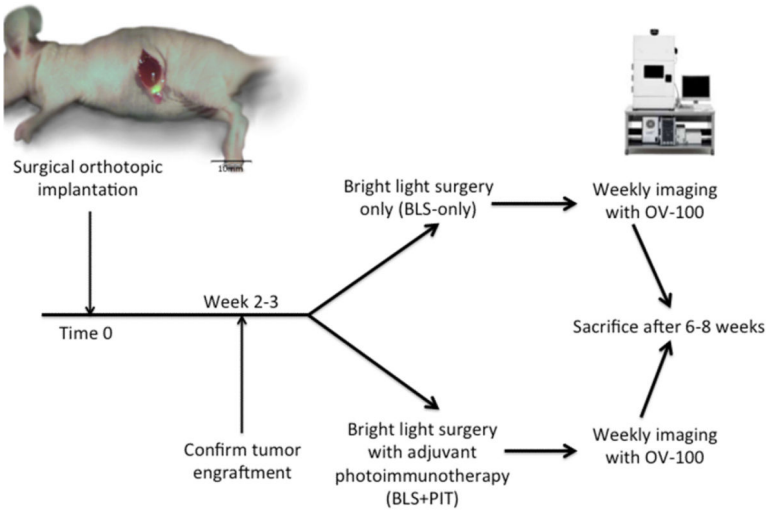

Figure 1. Experimental protocol

After orthotopic implantation of BxPC3-GFP, the mice were divided into 2 groups with both groups undergoing BLS and the treatment group receiving adjuvant PIT. Mice were serially imaged with the OV-100 weekly via left lateral incision and tumor exposure.

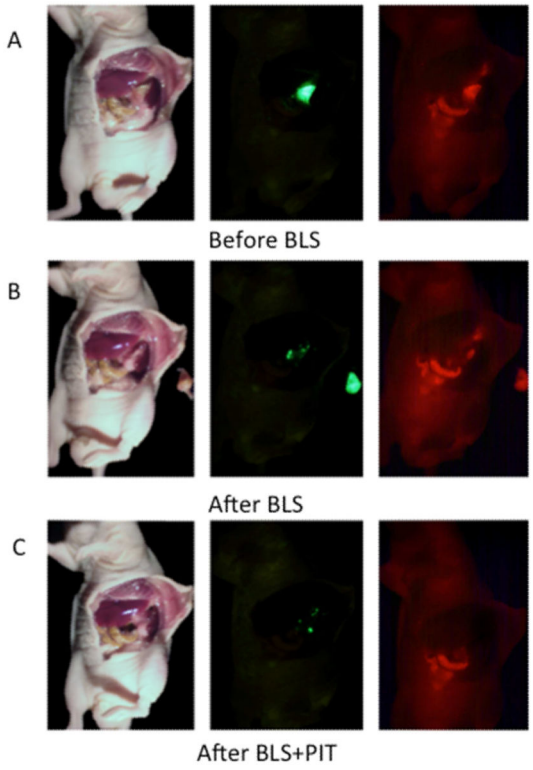

Figure 2. Perioperative images

Panel A demonstrates pre-excision images with colocalization of the GFP and IR700 signals prior to tumor excision. Panel B demonstrates the presence of residual disease after tumor excision with BLS, mimicking the clinical scenario. Panel C demonstrates the same mouse after BLS+PIT with a decrease in the amount of viable tumor and absence of the IR700 signal.

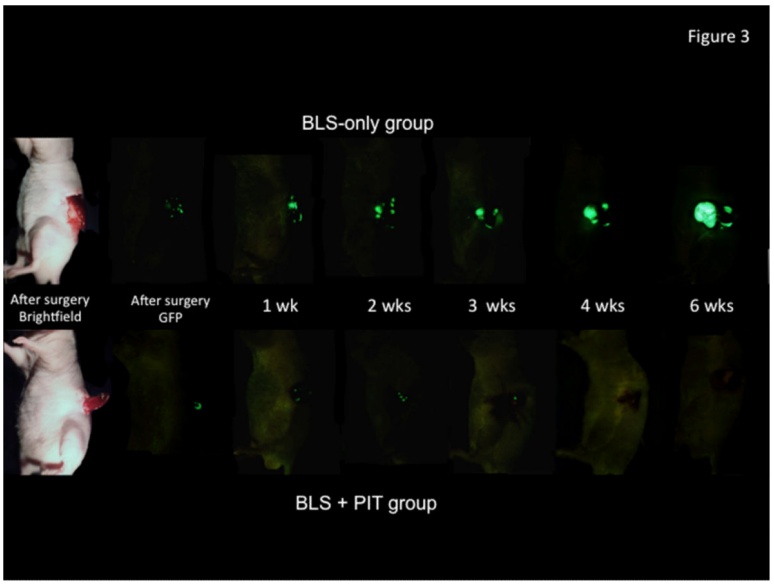

Figure 3. Weekly imaging of pancreatic tumor

Top panel shows an example of weekly imaging a mouse after BLS-only while the bottom panel shows representative weekly imaging after BLS+PIT. Note the disappearance of tumor after 3 weeks after BLS+PIT in contrast to the BLS-only group with a consistent increase in tumor size weekly.

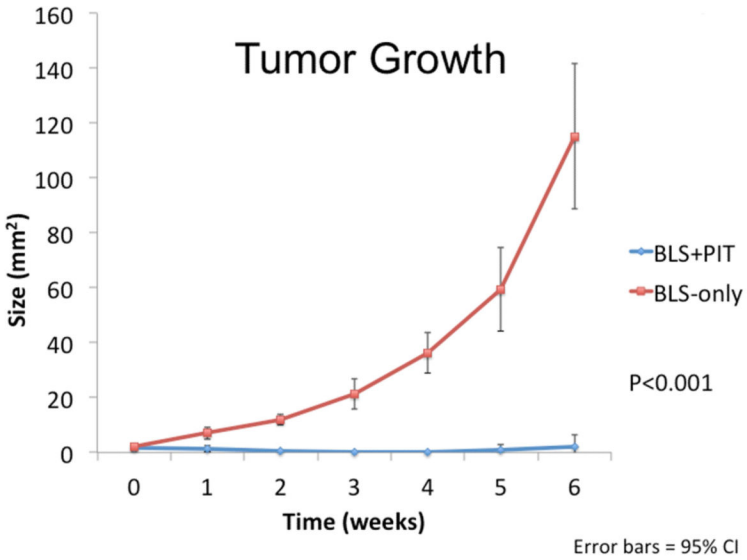

Figure 4. Graphical representation of pancreatic tumor growth over the course of the experiment

Plot of the average tumor area in each group every week. Error bars are included with 95% CI. A clear distinction in growth pattern is seen between the two groups with a significantly higher tumor burden in the BLS group when compared to the BLS-PIT group ($p<0.001$).

Table 1

	Local	Metastatic
BLS-only	7/7 (100%)	6/7 (85.7%)
BLS + PIT	1/7 (14.3%)	2/7 (28.6%)
Chi-square test	p=0.001	p=0.03