Effects of Chemoradiotherapy on Pancreatic Cancer

A Major Qualifying Project Report:

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Degree of Bachelor of Science

by

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February 10, 2007

Dear Professor Rulfs:

Attached is one copy of the Major Qualiying report: ***Effects of Chemoradiotherapy on Pancreatic Cancer,*** Project Number JXR 0435.

Sincerely,

Jillian F. Wise

ABSTRACT

Despite technical advancements in radiation and chemotherapy, pancreatic cancer remains resistant to treatment, with poor survival rates. Using both cell and animal models, we investigated combined radiation and chemotherapy regimens for their effectiveness in reducing tumor growth. Preliminary data suggests combination therapy may be more efficacious than either therapy alone, reducing *in vitro* growth of a pancreatic cell line and *in vivo* tumor size. Markers of angiogenesis suggest combined treatment may also result in a reduction in tumor vasculature.

INTRODUCTION

For several years now the survival and mortality rate of pancreatic cancer patients have remained relatively constant. [1] The American Cancer Society estimated that there would be 33,730 new cases of pancreatic cancer in the United States in 2006. [1] Although the occurrence rate is low, out of these new diagnoses there will be an estimated 32,300 deaths, representing the fourth most common cause of cancer mortality. [1] Currently, for all stages of pancreatic cancer combined, the 1-year survival rate is around 24%, and the overall 5-year survival rate has remained dismally poor at <5%. [1]

Pancreatic cancer typically has a late onset of symptoms and the diagnosis usually comes after metastasis has already occurred. For the patients who are asymptomatic, do not detect the cancer at an early stage, and present with late stage disease, complete surgical resection remains the only curative treatment for pancreatic cancer. Unfortunately, only about 15%-20% of cases of pancreatic cancer are amenable to surgical resection at the time of diagnosis. Regrettably, only 16% of these patients have a 5-year survival. [1]

Of the remaining 80%-85% of patients, 45% present with metastatic disease, with a median survival of 3-6 months. [2] For these patients, the therapies that exist consist of chemotherapy and radiation. The most common therapy administered is 5FU (fluorouracil) or gemcitabine chemotherapy. In addition, patients can receive a combination treatment or chemoradiotherapy. [2] A number of studies have shown that both survival and palliative benefit can be improved when radiotherapy (RT) is combined with chemotherapy in certain types of cancer. [3] One clinical trial by Thierry was done on 353 patients with non small cell lung cancer. The combined chemoradiotherapy was able to increase the two year survival of the patients by 7%. Other cancers have also shown benefits from combined chemoradiotherapy. In 2006, a clinical study was done on patients with advanced squamous cell carcinoma of the head and neck comparing radiation alone to chemoradiotherapy. The results showed that chemoradiotheray was a superior postoperative treatment. [4] Cervical cancer has also been shown to be reduced more effectively by chemoradiotherapy. A study performed in 2000 on cancer of the cervix showed that there was significant increased tumor shrinkage with concurrent chemoradiotherapy versus radiation alone. [5] However, despite refinements in RT techniques and the use of radiosensitizing agents (chemotherapies), pancreatic cancer remains a radiation-and chemotherapy-resistant disease with high rates of localized and metastatic failures. [2]

In this study a previously created L3.6pl cell line is used as a model of the metastatic form of the cancer. Not all cancer cells can survive in a specific environment, as stated by Stephen Paget in his seed and soil theory. [6] Therefore, a model was needed that could grow in the pancreas of mice, but also represent pancreatic metastatic cancer which produces lesions in the liver. To begin the creation of the pancreatic model the L3.6pl line was created from a COLO 357 human pancreatic adenocarcinoma cell line. In order to assure that the cell line would grow in the mouse pancreas and become metastatic, COLO 357 cells were orthotopically implanted in male athymic nude mice (BALB/c) pancreas and spleen. The spleen injections resulted in lesions in the liver. These lesions established that the cell line could survive in circulation and in the liver environment. These spontaneous metastases demonstrate that the cell line can effectively proliferate, induce angiogenesis, detach, move, invade the circulation, aggregate, and

arrest in the liver, and produce growth factors in this environment. With the aim of guaranteeing that the line would repeatedly produce metastases in the pancreas of mice, the spontaneous metastases were removed from the mice and re-injected into the pancreas of another set of nude mice. The liver lesions were once again harvested and the cells were grown in culture and then were put through three more selection cycles of re-injection. The final cell line was designated L3.6pl.

This cell line's doubling time is approximately 16 hours. It has high MMP-9 and MMP-2 mRNA levels. MMP-9 and MMP-2 are type IV collagenase enzymes that are responsible for degrading the extracellular matrix around the tumors. The detachment allows malignant cells from the primary tumor to detach and enter circulation. (Bruns, 1999) In addition, the expression of E-cadherin, a cell surface glycoprotein, is low in the L3.6pl cell line. E-cadherin is responsible for cell to cell adhesion in the pancreatic cells, therefore lower amounts allow the cells to more easily detach and enter circulation. Interlukein 8 (IL-8) expression is also abnormally high in the L3.6pl cell line along with VEGF/VPF and bFGF. IL-8, VEGF/VPF, and bFGF are angiogenesis factors that can initiate and stimulate the growth of the vascular system around tumor sites. With additional circulation increased malignancy is possible. [7] All of these factors help in the growth of endothelial cells and circulation pathways in cancerous cells.

In this study, we hypothesized that the use and therapeutic effects of radiation combined with chemotherapy can be improved by determining the optimal dose and schedule of these two regimens in human pancreatic cancer cells *in vitro* and in human tumors growing in the pancreas of athymic nude mice. By using *in vitro* testing, we determined the optimal dosage and schedule for chemoradiotherapy in highly aggressive and metastatic pancreatic cancer cells. Although we established that there is an optimal dose schedule for chemoradiotherapy in pancreatic cancer cells grown *in vitro,* we hypothesized that the effects may not be identical *in vivo* due to the microenvironment of the pancreas. Therefore, we undertook *in vivo* studies in orthotopic animal models in order to determine the effect of chemoradiotherapy on human pancreatic cancer growing in the pancreas (and spreading to the liver) of nude mice. These experiments provided information on the effects of the tumor microenvironment on treatment regimens.

MATERIALS AND METHODS

Pancreatic cancer cell lines and culture conditions. The highly metastatic human pancreatic cancer cell line L3.6pl [7] was obtained from Dr. Cheryl Baker, M. D. Anderson Cancer Center Orlando. L3.6pl was maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% sodium pyruvate, 2% L-glutamine, and a 2% penicillin-streptomycin mixture. Adherent monolayer cultures were maintained on polystyrene and incubated at 37^{o}C in a mixture of 5% carbon dioxide and 95% air. The cultures were free of *Mycoplasma* and the following pathogenic murine viruses: reovirus type 3, pneumonia virus, K virus, Theiler's encephalitis virus, Sendai virus, minute virus, mouse adenovirus, mouse hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by M. A. Bioproducts, Walkersville, MD). The cultures were maintained for no longer than 10 weeks, or passage 12, after recovery from frozen stocks that were frozen from below passage 4.

Reagents. Gemzar, Gemcitabine HCl, [2′-deoxy-2′,2′-diflurocytidine monohydrochloride (β-isomer)], a nucleoside analogue, (Eli Lilly Indianapolis, IN) was kept at room temperature and dissolved in 0.9% NaCl on the day of use. MTT assay kit, purchased from the ATCC (Manassas, VA), was protected from light and stored at 4^{o}C. The following antibodies were used: rat anti-mouse CD31/PECAM (platelet/endothelial adhesion molecule)-1 (PharMingen International, San Diego, CA); rabbit anti-proliferative cellular nuclear antigen (PCNA) clone PC-10 (DAKO A/S, Copenhagen, Denmark); peroxidase-conjugated goat anti-rat IgG, and peroxidase-conjugated goat anti-rabbit IgG (Jackson Research Laboratories, West Grove, CA). Other reagents used for immunohistochemical analysis included Hoechst Dye 3342 (molecular weight 615.9 g/mole; Hoechst, Warrington, PA), stable 3,3'-diaminobenzidine (Research Genetics, Huntsville, AL), Gill's hematoxylin (Sigma Chemical, St. Louis, MO), and pepsin (Biomeda, Foster City, CA). The terminal deoxynucleotidyl transferase-mediated dUTP-biotin end-labeling (TUNEL) assay was performed using a commercial apoptosis detection kit (Promega, Madison, WI). Polyclonal rabbit anti-VEGF/vascular permeability factor (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-rabbit IgG F (ab')2. Other reagents used for immunohistochemical (IHC) analysis include Hoechst Dye 3342 MW 615.9 (Hoechst, Warrington, PA), stable 3,3'-diaminobenzidine (DAB) from Research Genetics (Huntsville, AL), Gill's hematoxylin from Sigma Chemical Co. (St. Louis, MO), and pepsin from Biomeda (Foster City, CA).

Radiation.

In vitro experiments involving ionizing radiation were conducted in 96 well plates from Corning Incorporated (Costar), Corning NY. The treatments were delivered at a dose rate of 12 Gy/min using a 6 MV photon beam from a Varian 2100c linear accelerator. Beam collimation was accomplished using a 1.5-cm stereotactic cone. 12 Gy was delivered using 1-cm tissue-equivalent bolus to optimize dose.

Experiments involving ionizing radiation to the mice were conducted by delivering a dose rate of 4 Gy/min using a 6 MV photon beam from a Varian 2100c linear accelerator. Beam collimation was accomplished using a 1.5-cm stereotactic cone. 5 Gy was delivered at a depth of 5 mm in each mouse, using 1-cm tissue-equivalent bolus to optimize tumor dose.

***In Vitro* Cytotoxicity Assay.** 2 X 10^4 human L3.6pl cells in 200µl of 10% FBS DMEM were seeded into 38-mm^2 wells of flat-bottomed 96-well plates and allowed to adhere overnight. In the first set of experiments, the spent medium was removed and the cultures were refed with new medium (negative control), medium containing increasing concentrations of gemcitabine (0-5 µm) in triplicate treatments or new medium followed by administration of ionizing radiation at a dose of 12 Gy/2 mins. All plates were immediately returned to the incubator and 24, 48 and 72 hrs later, the number of metabolically active cells was determined by MTT assay. In a second set of experiments, the medium was removed and cultures were refed with medium containing the IC_{50} of gemcitabine of .011µm with or without radiation. All plates were immediately returned to the incubator and 24 hr after the last treatment, the number of metabolically active cells was determined by MTT assay. Briefly, the cell proliferation assay was performed according to the protocol of the commercially available MTT assay kit (ATCC, Manassas, VA). 10 µl of MTT reagent was added to each well and the plates were returned to the incubator. After 2 hours incubation, 100 µl of detergent was added to each well and the plate was incubated overnight at room temperature. The conversion of MTT to formazan by metabolically viable cells was monitored by measuring the absorbance at 562 nm using a BioTek ELx 808 Ultra Microplate reader (BIO-TEK instruments, Inc. Winnoski, VT). Cell proliferation was determined using a standard curve from 1 to 100,000 cells. Growth inhibition was calculated from the formula:

$$\text{Cytostasis (\%)} = [1-(A/B)] \times 100,$$

where A is the absorbance of treated cells and B is the absorbance of the control cells.

Animals. Male athymic nude mice (NCI-nu) were purchased from the Charles River Laboratories (NCI-Frederick Animal Production Area, Frederick, MD). The mice were housed and maintained in the Ventilated/IsoCage system (Techniplast USA, Exton, PA) under specific pathogen-free conditions. The facilities were in accordance with current regulations and standards by the United States Department of Agriculture, United States Department of Health and Human Services, and the NIH. The mice were used in accordance with institutional guidelines when they were 8-12 weeks old.

Orthotopic pancreatic injections. For *in vivo* injection, cells were harvested from subconfluent cultures by a brief (1-3 minute) treatment with 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% FBS and the cells were washed and resuspended in 10% FBS DMEM. Only single-cell suspensions of greater than 90% viability, via trypan-blue exclusion, were used for injection. Male nude mice were anesthetized with 50 mg/kg Xylazine/100 mg/kg Ketamine mix. A small left

abdominal flank incision was made and the spleen and pancreas were exteriorized. 1x 10^6 tumor cells were injected subcapsular in a region of the pancreas just beneath the spleen with a 27-gauge needle, 1-ml disposable syringe. To prevent intraperitoneal leakage, a cotton swab was held for one minute over the site of injection. Both layers of the abdominal wound were closed with wound clips (Auto-clip; Clay Adams, Parsippany, NJ). A successful subcapsular intrapancreatic injection of tumor cells was identified by the appearance of a fluid bleb without intraperitoneal leakage. The mice were sacrificed when the controls became moribund (5-6 weeks) or at predetermined times for pharmacokinetic determinations. The size and weight of the primary pancreatic tumors, the incidence of regional lymph node metastasis, and the presence or absence of liver metastases were recorded. Histopathology confirmed the identity of disease. For IHC and histology staining procedures, one part of the tumor tissue was fixed in formalin and embedded in paraffin, and another part was embedded in OCT compound (Miles, Elkhart, IN), frozen rapidly in liquid nitrogen, and stored at -70^{o}C.

Treatment of Established Human Pancreatic Tumors with Gemcitabine and Radiation. Seven days after implantation of L3.6pl human pancreatic cancer cells into the pancreas, mice were randomized to receive one of four treatments (n= 10 mice per group): (1) twice-weekly intraperitoneal (i.p.) administration of vehicle solution alone (phosphate buffered saline (PBS); control group); (2) twice-weekly i.p. administrations of 100 mg/kg gemcitabine; (3) once-weekly administration of radiation at 5Gy/2 min; (4) twice-weekly gemcitabine combined with radiation. Treatments continued for 5 weeks. The mice were sacrificed on day 35 and subjected to necropsy. The volumes of the pancreatic tumors and the incidence of liver metastases were recorded.

Necropsy Procedures and Histological Studies. Mice were sacrificed and their body weights were recorded. Primary tumor volume (measured by caliper), weight, and incidence of regional (celiac or para-aortal) lymph node and liver metastases were recorded. Visible liver metastases were counted with the aid of a dissecting microscope and recorded, and the tissues were processed for hematoxylin/eosin (H&E) staining. Tissue not homogenized immediately for immunoblot analysis was snap-frozen in liquid nitrogen and immediately stored at -80^{o}C. For immunohistochemical staining, a part of the tumor was embedded in OCT compound (Miles, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at -80 ^{o}C.

Immunohistochemical Analysis. Frozen tissues used for identification of CD31/PECAM-1 were sectioned (8-10 μm), mounted on positively charged Plus slides (Fisher Scientific,UK), and air-dried for 30 min. The sections were fixed in cold acetone for 5 min, followed by 1:1 acetone:chloroform (vol:vol) for 5 min, and then acetone for 5 min. The sections were then washed with PBS and immunohistochemical staining for CD31 was performed as previously described [8]. A CD31-positive reaction was visualized by incubating the slides in stable 3,3'-diaminobenzidine (DAB) for 10-20 min. The sections were rinsed with distilled water, counterstained with Gill's hematoxylin for

1 min, and mounted with Universal Mount (Research Genetics, Huntsville, AL). Control samples were exposed to secondary antibody alone.

Paraffin-embedded tissues were used for identification of PCNA. Sections (4-6 μm thick) were mounted on positively charged Superfrost slides (Fischer Scientific, Co., Houston, TX) and dried overnight. Sections were deparaffinized in xylene and then treated with a graded series of alcohol [100, 95, and 80% ethanol (v/v) in double distilled H_20] and rehydrated in PBS (pH 7.5) Sections were treated with 10 mM citrate buffer, pH 6.0 and microwaved 10 min for "antigen' retrieval." Sections were blocked with 3% H_20_2 in PBS for 12 min and washed with PBS (3 x 5 min). Sections analyzed for PCNA were incubated with protein blocking solution containing 5% normal horse serum and 1% normal goat serum for 20 minutes and were then incubated with the appropriate primary antibody; anti-PCNA (1:100) overnight at 4ºC. A positive reaction was visualized by incubating the slides in stable 3,3'-diaminobenzidine (DAB) for 10-20 min. The sections were rinsed with distilled water, counterstained with Gill's hematoxylin for 1 min, and mounted with Universal Mount (Research Genetics, Huntsville, AL). Control samples were exposed to secondary antibody alone.

Paraffin-embedded tissues were used for identification of VEGF. Sections (4-6 □m thick) were mounted on positively charged Superfrost slides (Fischer Scientific, Co, Houston, TX) and dried overnight. Sections were deparaffinized in xylene and then treated with a graded series of alcohol [100, 95,and 80% ethanol (v/v) in double distilled H_20] and rehydrated in PBS (pH 7.5). Tissues were then treated with pepsin (Biomeda) for 15 min at 37ºC and washed with PBS (36)**.** A positive reaction was visualized by incubating the slides with stable 3,3'-diaminobenzidine for 10-20 min. The sections were rinsed and distilled water, counterstained with Gill's hematoxylin for 1 min., and mounted with Universal Mount (Research Genetics). Control samples exposed to secondary antibody alone showed no specific staining.

Immunostained sections were examined using a 40× objective (Zeiss Plan-Neofluar) on an epifluorescence microscope equipped with narrow bandpass excitation filters mounted in a filter wheel (Ludl Electronic Products, Hawthorne, NY) to individually select for green and red fluorescence. Images were captured using a cooled charge-coupled device camera (Photometrics, Tucson, AZ) and SmartCapture software (Digital Scientific, Cambridge, UK) on a Macintosh computer. Images were further processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA). Endothelial cells were identified by red fluorescence, and DNA fragmentation was detected by localized green and yellow fluorescence within the nuclei of apoptotic cells. Quantification of apoptotic endothelial cells was expressed as an average of the ratio of apoptotic endothelial cells to the total number of endothelial cells in 5–10 random 0.011-mm^2 fields at x 400 magnification.

Statistical Analysis. Pancreatic tumor volumes, numbers of CD31- and PCNA-positive cells, and percentages of apoptotic endothelial cells were compared by the unpaired Student's *t* test (or ANOVA). The incidences of liver metastasis between groups were compared using Fisher's exact test.

RESULTS

In Vitro Schedule of Chemoradiotherapy

Through the use of MTT analysis of pancreatic cells treated with varying doses of gemcitabine (chemotherapy), the optimal biological dose to eliminate 50% of the viable cells of the L3.6pl cell line was determined. The three trials are shown in Figure 1 a,b, and c. The optimal dosage of gemcitabine for the 24, 48, and 72 hour trials was 0.33, .011, and 6.80*10^-4 µm respectively by using the logarithmic equation for the best fit line from each graph as determined using Excel software package. The 72 hr gemcitabine (panel c) showed the most promising r- squared value; however the % control was stabilized at 0% at a very low does of gemcitabine. Therefore, the next best r-squared value was the 48 hour experiment (panel b). Therefore, in all subsequent experiments 0.11µm gemcitabine was used.

Figure 1: Gemcitabine Ic50

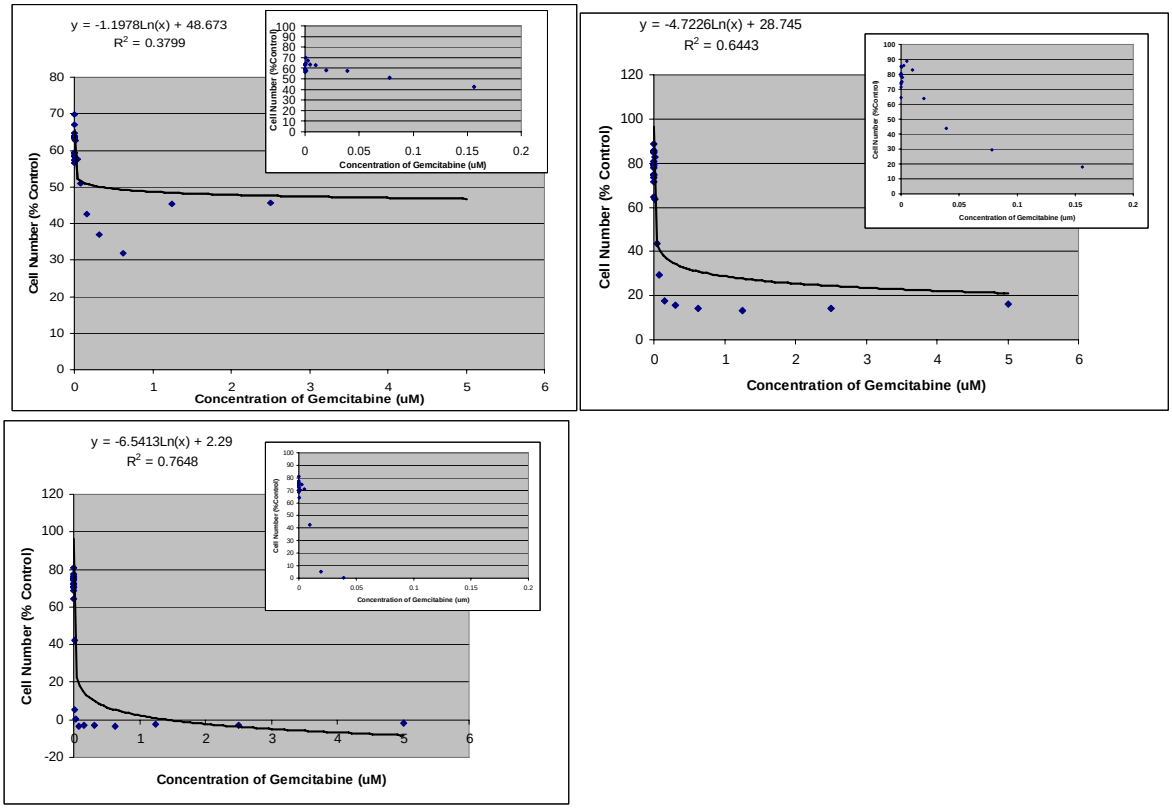

Figure 1: The cell number as a percent control versus chemotherapy concentration administered. Cell number was measured by MTT Assay (See Materials and Methods). Panel a is the effect on cell number of gemcitabine applied and measured 24 hours later. Panel b is the effect on cell number of gemcitabine applied and measured 48 hours later. Panel c is the effect on cell number of gemcitabine applied and measured 72 hours later. The insets of each panel represent the area from 0 to .2 µm gemcitabine for each time frame.

Next, the most effective schedule of administration of gemcitabine combined with radiation was evaluated. Figure 2 is a graphic representation of the chemoradiation therapy trials. The Varian linear accelerator was unable to do localized radiation and therefore internal negative control wells could not be included on the chemoradiotherapy plates. The radiation is able to affect the medium in which the cells are contained by

changing the pH, which is observed by a color change in phenol red containing medium. Therefore, comparing the chemoradiotherapies to a control without radiation may produce skewed results. In order to compare chemoradiotherapy to chemotherapy alone, internal control wells were kept, however they received radiation. When calculating the cell number as a percent control the control has received radiation and therefore the percent decrease is due only to the chemotherapy or the additive effect of the combined chemoradiotherapy.

Figure 2: Schematic Representation of Chemoradiotherapy schedules

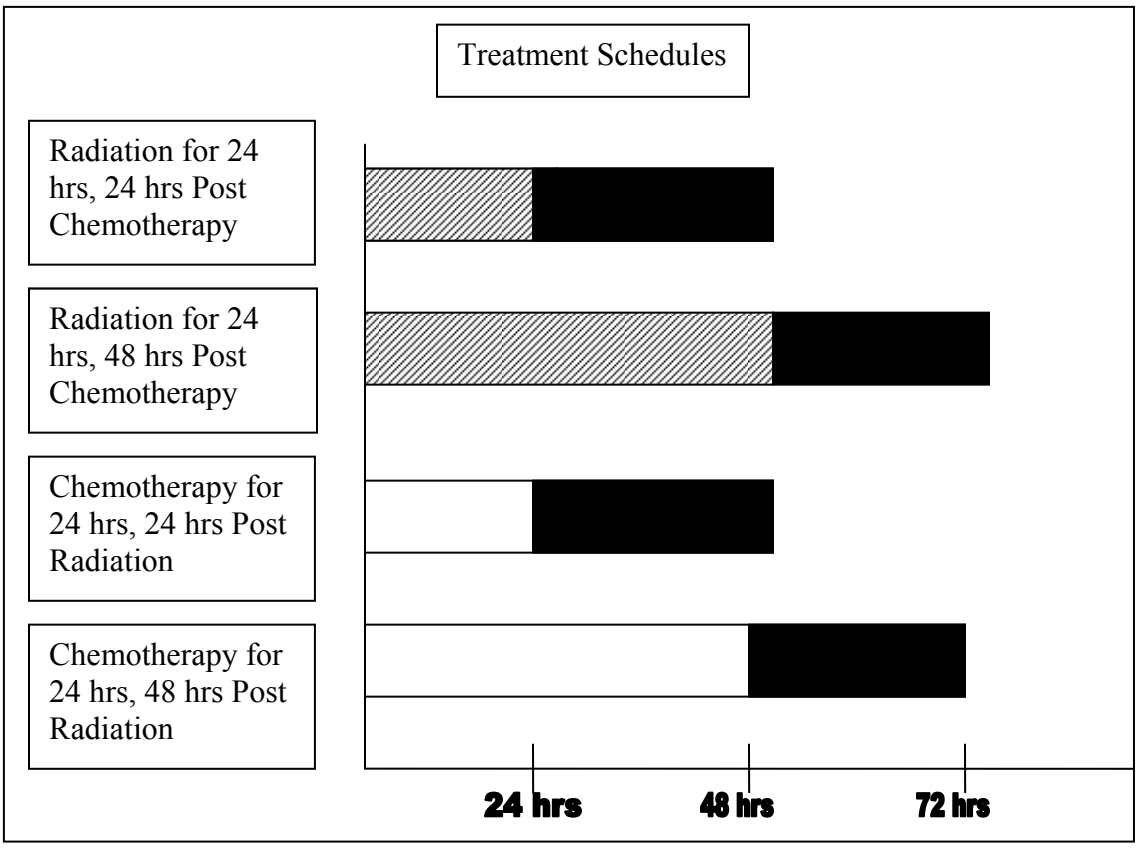

Figure 2: A schematic Representation of the combined chemoradiotherapy schedules. The striped boxes represent radiation, the white boxes represent chemotherapy, and the black boxes represent the combined therapy.

Table 1: MTT Results represented as a percent control. Chemoradiotherapies represented as a percent radiation control. n=1

Therapy	Cell Number as Percent Control
24 hr No Treatment Plate	100%
48 hr No Treatment Plate	100%
24 hr Radiation Plate	102%
48 hr Radiation Plate	97%
24 hr Chemotherapy Plate	84%
48 hr Chemotherapy Plate	61%
	Cell Number as Percent Radiation Control
Radiation for 24 hrs, 24 hrs Post Chemotherapy Plate	63%
Radiation for 24 hrs, 48 hrs Post Chemotherapy Plate	14%
Chemotherapy for 24 hrs, 24 hrs Post Radiation Plate	84%
Chemotherapy for 24 hrs, 48 hrs Post Radiation Plate	63%

We determined that administration of .011μM gemcitabine 48 hrs post administration of 12 Gy radiation caused a decrease in the cell count of human pancreatic cancer cells *in vitro* compared to chemotherapy or radiation alone (Table 1). Figure 3 is a comparison of the combined treatments versus the respective chemotherapy alone controls. The radiation for 24 hrs, 48 hrs post chemotherapy plate was omitted, because the chemotherapy alone control would have been 72 hours. However, a 72 hour chemotherapy control was not run and therefore, no comparison could be made. Nevertheless, prior experiments such as those in Figure 1 showed that 0.011 μm of chemotherapy would produce a decline in cell number by approximately 20%. Therefore, the 14% decrease in the combined therapy would likely not have represented a decrease from the potential chemotherapy alone plate.

Figure 3: Comparison of Chemotherapy and Chemoradiotherapy treatments

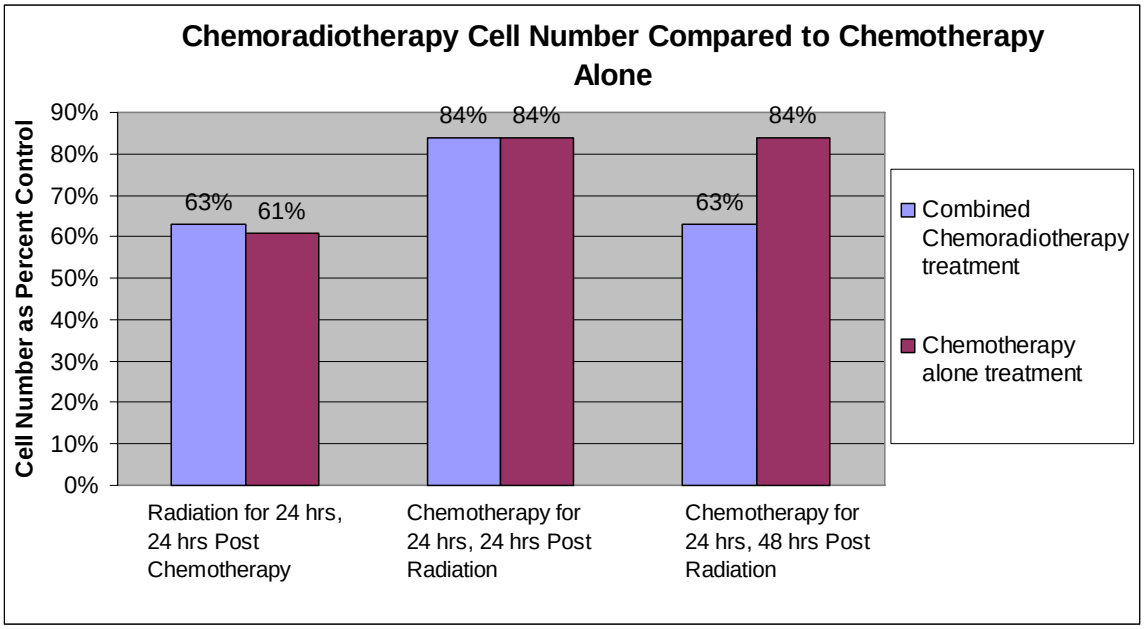

Figure 3: The cell number as a percent control of chemoradiotherapy treated cells versus chemotherapy treated cells. Cell number was measured by MTT Assay (See Materials and Methods) The first set of bars represent cell treated with radiation 24 hours post chemotherapy treatment, there was an insignificant change from the 48 hour chemotherapy treated cells. Bar two represents cells treated with chemotherapy 24 hours post radiation treatment, there was no change from the 24 hour chemotherapy treated cells. The last bar represents cells treated with chemotherapy 48 hours post radiation treatment, there was a 21% decrease in cell number from the 24 hour chemotherapy treated cells.

In Vivo Chemoradiotherapy Effects

Lastly, we evaluated the therapeutic effect of chemoradiotherapy in comparison to radiation and chemotherapy alone *in vivo*. L3.6pl cells were injected into the pancreas of nude mice. Seven days after injection the mice were randomized to receive one of four treatments: (1) twice-weekly i.p. administration of vehicle solution alone (control group); (2) twice-weekly i.p. administrations of 100 mg/kg gemcitabine; (3) once-weekly administration of radiation at 5Gy/2 min; (4) twice-weekly gemcitabine combined with radiation once weekly. The mice were treated for 5 weeks, sacraficed, and then necropsied. Results shown in Table 2.

Table 2: Necropsied Mice Pancreatic Tumor Data

Group	Body Weight (g)	Tumor Weight (g)	Tumor Volume (mm3)	Liver Mets (%)
Control	28.7+/- 1.4	1.2 +/- .3	249.7 +/- 90	75
Gemcitabine	28.7+/- .8	0.78 +/- .08	135.1 +/- 18.1*	43
Radiation	31.5+/- 1.1	0.41 +/- .12	60.3 +/- 28.5*	13
Gem/Rad	31.3 +/- 1.4	0.27 +/- .08	20.3 +/- 10.3*	17

*All treatments were found significantly different from the control group with a p value of < .01.

Necropsy showed that there was a tumor present in the pancreas of all the mice. Radiation and chemotherapy treatments alone decreased the tumor volume. The chemoradiotherapy also showed a decrease in tumor volume. An ANOVA showed a significant difference among treatment groups overall. A Student-Newman-Keuls post-hoc analysis (Table 3) showed the only significant difference was between all treatments

and the control ($p<.05$). However, the trend suggests that with larger sample sizes, significant differences might be observed between chemotherapy, radiation, and chemoradiotherapy. Therefore, further tests with larger groups are necessary.

Table 3: Student-Newman-Keuls Significance Test
Tumor Volume

	treatment	N	Mean Tumor Volume
			Treatment
Student-Newman-Keuls	both	6	20.333
	radiation	8	60.250
	chemo	7	135.143
	control	4	262.250
	Significance		0.052

Numbers shown are mean tumor volume for each treatment.

Additionally, IHC analyses were performed on the four different treatment groups to determine PCNA, CD31 and VEGF expression. The IHC analyses of the pancreas tumors revealed that tumor cell proliferation, represented by the PCNA staining, was barely detectable in the radiation and chemoradiotherapy treated mice. However, blood vessel growth represented by CD31, appeared to be unaffected in the radiation group while the amount of staining was reduced in the combination therapy groups. This was consistent with the VEGF expression which was high in the radiation group and low in the chemoradiotherapy. (Figure 4)

Figure 4: Immunohistochemistry Analysis of Necropsied Mice Pancreatic Tumors

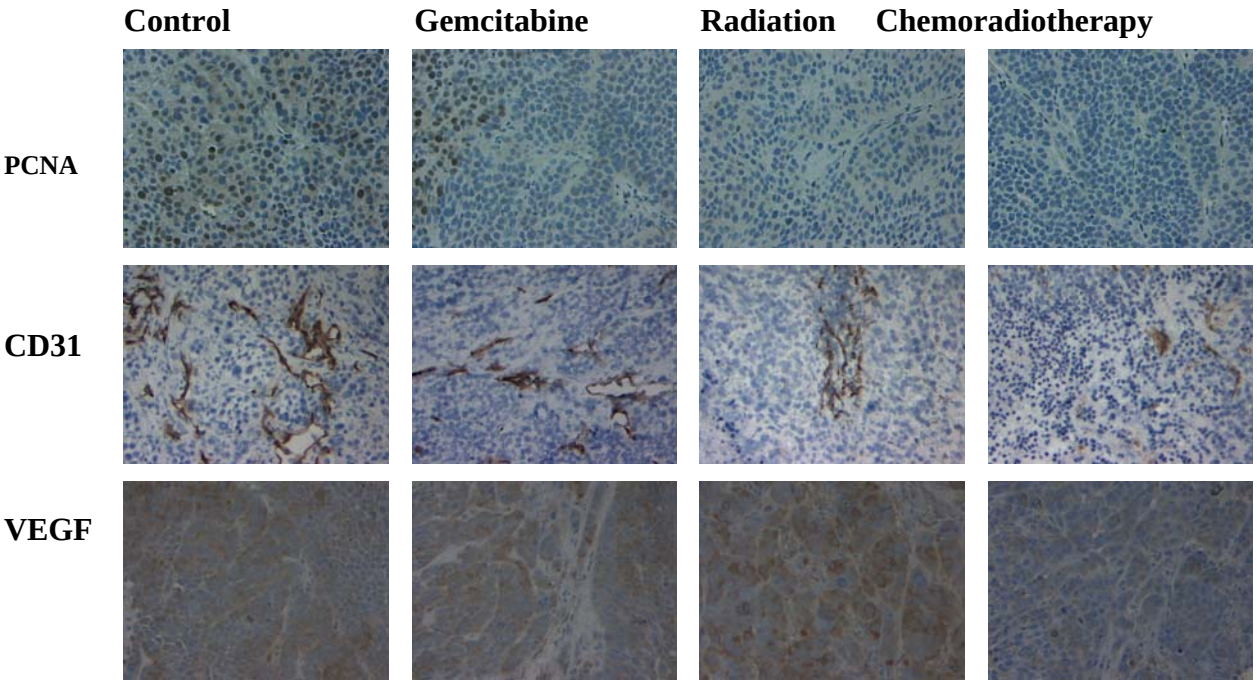

Figure 4: Immunohistochemistry analysis of mice pancreati . Immunohistochemistry performed as described in materials and methods.

DISCUSSION

Indeed the management of patients with locally advanced pancreatic cancer involves a multidisciplinary approach among the pancreatic surgeon, gastroenterologist, medical and radiation oncologist and the research team. The main consideration is whether a patient is ultimately a candidate for combined chemoradiotherapy (chemo-RT) or chemotherapy or radiation as a single agent. A number of studies have shown that both survival and palliative benefit can be improved when radiotherapy (RT) is combined with chemotherapy. However, the survival benefit is still modest and local control of the tumor remains a significant challenge. In this pilot study, we have investigated the effect of chemoradiotherapy on pancreatic cancer cells. Our data provide evidence that chemoradiotherapy treatments may be more effective in treating metastatic pancreatic cancer than either radiation or chemotherapy treatments alone by reducing tumor cell growth and decreasing the production of VEGF, which is responsible for the growth rate of blood vessel vasculature.

To determine the biological effects of varying chemoradiotherapies on cultured pancreatic cancer cells, we tested the treatment schedules by varying the time and schedule of radiation and chemotherapy administration. The cell number decrease in the combination therapies was calculated as a percentage of the internal control. The internal control on the plate received radiation, as it was not possible to administer radiation to only a section of the plate. Therefore, the cell number decrease was due to only the chemotherapy and the additive effects of the combined treatment. The only therapy that showed a significant change from the chemotherapy alone treatment was radiation for 48 hours followed by chemotherapy. This finding suggests that the scheduling of treatments can be an important factor in cytotoxicity. Perhaps the radiation administrations had damaged or mutated the DNA and given the cells time to divide, and therefore the daughter cells were more easily targeted by the chemotherapy.

To address whether the *in vitro* results would correlate to those *in vivo*, we evaluated the therapeutic effect of chemoradiotherapy in mice. Although the tumor volumes in the chemotherapy and chemoradiotherapy treatment groups were not significantly different, the extreme range in tumor size among the different treatment group suggests that more experiments should be performed to further investigate the potential effects of combined treatment regimens.

Vasculature in tumor cells has shown a significant correlation with the ability of a tumor to grow and increase in size. Vascular endothelial growth factors (VEGFs), produced by the tumor cells, induce the tumor vasculature. Therefore, the level of VEGFs was determined in each treatment group to determine whether chemoradiotherapy treatments could have an effect on tumor vasculature. The immunohistochemistry results showed a reduced staining of VEGF and CD31 in response to chemoradiotherapy. CD31 is a vasculature cell marker. There is an obvious decrease in blood vasculature in the chemoradiotherapy treatment groups as compared to the control and single treatment groups. The decrease in VEGF and vasculature in the tumors will limit the cells access to circulation and nutrients. Thus both tumor metastases and tumor cell growth will likely be limited by diminished tumor angiogenesis. This concept of anti-angiogenesis is a widely studied area in cancer therapies today, and this pilot study suggests that combined therapy regimens may contribute to anti-angiogenesis protocols.

Despite refinements is RT techniques and the use of radiosensitizing agents (chemotherapies), pancreatic cancer remains a radiation-and chemotherapy-resistant disease with high rates of local and distant failures. Evidently, a more effective treatment schedule is necessary in order to extend the median survival of patients diagnosed with pancreatic cancer. These results show that chemoradiotherapy given in optimal dosages may be a more effective cancer treatment than either treatment alone. The *in vitro* data clearly demonstrate that different treatment schedules of chemoradiotherapy can produce varying results. In addition, the *in vivo* results provided evidence for reduced VEGFs levels, reduced tumor vasculature, and decreased cell proliferation in the combined treatment group.

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