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CERIUM OXIDE NANOPARTICLES SENSITIZE PANCREATIC CANCER CELLS TO RADIATION BY PROMOTING ACIDIC PH, ROS AND JNK DEPENDENT APOPTOSIS

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Sciences in the College of Graduate Studies at the University of Central Florida Orlando, Florida

Spring Term 2013

Major Professor: Jihe Zhao
ABSTRACT

Side effects of radiation therapy (RT) remain the most challenging issue for pancreatic cancer treatment. In this report we determined whether and how cerium oxide nanoparticles (CONPs) sensitise pancreatic cancer cells to RT. CONP pretreatment enhanced radiation-induced reactive oxygen species (ROS) production preferentially in acidic cell-free solutions as well as acidic human pancreatic cancer cells. In acidic environments, CONPs favor the scavenging of superoxide radical over the hydroxyl peroxide resulting in accumulation of the latter whereas in neutral pH CONPs scavenge both. CONP treatment prior to RT markedly potentiated the cancer cell apoptosis both in culture and in tumors and the inhibition of the pancreatic tumor growth without harming the normal tissues or host mice. Mechanistically, CONPs were not able to significantly impact RT-induced DNA damage in cancer cells, thereby ruling out sensitization through increased mitotic catastrophe. However, JNK activation, which is known to be a key driver of RT-induced apoptosis, was significantly upregulated by co-treatment with CONPs and RT in pancreatic cancer cells in vitro and human pancreatic tumors in nude mice in vivo compared to CONPs or RT treatment alone. Further, CONP-driven increase in RT-induced JNK activation was associated with marked increases in Caspase 3/7 activation, indicative of apoptosis. We have shown CONPs increase ROS production in cancer cells; ROS has been shown to drive the oxidation of thioredoxin (TRX) 1 which results in the activation of Apoptosis Signaling
Kinase (ASK) 1. The dramatic increase in ASK1 activation following the co-treatment of pancreatic cancer cells with CONPs followed by RT in vitro suggests that increased the c-Jun terminal kinase (JNK) activation is the result of increased TRX1 oxidation. The ability of CONPs to sensitize pancreatic cancer cells to RT was mitigated when the TRX1 oxidation was prevented by mutagenesis of a cysteine residue, or the JNK activation was blocked by an inhibitor. Additionally, angiogenesis in pancreatic tumors treated with CONPs and RT was significantly reduced compared to other treatment options. Taken together, these data demonstrate an important role and mechanisms for CONPs in specifically killing cancer cells and provide novel insight into the utilization of CONPs as a radiosensitizer and therapeutic agent for pancreatic cancer.
This work is dedicated to the memory of my grandfather

who was lost to pancreatic cancer.

Raymond McCarthy

March 18, 1930 – January 10, 1989
ACKNOWLEDGMENTS

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TABLE OF CONTENTS

LIST OF FIGURES..........................................................................................................................xi
LIST OF TABLES...........................................................................................................................xii
LIST OF ABBREVIATIONS..............................................................................................................xiii
CHAPTER 1: GENERAL INTRODUCTION.........................................................................................1
Cancer .............................................................................................................................................1
Pancreatic Cancer ............................................................................................................................1
Current Treatment Options ............................................................................................................2
  Surgery ..........................................................................................................................................3
  Chemotherapy ...............................................................................................................................3
  Ionizing Radiation Therapy ...........................................................................................................4
  Combination Therapy: Chemoradiation .........................................................................................5
Nanoparticle Based Cancer Therapies ...............................................................................................6
  Cerium Oxide Nanoparticles .......................................................................................................7
CHAPTER 2: SENSITIZATION OF PANCREATIC CANCER CELLS TO RADIATION
BY CERIUM OXIDE NANOPARTICLE-INDUCED ROS PRODUCTION ........................................14
Introduction .....................................................................................................................................14
Methods and Materials...................................................................................................................16
  Cell Culture and Reagents ..........................................................................................................16
  Transmission Electron Microscopy of CONPs ..............................................................................17
  ROS Imaging ...............................................................................................................................17
  Cell Viability Assays ..................................................................................................................18
  Clonogenic Assays .....................................................................................................................18
  Hydrogen Peroxide Assays .........................................................................................................18
  Intracellular Acidity Assay .........................................................................................................19
  Superoxide Radical and Hydrogen Peroxide Scavenging Analysis ............................................19
  Orthotopic Injection of Pancreatic Cancer Cells in Athymic Nude Mice .....................................20
  Therapy of Established Human Pancreatic Carcinoma Tumors Grown in the Pancreas of Nude Mice ..........................................................................................................................20
  Histologic Analysis of Tumors .....................................................................................................21
  Statistical Analysis .....................................................................................................................22
Results ..........................................................................................................................................22
CONPs facilitate RT-Induced Hydrogen Peroxide Production Favoring Lower than Normal pH ................................................................. 22
CONPs Increase RT-Induced ROS Levels in Pancreatic Cancer Cells .......... 24
CONPs Enhance RT-Induced Cell Death in Pancreatic Cancer Cells .......... 26
CONPs Enhance Growth Inhibition and Apoptosis Induced by RT in vivo in Human Pancreatic Tumors .......................................................... 26
Discussion .......................................................................................... 27
Conclusion ......................................................................................... 31
Figures and Tables ........................................................................... 32
CHAPTER 3: CERIUM OXIDE NANOPARTICLES DO NOT INCREASE RADIATION-INDUCED DNA DAMAGE IN PANCREATIC CANCER CELLS .............. 39
Introduction ....................................................................................... 39
Methods and Materials ...................................................................... 40
Cell Culture and Reagents ................................................................. 40
Comet Assay .................................................................................... 41
Results ................................................................................................ 41
CONPs Do Not Impact RT-Induced DNA Damage in Pancreatic Cancer Cells in vitro ....................................................................... 41
CONPs Inhibit RT-Induced DNA Damage in Normal Pancreatic Epithelial Cells in vitro ....................................................................... 42
Discussion ......................................................................................... 42
Conclusion ......................................................................................... 45
Figures ............................................................................................... 46
CHAPTER 4: CERIUM OXIDE NANOPARTICLES SENSITIZE CANCER CELLS TO RADIATION BY INDUCING ROS ACTIVATION OF JNK AND APOPTOSIS ....... 47
Introduction ....................................................................................... 47
Methods and Materials ...................................................................... 49
Cell Culture ....................................................................................... 49
Generation of Stable Cell Lines ............................................................. 50
Phospho-ELISA .................................................................................. 50
Antibodies, Drugs, and Western Blot ...................................................... 51
P-ASK1 Immunostaining ..................................................................... 51
Caspase Activation Analysis ................................................................. 52
JNK Inhibitor Studies ......................................................................... 52
Orthotopic Injection of Pancreatic Cancer Cells in Athymic Nude Mice .......... 53
Therapy of Established Human Pancreatic Carcinoma Tumors Grown in the Pancreas of Nude Mice ................................................................. 54
Immunohistochemical Analysis ..................................................................... 54
Statistical Analysis ......................................................................................... 55
Results ........................................................................................................... 56
CONPs Drive RT-Induced JNK Activation in Cancer Cells ......................... 56
CONP-Driven JNK Activation Mediates RT-Induced Apoptosis in Pancreatic Cancer Cells................................................................. 57
CONPs Drive RT-Induced ASK1 Activation in Pancreatic Cancer Cells ........ 58
CONPs Increase JNK Activation and Apoptotic Signaling in Vivo ............... 59
JNK Inhibition Mitigates CONP-Induced Radiosensitization of Pancreatic Cancer Cells ..................................................................................... 60
TRX1 Oxidation is Required for CONP-Induced Radiosensitization of Pancreatic Cancer Cells ................................................................. 61
Discussion ...................................................................................................... 62
Conclusion ....................................................................................................... 66
Figures ............................................................................................................. 67
CHAPTER 5: CERIUM OXIDE NANOPARTICLES DOWNREGULATE ANGIOGENESIS IN PANCREATIC TUMORS ................................................................. 73
Introduction ...................................................................................................... 73
Methods and Materials .................................................................................. 75
  Antibodies and Reagents ........................................................................... 75
  Orthotopic Injection of Pancreatic Cancer Cells in Athymic Nude Mice .... 75
  Therapy of Established Human Pancreatic Carcinoma Tumors Grown In the Pancreas of Nude Mice ............................................................. 76
  Staining for Angiogenesis ......................................................................... 76
Results ............................................................................................................. 77
  Combination Therapy With CONPs and RT Decreases Angiogenesis in Pancreatic Tumors ................................................................. 77
Discussion ...................................................................................................... 78
Conclusion ....................................................................................................... 79
Figures ............................................................................................................. 80
CHAPTER 6: CONCLUSION ............................................................................. 81
Impact ......................................................................................................................... 81
Moving Forward ........................................................................................................ 82
  Pancreatic Cancer .................................................................................................... 82
  Cerium Oxide Nanoparticle Therapies ................................................................. 83
ENDNOTES .............................................................................................................. 85
PUBLICATIONS ......................................................................................................... 85
APPENDIX A: COPYRIGHT PERMISSION .............................................................. 86
APPENDIX B: SUPPLEMENTAL FIGURES .............................................................. 96
APPENDIX C: PROTOCOLS ...................................................................................... 107
APPENDIX D: IACUC APPROVAL LETTERS .......................................................... 130
APPENDIX E: DISSERTATION DEFENSE ANNOUNCEMENT ............................... 133
REFERENCES .......................................................................................................... 136
LIST OF FIGURES

Figure 1. Acidic pH is relatively resistant to H₂O₂ production compared to neutral pH. 32
Figure 2. At neutral pH CONPs generally decrease RT-induced H₂O₂ production........ 33
Figure 3. Under acidic conditions CONPs enhance H₂O₂ production and lose H₂O₂ scavenging activity. ................................................................. 34
Figure 4. CONP treatment prior to, but not post, RT increases RT-induced ROS levels in acidic pancreatic cancer cells and decreases RT-induced ROS levels in neutral pancreatic normal cells................................................................. 35
Figure 5. CONP pre-treatment selectively sensitizes pancreatic cancer cells to RT-induced cell death in culture................................................................. 36
Figure 6. CONPs enhance tumor cell apoptosis in vivo. ........................................ 37
Figure 7. CONPS do not alter RT-induced DNA damage in pancreatic cancer cells. ... 46
Figure 8. CONPs drive RT-induced JNK activation in pancreatic cancer cells......... 67
Figure 9. CONP-enhanced JNK activation induces apoptosis in pancreatic cancer cells. .............................................................................................................. 68
Figure 10. CONP pre-treatment increases RT-induced ASK1 activation in pancreatic cancer cells. ............................................................................................................. 69
Figure 11. CONP-induced JNK activation results in apoptosis in pancreatic tumors. ... 70
Figure 12. CONPs are unable to drive RT-induced apoptosis in the absence of JNK function............................................................. 71
Figure 13. CONPs are unable to drive sensitization in the absence of TRX1 oxidation. 72
Figure 14. CONP treatment decreases blood vessel formation in pancreatic tumors. .. 80
LIST OF TABLES

Table 1. CONPs increase sensitivity to radiation-induced inhibition of human pancreatic tumor growth. ........................................................................................................................................... 38
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>ASK1</td>
<td>Apoptosis Signaling Kinase 1</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Tissue Culture</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>CD-31</td>
<td>Cluster of Differentiation 31</td>
</tr>
<tr>
<td>Ce</td>
<td>Cerium</td>
</tr>
<tr>
<td>CeO₂</td>
<td>Cerium Oxide</td>
</tr>
<tr>
<td>CONPs</td>
<td>Cerium Oxide Nanoparticles</td>
</tr>
<tr>
<td>ctrl</td>
<td>Control</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Minimal Essential Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Regulated Kinase</td>
</tr>
<tr>
<td>FR</td>
<td>Folate Receptor</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3 Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hemotoxylin and Eosin</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Buffered Saline Solution</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>hrs</td>
<td>Hours</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>JNK</td>
<td>C-jun Terminal Kinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Image</td>
</tr>
<tr>
<td>NIH</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>O</td>
<td>Oxygen</td>
</tr>
<tr>
<td>p-ASK1</td>
<td>Phosphorylated Apoptosis Signaling Kinase 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>p-JNK</td>
<td>Phosphorylated c-Jun Terminal Kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RT</td>
<td>Radiation Therapy</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>SAHA</td>
<td>Suberoylanilide Hyroxamic Acid</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide Dismutase</td>
</tr>
<tr>
<td>SOD2</td>
<td>Superoxide Dismutase 2</td>
</tr>
<tr>
<td>SPIONs</td>
<td>Superparamagnetic Iron Oxide Nanoparticles</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TiO₂</td>
<td>Titanium Oxide</td>
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</table>
TRX1  Thioredoxin 1
VEGF  Vascular Endothelial Growth Factor
Δ     change
CHAPTER 1: GENERAL INTRODUCTION

Cancer

Cancer, which remains one of the leading causes of death, is responsible for causing one out of every four deaths in the United States each year (1). Over the last 40 years, medical and technological advances have allowed for improvements in early diagnosis and the subsequent treatment of patients. As a result, the 5-year survival rate for most cancers has markedly increased in the last few decades (1). Regardless, in 2012 alone, over 1.6 million new cases of cancer were expected and over 500,000 lives were lost to the disease (1). Therefore, development of new and more effective therapies remains essential and challenging.

Pancreatic Cancer

While the prognosis for other disease sites, such as breast and colon, has significantly improved in recent years, pancreatic cancer patients still face a dismal outlook (2); pancreatic cancer survival has not shown much improvement over the last 30 years (1). Pancreatic cancer, while representing only 6% of the new cancer diagnoses in the United States, is projected to cause 13% of all cancer related deaths and claimed nearly 40,000 lives in 2012 alone (1). Pancreatic cancer remains one of the few cancers where the number of new diagnoses each year and number of annual projected deaths from the disease are nearly identical (1), illustrating the devastating nature of the disease, as well as the inadequacy of the current treatment options after diagnosis.
Pancreatic cancer is characterized by several symptoms, often as common as abdominal pain, back pain, or the development of diabetes (3). Other symptoms, such as painless jaundice or the pain associated with the compression of the coeliac and mesenteric nerves, are more readily associated with pancreatic cancer and generally result in a quick diagnosis (3). Tissue biopsy to confirm the diagnosis is usually obtained after the identification of a pancreatic mass (3). Once the cancer is officially diagnosed, treatment options are assessed.

Current Treatment Options

Cancer treatment can have multiple goals. In patients diagnosed with the early stages of the disease, doctors strive to cure the disease. In patients with advanced, late stage cancers, the treatments are often palliative, intended to extend the life of a patient and increase comfort, rather than actually remedy the disease (4). However, a common goal between the two types of treatment is the maintenance of quality of life for patients, both during treatment and potentially after treatment (4). It is estimated that pain is a symptom of up to 70% of cancers, with pain stemming from primary tumor growth and metastasis (5). Additionally, patients often face adverse side effects from treatment, including pain, nausea, fatigue, hair loss, and skin irritation. Managing these factors has become increasingly important as the intensity of treatment has increased over the last few decades.

Pancreatic cancer is one disease that is particularly difficult to treat, as pancreatic cancers are often resistant to many of the currently available treatments (3).
Over 75% of pancreatic cancer patients present with inoperable disease as a result of the fact that pancreatic cancer generally disseminates to distant sites early in the disease progression (3). The primary tumor is not usually the killer in pancreatic cancer patients; nearly all pancreatic cancer patients develop metastases and die of the devastating effects of uncontrolled metastatic growth (3). Despite the number of advances in cancer treatment and the vast array of available drugs, treatments are still generally separated into three main categories: surgery, chemotherapy, and radiation therapy.

**Surgery**

Surgical resection remains the gold standard for cancer treatment. However, successful resection does not correlate with increased survival in pancreatic cancer patients (3). Most pancreatic cancer patients that undergo surgical resection die from recurrence of the disease in distant metastatic sites, indicating that surgery alone is an inadequate treatment for pancreatic cancer (2). Preoperative and postoperative chemoradiation has been added to the treatment plan for many patients, with some success (3).

**Chemotherapy**

Chemotherapy treats cancer by attempting to kill cancer cells with cytotoxic drugs. Currently, pancreatic cancer patients receive treatment including fluorouracil, cisplatin, or paclitaxel, with gemcitabine being the standard for metastatic pancreatic cancer (3). However, selectivity of chemotherapy agents remains a significant problem
with nearly 50% of patients requiring hospitalization with this approach due to extreme drug toxicity (3). Chemotherapeutic agents kill cancer in a number of ways, from binding and crosslinking DNA to pyrimidine analogues which get incorporated into DNA and induce cell cycle arrest. Reports indicate that current chemotherapy options will not cure metastatic pancreatic cancer (3) which, as discussed, characterizes the majority of patients at the time of diagnosis. Therefore, the potential benefits of chemotherapy must be dutifully considered against the toxic side effects. New, more effective chemotherapy agents could have a substantial impact on pancreatic cancer treatment.

*Ionizing Radiation Therapy*

Ionizing radiation therapy (RT) is a commonly used tool to treat various types of cancer, with more than half of all cancer patients receiving RT at some point during treatment (6). RT kills cells by transferring energy to cellular components to break down chemical bonds resulting in DNA damage, protein damage, and the generation of reactive oxygen species (ROS) (6). RT has shown distinct survival benefits in most cases (6) and, although not yet thoroughly investigated, studies have suggested that RT decreased the pain associated with advanced pancreatic cancer in more than 50% of patients receiving the treatment (3).

However, radiation has also been associated with negative effects for cancer patients; pain and nausea are often associated with the treatment. Radioprotectants, compounds designed to protect normal tissues from the harmful side effects of RT thereby increasing the therapeutic window, have shown some benefits. Yet, Amifostine
continues to be the only radioprotective agent actively used in clinics (7). Amifostine is itself associated with toxic side effects and requires repeated dosing. There is a clear need for new radiation adjuvants.

Conflicting opinions exist about the long term effects of RT, with several reports indicating that the population of cells which survive RT develops a more aggressive phenotype characterized by survival, invasion, and metastasis (6). Additionally, radiation has been shown to promote survival by driving angiogenesis in tumors (6). Other non-targeted responses to RT have also become a concern, as RT has been shown to activate pathways not associated with DNA damage. However, the identification and characterization of the non-targeted responses could provide novel targets to improve RT for patients.

*Combination Therapy: Chemoradiation*

The combination of chemotherapy and radiation therapy, commonly referred to as “chemoradiation,” is the standard treatment for locally advanced and inoperable pancreatic cancer (3). Research suggests that the combination of radiation therapy with chemotherapy agents enhances the efficacy of treatment (6). Chemoradiation offers significant survival, in some cases as much as doubling the resulting survival time when a chemotherapy agent is added to existing radiation treatment options (3). Chemoradiation is also associated with palliative benefits, thereby improving the quality of life of many pancreatic cancer patients (3).
One of the main goals for combining RT and chemotherapy is to sensitize cancer cells to the toxic effects of treatment (radiosensitization). Radiosensitizers act through numerous mechanisms. Suberoylanilide hydroxamic acid (SAHA), which is a histone deacetylase inhibitor, relaxes the DNA structure and increase exposure to RT (8). Other chemotherapy agents, such as Gemcitabine, have also been associated with sensitization of pancreatic cancer cells to RT (3). The approach of combining chemotherapeutics and radiation has shown promise in the treatment of many cancers. However, current chemoradiation options rarely control advanced pancreatic cancers for extended periods of time, with tumor progression and metastasis occurring in most patients within several months of the completion of treatment (3). Therefore, there is a desperate need for novel therapies and adjuvants in the treatment of pancreatic cancer to improve and extend the lives of patients.

Nanoparticle Based Cancer Therapies

In recent years, nanotechnology has become a main focus of biomedical research; nanoparticles applications include drug delivery systems, luminescent biomarkers, and tissue engineering, among others (9). Nanomedicine, as the application of nanoparticles to medicine is commonly referred, aims to overhaul the current treatment of diseases with more effective therapies (10). Nanoparticle encapsulation has been shown regulated tissue and cellular distribution of many drugs (11). Tumors also possess abnormal physiological pathologies, such as hypervascularization, that result in unique interactions with nanoparticles (11).
Therefore, converting current cytotoxic agents into nanoparticle based therapies is of particular interest in the context of cancer for the potential to increase tumor growth inhibition and cell death, yet reduce systemic side effects (11).

Beyond the common cytotoxic agents, several inorganic nanoparticles have shown biocompatibility and are currently being investigated in the context of cancer treatment. Superparamagnetic iron oxide nanoparticles (SPIONs) and titanium oxide (TiO\textsubscript{2}) nanoparticles have shown promise in testing as magnetic resonance imaging (MRI) contrast agents to detect tumors (10). Research indicates gold nanoparticles possess inherent anti-tumor and anti-angiogenic properties (10). Additionally, gold nanoparticles have been developed as targeted drug delivery systems in cancers overexpressing epidermal growth factor receptors (EGFR) or folate receptors (FR) (12). The class of inorganic nanoparticles and the applications for cancer are rapidly being explored with largely positive results.

*Cerium Oxide Nanoparticles*

In particular, cerium oxide nanoparticles (CONPs), which consist of a cerium (Ce) core surrounded by an oxygen (O) lattice, have shown promise in a number of cancer related applications. Initial interest in CONPs stemmed from the ability of surface oxygen vacancies to interact with and potentially modulate free radicals. CONPs have since been shown to display a number of antioxidant behaviors, including superoxide dismutase (SOD) activity (13), catalase mimetic activity (14), nitric oxide radical scavenging (15), and hydroxyl radical scavenging (16). However, environmental
conditions appear to play an important role in the determination of activity, as CONPs also possess direct oxidant behavior (17). To date, pH is one of the few factors shown to drive whether CONPs act as oxidants or antioxidants (18,19). Surely, there are other, yet unidentified, factors that will also play a role in determining the manifestation of radical modulation by CONPs.

**Uptake and Localization**

CONPs have been shown to enter mammalian cells in both normal and diseased states (20-22), with significant uptake occurring within 3 hours of exposure in culture (23). CONPs appear to take multiple routes into cells. Uptake has been suggested to occur via receptor-mediated endocytosis in both lung cancer and normal lung cell lines (24). Other studies have shown CONP uptake via clathrin-mediated and calveolae-mediated endocytic pathways (23). Particle size and surface charge have been shown to influence cellular uptake of CONPs, but the impact of factors such as pH and cell type has yet to be fully elucidated in the context of CONP uptake.

There is also some debate about the fate of CONPS once inside the cell. Some studies show CONPs accumulate in the cytoplasm without translocation to the nucleus (20,21). Other studies have demonstrated CONPs accumulate primarily in the perinuclear space (25), while still others detected CONPs co-localized with the mitochondria, endoplasmic reticulum, lysosomes, as well as diffuse throughout the cytoplasm and nucleus (23). Particle size and surface charge also appear to be determinants of CONP cellular localization (18). As the differential pH of various sub-
cellular localizations has been shown to be a determinant CONPs' anti- or pro-oxidant activity (18), discovering where CONPs predominantly reside in the cell or why they localize to different sub-cellular locations in different cell types may provide further insight into their function. Manipulation of CONPs to target specific cells or sub-cellular locations is a path that has yet to be fully elucidated and exploited.

_Biodistribution and Biopersistence_

Several reports have shown CONPs (<10 nm) to be well tolerated by animals without inducting overt toxicity or an immune response across a range of doses (26-28). When administered intravenously (IV) or intraperitoneally (IP), studies show that CONPs accumulate primarily in the spleen and liver, to a lesser extent in the lungs and kidneys, but not in the heart or brain (26,29). Tissues such as the breast and pancreas have not been analyzed for retention, yet nearly half of the injected CONPs remained in undetermined locations within the body (29). Further, CONPs were not readily cleared, persisting in the animals at least 30 days without any appreciable CONP concentration in the urine or feces (26,29), suggesting that other CONP destinations within the body have yet to be identified.

_Toxicity_

Despite the apparent lack of toxicity in animal models, reports provide conflicting data about the toxicity of CONPs _in vitro_, likely attributable to the impact of undetermined cellular and environmental factors on the manifestation of anti- or pro-oxidant behavior. CONPs are toxic to bronchial epithelial lung fibroblasts in culture (25),
but non-toxic to mammary epithelial cells (30), macrophages (31), or immortalized keratinocytes (23). In normal cells to which they are not toxic, the physiological pH is an environment which enables canonical radical scavenging by CONPs. Therefore, CONPs introduced prior to ROS insult confer protection from the effects of oxidative stress in vitro and in vivo (32-35).

However, published data indicates that CONPs are toxic to several types of human cancer cells in vitro, including squamous cell carcinoma (20) and alveolar epithelial cancer cells (36). Cellular toxicity is attributed to the generation of ROS (19,20) and the induction of oxidative stress (36), at least in part by the inherent oxidase activity of the nanoparticle core at acidic pH similar to that of cancer cells (17). In particular, CONP treatment has been shown to induce glutathione oxidation, lipid peroxidation, and membrane damage in lung cancer cells (36). Further experiments have demonstrated that generation of CONPs with a negative surface charge can induce preferential accumulation in acidic lysosomes within the cell, resulting in increased toxicity selectively in cancer cells (18).

Cancer Treatment

Anti-invasive Properties:

In addition to CONPs’ toxicity to cancer cells in vitro and in vivo, studies have shown polymer-coated CONPs to also manipulate tumor-stroma interactions to the detriment of tumor progression and invasion (20). Polymer coating of CONPs increases aqueous solubility (18), yet does not appear to impact CONP redox activities (17,20).
Epithelial-stromal signaling is largely mediated by myofibroblasts, which play a key role in the expression of extracellular matrix components, including α-smooth muscle actin and collagen, to facilitate tumor invasion and angiogenesis (37). With the transition from fibroblast to myofiberblast driven by transforming growth factor beta 1 (TGFβ1) - induced ROS-dependent expression of α-smooth muscle actin, data shows CONPs possess the ability to modulate myofibroblast formation (20). Pre-treatment with CONPs mitigated both TGFβ1-induced α-smooth muscle actin expression in fibroblasts and the corresponding myofibroblast transition (20). As some myofibroblasts localize to the invasion front of tumors, CONP treatment diminished the ability of myofibroblasts to induce invasion by squamous tumor cells in vitro (20). Interestingly, CONPs were also able to decrease the intrinsic ability of cultured squamous tumor cells to invade, even in the absence of any myofibroblast stimulation (20). Taken together, these data demonstrate the direct negative effects of CONPs on cancer cells, as well as their ability to modulate the tumor environment and indirectly inhibit tumor cell invasion.

Radioprotection

The ability of CONPs to modulate ROS has led to their exploration for the improvement of current cancer treatments, mainly radiation therapy (RT). As discussed, in addition to surgery and chemotherapy, RT remains a mainstay in the treatment of cancer, with nearly half of all cancer patients receiving RT at some point during treatment (38). Despite the many harmful side effects are associated with the RT, including fatigue, nausea, and dermatitis, few radiation adjuvants are available to
mitigate these painful outcomes. For example, Amifostine, which remains the only clinically available radio-protectant (38), is itself associated with nausea and hypotension (39). The dual capabilities of CONPs to act as an antioxidant in normal cells, yet oxidant in cancer cells, supports the role of CONPs as an adjuvant for RT that could significantly impact patient quality of life.

In line with the protection from other methods of inducing oxidative stress, several publications have shown that treatment with CONPs prior to RT exposure decreases the RT-induced cell damage and death in normal tissues of the gastrointestinal tract (28), lung (27), breast (30), and head and neck (7). Mechanistically, CONP radical scavenging inhibited the resulting caspase 3 activation in irradiated colonic crypt tissue (28), as well as capsase 3 and 7 activation in irradiated lung fibroblasts in culture (27). CONPs also increased super oxide dismutase 2 (SOD2) expression up to two-fold in a dose dependent manner in normal human colon cells in vitro, while increasing SOD2 expression by 40% in colonic crypt cells from mice treated with CONPs (28). Together, these data indicate that CONPs protect normal cells both directly, by scavenging cellular ROS, and indirectly, by priming cells to respond to ROS insult.

**Radiosensitization**

Conversely, in cancer cells with acidic pH, pre-treatment with CONPs has been predicted (18) to enhance the ability of RT to induce cell death. As acidic pH has been shown to inhibit the catalase activity of CONPs (20), it is suggested that CONPs in
cancer cells are only capable of catalyzing the conversion of highly unstable superoxide to far more stable H₂O₂. Without the ability to act as a catalase mimetic and remove H₂O₂, CONPs could enhance the toxicity of RT in cancer cells by encouraging the accumulation of stable ROS in the cell, which has been shown to induce cell death. Overall, it is suggested that CONPs modulate ROS in cancer cells such that, not only are there direct toxic effects, but the therapeutic properties of CONPs extend to radio-sensitization of cancer cells and potentially sensitization to other ROS-inducing therapies.
CHAPTER 2: SENSITIZATION OF PANCREATIC CANCER CELLS TO RADIATION BY CERIUM OXIDE NANOPARTICLE-INDUCED ROS PRODUCTION

Introduction

Pancreatic cancer carries an extremely poor prognosis, with 90% of pancreatic cancers being malignant and the 5-year survival rate after diagnosis hovering at 5% (40). Less than 20% of patients are candidates for surgical resection (40); therefore, chemotherapy and radiation therapy (RT) remain the only other treatment options. Ionizing radiation used in RT induces the radiolysis of water which generates reactive oxygen species (ROS), such as superoxide and hydroxyl radicals. These molecules play an important role in the subsequent cellular events such as DNA damage potentially leading to apoptosis. Unfortunately, RT induces side effects, including skin irritation, loss of appetite, fatigue, and nausea, as well as the pain associated with these conditions.

Research to reduce the unwanted side effects of RT has yielded two categories of compounds: radiation protectants and radiation sensitizers. Radiation protectants selectively protect normal tissue from the harmful impact of ionizing radiation, while radiation sensitizers, such as histone deacetylase (HDAC) inhibitors, selectively increase the damage ionizing radiation induces in cancer cells. Side effects associated with currently available protectants such as Amifostine include nausea, vomiting, and hypotension (39), as well as high costs, increasing the cost of the overall treatment (41). Suberoylanilide hydroxamic acid (SAHA), an HDAC inhibitor currently in the late stage clinical trials as a radiation sensitizer, has been associated with fatigue, dehydration,
nausea, and vomiting (42). Therefore, creation and identification of novel compounds that improve the efficacy and therapeutic index of RT would directly improve cancer treatment. Yet, as evidenced by the disparity between the number of patients receiving RT and how few radiation protection/sensitization compounds exist, identifying viable adjuvants has proved elusive.

Nanoparticle based therapies for cancer treatment is a rapidly growing field, with recent publications highlighting the ability of Ag microspheres (43) and folic acid-conjugated silica-modified gold nanorods (44) to act as radiosensitizers. Cerium oxide nanoparticles (CONPs) have been used as an adjuvant to improve RT in pre-clinical trials. Wide range CONP applications stem from the surface chemistry of the nanoparticles. The valence state and oxygen defects allow CONPs to act as auto-regenerative redox status modulators (45). Recently, CONPs have been shown to be capable of entering mammalian cells (46) and have implications in biological systems.

The antioxidant behavior of CONPs has been employed to treat diseases of the central nervous system (47), repair spinal cord injuries (47), and extend the life of neurons in vitro (48). The antioxidant properties of CONPs allow them to decrease the accumulation of ROS and prevent subsequent ROS-induced apoptosis in normal cells (34). Biochemically, CONPs have been shown to act as either a superoxide dismutase (SOD) mimetic (49), converting superoxide to hydrogen peroxide (H$_2$O$_2$), or a catalase mimetic (14), converting H$_2$O$_2$ to water. It is through these mechanisms that previous work suggests CONPs are able to protect normal tissue from radiation-induced damage.
in the lungs (27), breast (30), and gastrointestinal tract (28). Most recently, CONPs were shown to scavenge hydroxyl radicals (16) and possess intrinsic oxidase activity (17), as well as cytotoxic and anti-invasive properties in melanoma cells (20,24,25,36). Due to the array of radical interactions (both pro- and antioxidant) now established, CONPs can no longer be strictly characterized as free radical scavengers and must be viewed as free radical modulators.

This study examines the ability of CONPs to drive ROS accumulation, as well as the subsequent impact on pancreatic cancer cell survival in vitro and in vivo. Our data demonstrate that the pro-oxidant activity of CONPs drives radiation-induced radical production selectively in pancreatic cancer cells resulting in radiation sensitization to apoptotic death and growth inhibition. These results identify CONPs as a potentially novel radiation sensitizer for the treatment of human pancreatic cancer.

Methods and Materials

Cell Culture and Reagents

The normal pancreatic cells (hTERT-HPNE) were obtained from American Type Culture Collection (ATCC) and maintained in 3:1 glucose free DMEM:M3 Base medium, supplemented with 10% fetal bovine serum and 100 µg/mL gentamycin. The human pancreatic cancer cell line L3.6pl (50) was cultured in DMEM, supplemented with 10% fetal bovine serum and 100 µg/mL penicillin-streptomycin mixture (GIBCO) and maintained at 37°C and 5% CO₂. CONPs were purchased from NanoScale Corporation
(Manhattan, KS) or synthesized as previously described (51). Hydrogen peroxide 
(H₂O₂) was purchased from Sigma Aldrich (St. Louis, MO).

*Transmission Electron Microscopy of CONPs*

The sizes and shapes of the nanoparticles were determined by high-resolution 
transmission electron microscopy (TEM) as previously described (52,53).

*ROS Imaging*

L3.6pl and hTERT-HPNE cells were plated in 6-well plates (5 x 10⁴/well) and 24 
hours later treated with 10 μM CONPs in fresh media for 24 hours. Subsequently, cells 
were exposed to 5 Gy RT using a 160-kV cell culture and small animal irradiator 
(Kimtron Inc., Woodbury, Connecticut). ROS production was determined 0.5 and 24 
hours post RT by Image-iT LIVE Green ROS Detection Kit (Invitrogen) according to 
manufacturer’s protocol. The kit provides carboxy-H₂DCFDA. The carboxy-H₂DCFDA 
permeates live cells and is cleaved by cellular esterases into carboxy-DCFH. In the 
presence of cellular ROS, the carboxy-DCFH is then oxidized to produce carboxy-DCF, 
resulting in the emission of a bright green fluorescence. Alternatively, cells were 
cultured in 6 well plates for 24 hours followed by exposure to RT. 24 hours post RT, the 
media was replaced with fresh media containing CONPs (10 μM). ROS production was 
determined 3 and 24 hours post addition of CONPs. Photographs are representative
images from triplicate experiments, which were quantified using NIH ImageJ software to determine the number of fluorescent cells per field of view.

Cell Viability Assays

L3.6pl or hTERT-HPNE cells were plated (2 x 10³/well) and grown in 96-well plates for 24 hours and then treated with CONPs (10 µM) for an additional 24 hours, followed by exposure to 5 Gy RT. Cell viability was determined 96 hours post RT by the Cell Titer-Glo Luminescent Cell Viability Assay from Promega (Madison, Wisconsin) and an Optima Fluor Star Luminometer (BMG Lab Tech, Durham, NC) following the manufacturers' protocols.

Clonogenic Assays

L3.6pl cells were plated (1 x 10⁶/10-cm dish), grown for 24 hours and then treated with CONPs (10 µM) for an additional 24 hours. Immediately after exposed to 5 Gy RT, the cells were trypsinized and re-seeded (100 cells/well) into 6 well dishes. One week later, cells were stained with 6.0% gluteraldehyde (vol/vol), 0.5% crystal violet (wt/vol) in water and photographed colonies of greater than 50 cells were counted (54).

Hydrogen Peroxide Assays

H₂O₂ production by ionizing radiation (0-30 Gy), CONPs (0-200 µM) or the combinations of both was determined in 50 µL of water or phosphate-buffered saline
(PBS) at various pH values (pH 7.4, pH 5, and pH 3) by Amplex Red Assay (Invitrogen) following the manufacturer’s protocol. The combination treatments include two strategies: 1) irradiating 50 µL of CONP suspension and determining the time-course (0-25 h) of H₂O₂ production, and 2) irradiating 48 µL of water or PBS first, waiting for 1 or 24 hours and then adding 2 µL of CONP prior to determining the H₂O₂ production at a desired time point.

*Intracellular Acidity Assay*

L3.6pl or hTERT-HPNE cells were seeded at 500,000 cells per 6-cm dish and grown overnight. The medium was then removed and cells were incubated in 500 µL HBSS containing 1 µM BCECF-AM (Invitrogen) for 45 minutes. Cells were then trypsinized, washed twice with fresh media, and re-suspended in 1 mL HBSS. Flow cytometry was used to determine cellular fluorescence (as defined by the ratio of FITC/APC). Decreased fluorescence indicates increased intracellular acidity.

*Superoxide Radical and Hydrogen Peroxide Scavenging Analysis*

SOD mimetic activity of CONPs with different surface valance state to scavenge superoxide radical at neutral (pH 7) and acidic pH (pH 3) was determined using a SOD assay kit (Sigma-Aldrich, Kit #19160-1KTF) according to the manufacturer’s instructions (55). Catalase mimetic activity of CONPs to scavenge hydrogen peroxide under the
same conditions was determined using an Amplex Red hydrogen peroxide assay kit (Life Technology, Cat # A22188) according to the manufacturer’s instruction.

Orthotopic Injection of Pancreatic Cancer Cells in Athymic Nude Mice

Female athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute Frederick Cancer Research and Development Center. The mice were housed and maintained in specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the National Institute of Health. The mice of 8 to 12 weeks of age were used in accordance under institutional guidelines with approved IACUC protocol. Human care of the mice was thoroughly considered. To develop tumors, L3.6pl cells were harvested from culture dishes and injected as previously described (56-58).

Therapy of Established Human Pancreatic Carcinoma Tumors Grown in the Pancreas of Nude Mice

Immediately following injection of cancer cells (1 x 10^6) into the pancreas, the mice were randomized into two groups (n = 20) as follows: (a) twice weekly intraperitoneal (i.p.) injections of saline in control groups; (b) twice weekly (Tuesday and Thursday) i.p injections of CONPs (15 µM; 0.01 mg/kg). Two weeks later each group was randomized into 2 sub-groups (n = 10) as follows: (c) continued with twice weekly
i.p injections of saline (for control group) or twice weekly i.p. injections of CONPs; (d) twice weekly i.p. injections of saline or CONPs and thrice-weekly administration of 5 Gy radiation for 2 weeks (30 Gy total). All treatment groups continued to receive twice weekly i.p. injections of saline (control) or CONPs for 2 additional weeks (for a total of 6 week treatment). Mice were sacrificed at 8 weeks (day 56) and subjected to necropsy. Primary tumors in the pancreas were excised and weighed. Tumor volumes were determined by liquid displacement analysis.

*Histologic Analysis of Tumors*

For hematoxylin and eosin (H & E) staining, tissues were fixed in formalin, embedded in paraffin, and serially sectioned at 200 µm. H & E staining was performed based on standard protocol. Paraffin-embedded tissues were used for TUNEL staining. TUNEL-positive cells were detected using the DeadEnd Colorimetric TUNEL System (Promega, Madison, WI). Immunohistological microscopy was performed using 10x and 40x objectives on a Nikon E400 microscope (Nikon Instruments, Melville, NY). Routine procedures were used to capture images. Immunopositive cells for TUNEL staining were observed over 10 individual slides for each condition and quantified using the NIH ImageJ software to determine the number of TUNEL positive cells per field of view. Independent review by the Pathology Department at Orlando Health/MD Anderson Cancer Center Orlando confirmed the results.
Statistical Analysis

All the experiments were completed in three triplicates and the data are presented as mean ± standard deviation (SD). The data presented in Table 1 are presented as mean and range. Statistical analysis was performed using Student's t-test, and P value was calculated based on two-tailed test. P < 0.05 was considered statistically significant. GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA) was used for all statistical analyses.

Results

CONPs facilitate RT-Induced Hydrogen Peroxide Production Favoring Lower than Normal pH

To characterize H$_2$O$_2$ induction in vitro by RT, water or PBS of varying pH (pH 7.4, pH 5 and pH 3) was exposed to serial doses of ionizing radiation (5-30 Gy) and relative H$_2$O$_2$ production was determined (Figure 1A). The results show a RT dose-dependent increase in H$_2$O$_2$ production immediately following RT exposure in all conditions to a similar degree. The basal as well as post-RT levels of H$_2$O$_2$ in acidic buffer were considerably lower than those at neutral pH, suggesting that the autodismutation activity was lower at acidic pH. In acidic pH CONPs produced H$_2$O$_2$ while scavenging superoxide radicals with an equation of O$_2^{-}$ + CeO$_{2-x}$(Ce$_{3+}$) +2H$^+$ → H$_2$O$_2$+ CeO$_2$ (Ce$_{4+}$), indicating that in acidic pH, CONPs produce H$_2$O$_2$ while scavenging...
superoxide radical. The size and shape of the CONPs were also determined by TEM (Figure 1B).

The impact of CONPs on radiation-induced radical production has been suggested but not documented. To characterize this, we first tested if prior presence of CONPs affects H$_2$O$_2$ induction at neutral pH by RT (Figure 2A & 2B and Figure S1A & S1B). CONPs suspended in water at serial concentrations (up to 200 µM) were exposed to 3 or 5 Gy RT. We noticed that CONPs alone, regardless of concentration, did not alter baseline H$_2$O$_2$ levels in water (pH 7.4) (data not shown). We found that the prior presence of CONPs prevented RT-induced H$_2$O$_2$ production in the water compared to non-CONP treated groups, although a slight increase in H$_2$O$_2$ levels was observed with CONPs at a concentration less than 10 µM. Similar changes were observed when CONPs were added 1 hour (Figure 2C & 2D and Figure S1C & S1D) or 24 hours (Figure 2E & 2F and Figure S1E & S1F) after RT. It should be noted that the H$_2$O$_2$ induction at neutral pH (7.4) is comparable between water and PBS solution (Figure S2), and that the presence of CONPs in the assay reaction or removal of CONPs from the reaction does not change the readout for H$_2$O$_2$ levels (Figure S3). These results suggest that the neutral pH environment seems to favor the catalase-mimetic H$_2$O$_2$ scavenging activity over the SOD-mimetic H$_2$O$_2$ producing activity of CONPs.

The neutral pH mimics the cellular environment of a normal cell. A cancer cell such as the L3.6pl human pancreatic cancer cells usually maintains an acidic intracellular pH (Figure S4). To assess impact of CONPs on RT-induced H$_2$O$_2$ production at acidic pHs, PBS-buffered solution at pH 5 or pH 3 was incubated with
CONPs for 24 hours prior to RT. We found that under these acidic conditions, treatment with CONPs helped maintain the persistent high levels of H$_2$O$_2$ (Figure 3A-3D and Figure S5). Combined with the data obtained at the neutral pH, these results indicate that the acidic environment favors the SOD-mimetic H$_2$O$_2$ producing (or superoxide radical scavenging) activity over the catalase-mimetic H$_2$O$_2$ scavenging activity of CONPs. It has recently been suggested that the switch between the SOD-mimetic and catalase-mimetic activities of CONPs depends upon the oxidation state of CONPs with Ce$^{3+}$ state favoring the H$_2$O$_2$ production and Ce$^{4+}$ state favoring the H$_2$O$_2$ scavenging. To determine the potential effect of pH on the role of CONPs of either oxidation state, we added the Ce$^{3+}$ or Ce$^{4+}$ CONPs into water solution of pH 7, pH 5 or pH 3 and examined their superoxide radical or H$_2$O$_2$ scavenging activity (Figure 3E & 3F). The Ce$^{3+}$ CONPs showed high activity on superoxide scavenging (Figure 3E) but little or no activity on H$_2$O$_2$ scavenging regardless of pH changes (Figure 3F). Conversely, the Ce$^{4+}$ CONPs showed little or no activity on superoxide scavenging at both pHs (Figure 3E) but high activity on H$_2$O$_2$ scavenging at pH 7 which decreased dramatically at pH 3 (Figure 3F). These results imply that the acidic environment plays a major role in accumulating H$_2$O$_2$, presumably through increasing the Ce$^{4+}$ to Ce$^{3+}$ switch or Ce$^{3+}$/Ce$^{4+}$ ratio.

**CONPs Increase RT-Induced ROS Levels in Pancreatic Cancer Cells**

Next, we investigated whether and to what extent CONPs might affect radiation-induced ROS production in pancreatic cancer cells and normal pancreatic cells. Our
results show a persisting increase (> 2-fold, at 0.5 hour through 24 hours post RT) in radiation-induced ROS production in L3.6pl cells pretreated with CONPs (10 µM) for 24 hours (sufficing for CONP uptake by cells (23,27,28) before 5 Gy RT exposure, as compared to cells exposed to radiation alone (Figure 4A & 4B; P=0.006). In sharp contrast, the same treatment led to a constant decrease (> 50%) in radiation-induced ROS production in hTERT-HPNE cells (Figure 4A & 4B P=0.006). The difference was even greater if the ROS levels were compared between the cancer and normal cells (Figure 4B; P < 0.001). Consistent with the buffer only based experiments shown in Figure 3, these results suggest that radiation action on pre-existing CONPs is critical for ROS production selectively in the cancer cells, presumably by increasing the Ce$^{3+}$/Ce$^{4+}$ ratio in the acidic cellular environment. This was further supported by the little effect of post-RT treatment of the cells with CONPs (Figure 4C & 4D). Notably, CONP treatment alone (Figure 4B, 0 hour post-RT) could also increase the ROS levels in the cancer cells but decrease it in the normal cells, suggesting an interesting possibility that the basal oxidation state or Ce$^{3+}$/Ce$^{4+}$ ratio of CONPs depends upon the acidity of the cellular environment. While the ROS data show CONPs do act as an antioxidant under the condition of normal cells, the radicals generated by RT seem to eventually overwhelm the antioxidant ability of the CONPs. This is evidenced by the ROS levels 24 hours versus 3 hours post RT, further supporting the potentially differential impact of RT on the Ce$^{3+}$/Ce$^{4+}$ ratio in the acidic cancerous versus neutral normal cellular environment. Nevertheless, the CONP-then-RT treatment seems to be the favorable strategy to enhance ROS levels selectively in the cancer cells.
CONPs Enhance RT-Induced Cell Death in Pancreatic Cancer Cells

L3.6pl and hTERT-HPNE cells were incubated with CONPs concentrations (10 µM) for 24 hours followed by 5 Gy RT and subject to cell viability assays (Figure 5A). CONPs alone induced cancer cell death (12.5%, P=0.0055) compared to control (no treatment) and sensitized L3.6pl cells to 5 Gy RT resulting in an additional 12.9% (P=0.0196) increase in cell death compared to cells exposed to radiation alone. Excitingly, CONP treatment caused neither statistically significant increase in cell death nor sensitization to RT in the normal cells. Consistent results were obtained by another independent, clonogenic assays (Figure 5B & 5C). These results indicate that CONPs alone can induce cell toxicity and enhance sensitization to RT selectively in acidic cancer cells.

CONPs Enhance Growth Inhibition and Apoptosis Induced by RT in vivo in Human Pancreatic Tumors

To determine if CONPs can increase pancreatic cancer cell sensitivity to RT in vivo, we examined the effect of CONPs on orthotopic L3.6pl tumor growth in athymic nude mice (Table 1). The data show that, compared to RT alone, the addition of CONP treatment to RT caused a dramatic decrease in tumor weight (P = 0.0112) and tumor volume (P = 0.0006). Interestingly, like RT alone, CONPs alone also resulted in a striking decrease in tumor volume although the change in tumor weight was marginal. While the mean tumor volume may suggest that the synergistic effects of the co-treatment were only marginally better than the CONPs or RT alone, it is not necessarily indicative of the efficacy of the co-treatment. In the co-treated group, 50% of the mice
had tumors with a volume less than 1000 mm$^3$, whereas no other group had a single mouse with a tumor that small. Body weight was not changed among all treatment groups as compared with control mice. These results suggest that CONPs can act synergistically to enhance RT-induced inhibition of the tumor growth without causing obvious undesired toxicity to the host.

To determine the mechanisms underlying the tumor growth inhibition, effects of CONPs (alone or in combination with RT) on apoptosis were analyzed on the tumors harvested from the different treatment groups. H & E (Figure 6A, panels a-d) and TUNEL staining (Figure 6A, panels e-h) revealed that there was no apoptosis in untreated control tumors (Figure 6Aa & 6Ae). Apoptosis was increased by 25 times in RT-treated tumors (Figures 6Ab, 6Af and 6B), 120 times in CONP-treated tumors (Figures 6Ac, 6Ag and 6B), and 180 times in CONPs plus RT treated tumors (Figures 6Ad, 6Ah and 6B), with all groups being statistically significantly different from one another following TUNEL quantification (Figure 6B). The apoptosis appeared to be restricted to the tumor tissue, suggesting that CONPs selectively sensitize tumor cells to and protect normal cells from RT-induced apoptosis.

Discussion

This is the first study to examine a potential role of CONPs as an adjuvant for radiation therapy to treat pancreatic cancer. The results demonstrate that CONPs selectively sensitize human pancreatic cancer cells to RT acting as pro-oxidant and induce apoptosis due to acidic tumor cell environment.
The fact that acidic environment allows lower basal levels of $\text{H}_2\text{O}_2$ and more resistance to RT induction of $\text{H}_2\text{O}_2$ than neutral environment (see Figure 1) suggests that the autodismutation activity is lower in acidic than neutral environment. This could make a significant, if not major, contribution to resistance to RT by (acidic) tumor cells as well as RT-induced toxicity to (neutral) normal tissues. Therefore, increasing SOD activity in tumor cells and $\text{H}_2\text{O}_2$ scavenging activity in normal cells appears to be a key to sensitizing cancer cells to RT.

RT alone is known to kill normal cells in addition to cancer cells, as also demonstrated in Figure 5A. This is not surprising given that cancer cells have developed some mechanisms such as high acidity that make them more resistant to RT than normal cells. This is also the very reason why RT sensitization therapy is critical so that the same dose of RT can kill much more cancer cells without increasing the side effect on normal cells. The pre-treatment with CONPs has achieved exactly such a goal of sensitizing the cancer cells selectively to the subsequent RT (Figure 5 and 6) without increasing the RT toxicity to the normal cells (Figure 5A). Previous studies have shown that CONPs are capable of acting as both a SOD mimetic to scavenge superoxide radicals (49) and a catalase mimetic to scavenge $\text{H}_2\text{O}_2$ (14,16), and that acidic pH promotes SOD mimetic activity of CONPs while inhibiting catalase mimetic activity, resulting in increased accumulation of $\text{H}_2\text{O}_2$ (20). Consistently, our results further demonstrate that the acidity-dependent differential role of $\text{Ce}^{3+}$ versus $\text{Ce}^{4+}$ oxidation state of CONPs is a critical mechanism in the regulation of the activity switch between SOD mimetic and catalase mimetic and subsequent $\text{H}_2\text{O}_2$ accumulation (see Figure 3).
This could explain why CONPs alone was almost as toxic as RT to the cancer cells but showed little or no toxicity to the normal cells (Figure 5A). This finding points out an important role of CONPs as a stand-alone therapeutic for cancer treatment. More importantly, our results also indicate that RT further increases the SOD mimetic activity and decreases the catalase mimetic activity of CONPs in acidic environment (see Figure 2 and Figure 3), suggesting that RT may induce a switch of oxidation states from Ce⁴⁺ to Ce³⁺ of CONPs favorably in acidic tumor environment, which is supported by the chemical reaction equation of O₂⁺ + CeO₂ₓ⁻(Ce³⁺) +2H⁺→ H₂O₂ + CeO₂ (Ce⁴⁺). Indeed, we have observed a few percent change in Ce³⁺/Ce⁴⁺ ratio at neutral pH induced by radiation (data not shown). It remains unknown and interesting how the SOD activity of Ce³⁺ and catalase activity of Ce⁴⁺ are differentially regulated by different pH and to what extent RT can switch the oxidation states from Ce⁴⁺ to Ce³⁺ at acidic pHs.

It is not known whether the acidic cellular environment also determines a preferential uptake of CONPs into cancer cells. The differential role and oxidation state of CONPs in acidic versus neutral pH environment make the implication of CONP uptake into cancer versus normal cells more complicated. Assuming that as postulated CONPs act primarily as a producer of H₂O₂ in (acidic) cancer environment and a scavenger of H₂O₂ in (neutral) normal tissues, one would wish both the cancer and normal cells take up sufficient amount of CONPs during the combination therapy. This would make CONPs a 'super sensitizer' of RT which not only sensitizes cancer cells to the therapeutic effect of RT but also desensitizes normal cells to the toxic side-effect of RT. On the other hand, RT could kill the cancer cells and normal cells by distinct
mechanisms which could interpret why CONPs reduce ROS levels in the normal cells (Figure 4A) but fails to reduce the killing effect of RT on the cells (Figure 5A). Indeed, in addition to apoptosis, mitotic cell death accounts for a significant portion of radiation induced cytotoxicity. When this death mechanism fails to kill, the survived cancer cells become more resistant to RT. This mechanism also poses a risk of aneuploidy and oncogenesis in the normal cells that have survived the undesired exposure to RT. Given that ROS pathways can induce both mitotic and apoptotic cytotoxicity, it will be interesting to determine whether or not CONP treatment also sensitizes the cancer cells to RT-induced mitotic cell death as well.

Previous work has documented the differential uptake of CONPs by lung cancer and normal cells in culture (16). However, it remains very challenging to assess CONP uptake in vivo or in situ by tumor versus normal tissues including its levels, distribution, oxidation states as well as subcellular versus extracellular localizations and so on. Furthermore, in addition to regulating ROS, CONPs likely play many other biological roles to be identified in the cells or tissues. Further addressing these 'nanodynamic' and 'nanokinetic' issues would enhance the interpretation of the pre-clinical therapeutic role of CONPs.

It would be interesting to test whether the H$_2$O$_2$ accumulation is the primary factor contributing to the tumor selective apoptosis (see Figure 6) and whether the apoptosis occurred to the cancer cells only, the tumor stromal cells only or both. One of the tumor-associated stromal cell types is vascular endothelial cells which are the building blocks
of tumor angiogenesis that is in turn essential for aggressive tumor growth as well as metastasis. Since all these processes are closely favored by hypoxia-induced acidification within tumor microenvironment, both intracellular and extracellular, of the vast majority types of cancer, CONPs could also play a role selectively against these processes as well of tumor progression of many cancer types including pancreatic cancer. Experiments are in progress to address these important questions.

Conclusion

In conclusion, this work demonstrates the novel role of CONPs to enhance RT-induced ROS production and cell death selectively in human pancreatic tumor cells while protecting normal tissues from the toxic side-effect of RT depending upon the environmental acidity. These findings suggest that CONPs may be further developed as a novel tumor tissue sensitizer and normal tissue protectant to increase the therapeutic index of RT for improving treatment of pancreatic cancer patients.
Figure 1. Acidic pH is relatively resistant to H$_2$O$_2$ production compared to neutral pH.

Water (pH 7.4) and PBS at indicated pHs were irradiated at indicated doses and H$_2$O$_2$ production was examined by Amplex Red Assay. *P<0.05 compared to water exposed to that RT dose. B, TEM analysis of CONPs. Left panel shows TEM image of the CONPs of size between 5-8 nm (inset, high magnification images). Right panel shows selected area of electron diffraction pattern of the CONPs where A (111), B (200), C (220), D (311), E (222) and F (400) are the different crystal planes of fluorite crystal structure.
Figure 2. At neutral pH CONPs generally decrease RT-induced H$_2$O$_2$ production.

(A & B) CONP suspensions of serial concentrations up to 200 μM in H$_2$O or PBS at neutral pH (Figure S2) were irradiated at indicated doses. H$_2$O$_2$ production was determined at indicated time points post-RT (response to all the concentrations and time-course response in Figure S1A-B). (C & D) H$_2$O was irradiated at indicated doses. After 1 hour CONPs were added up to 200 μM. H$_2$O$_2$ production was then determined at indicated time points post-RT (see response to all the concentrations in Figure S1C & S1D). (E & F) Water was irradiated at indicated doses. After 24 hours CONPs were added up to 200 μM. H$_2$O$_2$ production was then determined at indicated time points post-RT (see response to all the concentrations in Figure 2E & S1F).*P < 0.05 compared to 0 μM CONP at that time point.
Figure 3. Under acidic conditions CONPs enhance H2O2 production and lose H2O2 scavenging activity.

(A-D) CONPs of serial concentrations (up to 200 μM) were included in acidic PBS solutions at indicated pHs for 24 hours followed by RT at indicated doses. H2O2 production was determined at indicated time points post-RT (see response to all the concentrations and time course response in Figure S4 A-D). *P < 0.01 compared to 0 μM at the same time point. (E & F) CONPs with predominant Ce3+ and Ce4+ on the surface were included in water at indicated pHs for indicated periods of time before concentration of superoxide radical (E) or H2O2 (F) was determined using a SOD assay kit and Amplex Red assay kit, respectively as described in Methods.
Figure 4. CONP treatment prior to, but not post, RT increases RT-induced ROS levels in acidic pancreatic cancer cells and decreases RT-induced ROS levels in neutral pancreatic normal cells.

(A & B) The cancer (L3.6pl) and normal hTERT-HPNE cells were treated with (CONP) or without (Ctrl) CONPs for 24 hours followed by 5 Gy RT. ROS levels were then determined and compared between the cells at indicated times. Relative fold changes were normalized to the control groups. (C & D) The cells were treated with 5 Gy RT for 24 hours prior to CONP treatment. ROS levels were then determined and compared between the cells at indicated times. Relative fold changes are normalized to the control groups. *P < 0.001. The acidic cancer cellular environment relative to the neutral normal cellular environment was confirmed (see Figure S5).
Figure 5. CONP pre-treatment selectively sensitizes pancreatic cancer cells to RT-induced cell death in culture.

(A) Indicated cells were pre-treated with 10 μM CONPs for 24 hours followed by RT at 5 Gy. Cell viability was determined 96 hours post-RT. Cell death was normalized to untreated group. (B & C) L3.6pl cells were treated similarly as in A. Immediately after the treatment, cells were detached, replated and grown for 7 days before colonies were counted.
Figure 6. CONPs enhance tumor cell apoptosis in vivo.

(A-D) Histologic evaluations using hematoxylin and eosin (H&E) staining. (E-H) TUNEL staining of apoptosis cells in situ. Tumor cell implantation and treatment of mice are described in Table 1. Tumor tissues along with adjacent normal pancreatic tissues were collected at the time when mice were sacrificed. Formalin-fixed and paraffin-embedded, immediately adjacent tissue sections were used for the staining. Immunopositive cells for TUNEL staining were observed over 10 individual slides for each condition and quantified using the NIH ImageJ software to determine the number of TUNEL positive cells per field of view.
Table 1. CONPs increase sensitivity to radiation-induced inhibition of human pancreatic tumor growth.

<table>
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<tr>
<th>Treatment</th>
<th>Tumor</th>
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<tr>
<td>CONP</td>
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<td>1306†</td>
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<td>CONP + RT</td>
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*L3.6pl human pancreatic cancer cells (1 x 10⁶) were injected into the pancreas of nude mice. Groups of mice were treated with i.p injections: vehicle solution or CONP (15 µM: 0.01mg/kg) twice weekly for 6 weeks. Sub-groups of mice also received fractionated radiation therapy (5 Gy) three times per week (total 30 Gy) during weeks 3 and 4. All mice were sacrificed on day 32. Tumor weight and volume analysis were performed as described in methods.

†P < 0.05 compared to radiation treatment.

*P < 0.05 compared to control treatment.
CHAPTER 3: CERIUM OXIDE NANOPARTICLES DO NOT INCREASE RADIATION-INDUCED DNA DAMAGE IN PANCREATIC CANCER CELLS

Introduction

It is known that ionizing radiation therapy kills cells via multiple mechanisms, as irradiation has been shown to induce cell death through apoptosis, mitotic catastrophe, and even senescence (59). In cell types which are prone to apoptotic cell death, such as hematopoietic cells, apoptosis is the predominant cell death pathway induced by exposure to radiation (59). However, most solid human malignancies, such as pancreatic cancer, generally lose pro-apoptotic mechanisms over the course of tumor progression (59). As a result, mitotic catastrophe becomes the driving mechanism of cell death induced by ionizing radiation in solid tumors, especially of epithelial origin (60).

Mitotic catastrophe, or clonogenic cell death, occurs when unrepaired DNA damage does not properly arrest the cell cycle (61) and, as a result, the cell dies during or due to aberrant mitosis (59). Just as many malignant cells lose pro-apoptotic mechanisms, they often also become deficient in cell cycle checkpoints during transformation and progression, which contributes to the chance for mitotic catastrophe (61). The premature progression of the cell cycle through mitosis results in the abnormal division of chromosomes during cell division, often leading to giant cells with unusual nuclear morphology, multiple nuclei, or multiple micronuclei (59). The nuclear and
chromosomal abnormalities eventually doom the cell to death through delayed apoptosis or necrosis.

We have previously established that CONPs are able to sensitize pancreatic cancer cells to RT-induced cell death, but the mechanism by which that sensitization occurs remained unknown. The induction of DNA damage is the first step in a cell’s progression to mitotic catastrophe resulting from radiation exposure. If CONPs are able to induce DNA damage either by directly oxidizing DNA or by some interaction with the effects of RT, this could explain the sensitization phenomenon we have observed in pancreatic cancer cells. Therefore, this study determined the ability of CONP treatment to influence RT-induced DNA damage in pancreatic cancer cells as a potential mechanism of sensitization.

Methods and Materials

*Cell Culture and Reagents*

The normal pancreatic cells (hTERT-HPNE) were obtained from American Type Culture Collection (ATCC) and maintained in 3:1 glucose free DMEM:M3 Base medium, supplemented with 10% fetal bovine serum, 100 µg/mL gentamycin, and 1 g/L dextrose. The human pancreatic cancer cell lines L3.6pl (50) and Panc1, also obtained from ATCC, were cultured in DMEM. Both cell mediums were supplemented with 10% fetal bovine serum and 100 µg/mL penicillin-streptomycin mixture (GIBCO) and maintained at 37°C and 5% CO₂. CONPs were purchased from NanoScale Corporation (Manhattan, KS) or synthesized as previously described (51).
**Comet Assay**

Human pancreatic cancer cells (L3.6pl and Panc1) and normal human pancreatic epithelial cells (hTERT-HPNE) were plated in 10 cm dishes and let attach overnight. Cells were then treated with 0 or 10 µM CONPs. 24 hours later, cells were exposed to 0 or 5 Gy RT using a 160-kV cell culture and small animal irradiator (Kimtron Inc., Woodbury, Connecticut). Cells were trypsinized immediately following RT and subjected to single cell electrophoresis (CometAssay®, Trevigen) according to the manufacturer’s protocol, including control cells with predetermined amounts of DNA damage purchased from Trevigen with the kit.

**Results**

**CONPs Do Not Impact RT-Induced DNA Damage in Pancreatic Cancer Cells in vitro**

L3.6pl and Panc1 pancreatic cancer cells exposed to RT, CONPs, or the combination of CONPs followed by RT all displayed some degree of DNA damage, as indicated by the ratio of the amount of DNA in the comet head to the amount of DNA in the comet tail (Figure 7A-B). However, the combination therapy did not display additive effects; CONPs followed by RT did not induce a greater amount of DNA damage than RT alone. Additionally, the amount of DNA damage that resulted from exposure to RT correlated with the resistance of the cancer cell line to radiation-induced death. Panc1 cells, acknowledged to be more radiation resistant, showed less DNA damage demonstrated by the migration of DNA into the tail, than L3.6pl, which is a comparatively radiation-sensitive cell line. As this assay was completed immediately
following RT exposure, the effects of DNA repair in response to CONP treatment can be excluded.

**CONPs Inhibit RT-Induced DNA Damage in Normal Pancreatic Epithelial Cells in vitro**

In contrast to the lack of effect of CONPs on RT-induced DNA damage in pancreatic cancer cells, normal hTERT-HPNE cells were responsive to pre-treatment with CONPs prior to RT. Normal pancreatic epithelial cells that were pre-treated with 10 µM CONPs before exposure to 5 Gy RT displayed significantly less DNA damage, indicated by the tail moment, compared to cells exposed to RT alone immediately following RT exposure (Figure 7C). Further, CONPs alone did not induce any DNA damage in normal cells, indicating the non-toxic properties of CONPs in normal cells and the protective capabilities of CONPs in normal pancreatic epithelial cells exposed to RT.

**Discussion**

While differing reports exist about the impact of CONPs on cells in culture, CONPs have been shown to be toxic to various types of cancer cells (19,20,36). Several mechanisms for the toxicity to cancer cells have been suggested. CONPs have been shown to induce oxidative stress, glutathione oxidation, lipid peroxidation, and membrane damage in some cancer cells (19,36). However, CONP-induced DNA damage has not been implicated as a mechanism of CONP toxicity in any cell types.
As discussed, there is uncertainty about the fate of CONPs once inside a cell. Therefore, the intracellular proximity of CONPs to DNA remains unclear. Several studies have found that CONPs accumulate in the cytoplasm without translocation to the nucleus (20,21), yet CONPs have also been identified accumulating in the perinuclear space (25). Other groups were able to detect CONPs localized to the mitochondria, lysosomes, and endoplasmic reticulum, in addition to a diffuse distribution throughout the cytoplasm and nucleus (23). CONPs in the nucleus are far more likely to be able to induce DNA damage than CONPs compartmentalized elsewhere in the cell. As Figure 1 demonstrates, CONP treatment alone was able to induce moderate DNA damage selectively in pancreatic cancer cells, potentially due to inherent oxidase activity. However, CONP treatment did not induce any DNA damage in normal pancreatic epithelial cells. This difference may be attributable to the differential activity of CONPs at differing pH. CONPs at acidic pH similar to that of a cancer cell have been shown to increase H$_2$O$_2$ levels by performing only SOD mimetic activities. In contrast, CONPs at the neutral pH of a healthy cell reduce ROS levels by acting as both an SOD mimetic and a catalase mimetic to remove H$_2$O$_2$ from the cell (19). Therefore, only under acidic conditions found in cancer cells, CONPs may be able to directly induce a low level of DNA damage. The differential properties of CONPs in cancer and normal cells add to their potential therapeutic applications and further support their pursuit as a novel radiation adjuvant.

As expected, RT-induced DNA damage was evident in each of the cell types exposed to radiation. However, CONP and radiation treatment did not have a
synergistic effect on the cancer cells; co-treatment of cancer cells did not increase the amount of DNA damage above that induced by RT alone. In contrast, CONP treatment dramatically decreased the amount of initial DNA damage in normal pancreatic epithelial cells exposed to RT. The opposing responses suggest that radical scavenging by CONPs is sufficient to decrease DNA damage, but radical production by CONPs is not sufficient to induce DNA damage resulting in sensitization.

Of note, our other assays have also demonstrated protection of normal cells exposed to RT by CONPs, albeit transient. This assay was completed immediately following RT exposure, which would be well within the timeframe of transient protection. It would be interesting to determine if CONPs are able to influence the levels of DNA damage in normal cells, long term. However, later experiments that determine viability will suggest that long term protection of normal pancreatic epithelial cells is not achieved.

Despite the differential impact of CONPs to protect normal pancreatic cells from DNA damage, but not cancer cells, the lack of sensitization of cancer cells to DNA damage suggests that other cellular mechanisms must be involved. Therefore, a cellular response separate from mitotic catastrophe must be responsible for the CONP-induced sensitization of cancer cells to RT, likely apoptosis.
Conclusion

In conclusion, CONPs showed protection of normal hTERT-HPNE cells against the DNA damaging effects of radiation. However, although RT alone induced DNA damage, CONPs did not significantly impact RT-induced DNA damage in pancreatic cancer cells. Therefore, enhanced DNA damage is not the mechanism by which CONPs sensitize cancer cells to RT and a different mechanism or pathway must be responsible for the sensitization phenomenon observed in pancreatic cancer cells.
Figure 7. CONPS do not alter RT-induced DNA damage in pancreatic cancer cells.

Cells were treated with 0 or 10 µM CONPs for 24 hours, followed by exposure to 0 or 5 Gy RT. Cells were immediately tripsinized following RT and used for CometAssay®. (A) L3.6pl and (B) Panc1 pancreatic cancer cells did not show sensitization to RT-induced DNA damage by CONP treatment. (C) CONPs protected normal hTERT-HPNE cells from RT-induced DNA damage. *P<0.001.
CHAPTER 4: CERIUM OXIDE NANOPARTICLES SENSITIZE CANCER CELLS TO RADIATION BY INDUCING ROS ACTIVATION OF JNK AND APOPTOSIS

Introduction

Current predictions indicate that one in two men and one and three women in the United States will develop cancer at some point in his or her lifetime (4). Of the nearly 600,000 cancer related deaths in the USA each year (1), roughly 30,000 of those deaths are caused by pancreatic cancer (3). Only 15-20% of pancreatic cancer patients present with surgically resectable disease; roughly 20% of patients with surgically resectable disease survive to 5 years post operation (3). The main treatment suggested for patients with locally advanced, or surgically unresectable, pancreatic cancer is a combination of chemotherapy and radiation therapy (3). Chemoradiation has been shown to both extend survival and decrease the pain commonly associated with pancreatic cancer (3). However, current chemotherapy and radiotherapy agents for advanced pancreatic cancer have shown minimal lasting impact, as most patients show signs of progression and metastatic development within only a few months of completing treatment (3).

While surgical resection physically removes the tumor cells, chemotherapy and radiation therapy attempt to induce tumor cell death while the tumor is still inside a patient. It has been previously shown that tumor cell redox status plays a key role in response to death stimuli, with treatments that generate significant amounts of H$_2$O$_2$ more effectively inducing cell death execution (62). Cell death can be executed by cells
through multiple mechanisms in response to various signals, with the two main death mechanisms induced in cells by exposure to radiation therapy (RT) being mitotic catastrophe and apoptosis (59). Apoptosis, or programmed cell death, is an essential cellular process for the development and maintenance of normal cells and tissues in the body (63). Malfunctions in the apoptotic machinery have been linked to a variety of diseases, including cancer and worsening with the cancer progression. As cells move through the neoplastic progression, they lose sensitivity to many of the apoptotic stimuli, which may help explain why cancers in the early stages tend to be more sensitive to chemotherapies and radiotherapy (63). Therefore, especially for cancers such as pancreatic that are commonly diagnosed in late stages, it is critical to identify treatments that are able to overcome the resistance of cancer cells to the available treatment modalities.

Cerium oxide nanoparticles (CONPs) are a new compound currently being pursued in pre-clinical trials for their potential use as a cancer therapy. Originally tested for their ability to scavenge radicals and protect normal tissues from radiation-induced damage associated with treating cancer in the head and neck (7), intestine (28), lung (27), and breast (30), the application of CONPs has expanded beyond the mitigation of the side effects of other cancer therapies. Several reports have documented the inherent toxicity of CONPs to cancer cells of various origin, including alveolar epithelial cancer cells (23), hepatocellular carcinoma cells (64), and pancreatic carcinoma cells (19), among others. Studies have now established that CONPs can directly inhibit squamous carcinoma cell invasion, as well as indirectly inhibiting invasion by blocking
the formation of myofibroblasts (20). Other researchers have demonstrated CONP-induced inhibition of the growth and invasion in melanoma cells (65). A most recent publication also established the anti-angiogenic properties of CONPs in an ovarian cancer model (66). The testing of CONP-based cancer therapies is rapidly expanding, both as the primary treatment and as an adjuvant treatment for already established therapies.

Our previous publication demonstrates that CONPs possess inherent toxicity and the ability to sensitize pancreatic cancer cells to radiation-induced cell death, without negatively impacting the corresponding normal cells viability or radiosensitivity. CONPs were also shown to selectively increase RT-induced ROS production in pancreatic cancer cells. In the absence of significant changes in RT-induced DNA damage in cancer cells (Figure 1), redox controlled apoptotic signaling was implicated as the driver of CONP-induced radiosensitization. Herein, we establish one of the redox-responsive apoptotic pathways responsible for the radiosensitization of pancreatic cancer cells by CONPs.

Methods and Materials

Cell Culture

Normal pancreatic cells (hTERT-HPNE) were obtained from American Type Culture Collection (ATCC) and maintained in 3:1 glucose free DMEM:M3 Base medium, supplemented with 10% fetal bovine serum, 100 µg/mL gentamycin, and 1 g/L dextrose. The human pancreatic cancer cell lines Panc1 (ATCC) and L3.6pl was cultured in
DMEM. Both cell mediums were supplemented with 10% fetal bovine serum and 100 
µg/mL penicillin-streptomycin mixture (GIBCO), with cells maintained at 37°C and 5% 
CO₂.

Generation of Stable Cell Lines

Wild type TRX1 (Addgene plasmid 21283) and TRX1 His C32S and TRX1 His 
C35S (Addgene plasmids 21284 and 21285) constructs were transferred into the pKH3 
vector to add a HA-Tag (67,68) and then into the lentiviral pLVPZ vector encoding 
puromycin resistance. As previously described (69), the lentivirus was produced by 
transfecting 293FT cells with the vectors, the media collected from the transfected 
293FT cells were used to infect L3.6pl and Panc1 cells for three repeated times. 
Positively infected cells were then selected with and maintained in medium 
supplemented with 1 µg/mL puromycin. Protein expression was confirmed via western 
blot for the HA-Tag. Puromycin was removed prior to caspase or viability assays.

Phospho-ELISA

L3.6pl cells were seeded into 10 cm dishes and let attach overnight. Cells were 
then treated with 0 or 10 µM CONPs followed by exposure to 0 or 5 Gy RT 24 hours 
later. 72 hours post RT, cells were collected and cell lysate was subjected to screening 
with the PhosphoELISAArray Multi-Analyte Kit for key pathways associated with human 
cancer according to the manufacturer's protocol (SA Biosciences, FEM-5001).
**Antibodies, Drugs, and Western Blot**

Primary antibodies were purchased from Cell Signaling Technology (TRX #2429, p-ASK1 #3765, p-JNK1/2 #9251, JNK1/2 #9252, Cleaved Caspase-3 #9664, HA-Tag #3724, GAPDH #5174). Secondary antibodies were purchased from Invitrogen (Alexa Fluor® 488 anti-rabbit #A-11034) and Santa Cruz (anti-rabbit #sc-2004). CONPs were purchased from NanoScale Corporation (Manhattan, KS) or synthesized as previously described (51). For western blotting, cells were plated in 10 cm dishes and let attach overnight. Media was then removed and replaced with media containing 0 or 10 µM CONPs. 24 hours later, cells were exposed to 0 or 5 Gy RT with cell lysates collected 24-72 hours after radiation. Proteins were separated on 12.5% SDS-PAGE gels, transferred to nitrocellulose membranes using the iBlot system (Invitrogen), and blotted following standard procedures.

**P-ASK1 Immunostaining**

hTERT-HPNE, Panc1, and L3.6pl cells were seeded onto glass coverslips and let attach overnight. Media was then removed and fresh media containing 0 or 10 µM CONPs was added. 24 hours later, cells were exposed to 0 or 5 Gy RT. 4 hours post RT exposure, coverslips were washed 2X in ice-cold PBS (5 min) and fixed in 10% paraformaldehyde (20 min) at room temperature. Coverslips were then washed 3X in ice-cold PBS (5 min) and permeabilized with ice-cold acetone (5 min) at -20°C. Subsequent to 3 more washes (5 min) in ice-cold PBS, coverslips were blocked (5% bovine serum albumin (BSA) in PBS) for 1 hour at room temperature followed by
incubation in primary p-ASK1 antibody (1:50 - 1:100) at 4°C overnight (Cell Signaling, #3765). Coverslips were then washed 3X (5 min) in ice-cold PBST, followed by incubation Alexa Fluor® 488 anti-rabbit secondary (1:100 – 1:200) at room temperature for 1 hour (Invitrogen). Finally, coverslips were washed 3X (5 min) in ice-cold PBS and mounted using ProLong® Gold antifade reagent with DAPI. A 10X objective on a Nikon E400 microscope (Nikon Instruments, Melville, NY) was used to capture random fields on each slide (slides stained in triplicate). P-ASK1 positive cells were quantified using Image-J software (NIH).

Caspase Activation Analysis

hTERT-HPNE, Panc1, and L3.6pl cells were seeded onto white-walled, clear-bottomed, 96 well plates (2,000 cells/well) and let attach overnight. Media was then removed and media containing 0 or 10 µM CONPs was added. 24 hours later, cells were exposed to 0 or 5 Gy radiation. At 24-48 hours post radiation, caspase 3/7 activation was determined via Caspase-Glo® 3/7 Assay (Promega) following the manufacturer’s protocol. Caspase activity was normalized to cell viability, which was determined with CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Data was normalized to control (untreated) capsase levels for graphing.

JNK Inhibitor Studies

Panc1 and L3.6pl pancreatic cancer cells were seeded into 10 cm dishes and let attach overnight. Media was then removed and replaced with media containing 0 or 10 µM CONPs and 20 µM specific JNK inhibitor (EMD #420119), added at the same time.
Radiation treatment (0 or 5 Gy) and the subsequent analysis of caspase activation (Caspase-Glo® 3/7 Assay, Promega) normalized to viability (CellTiter-Glo® Luminescent Cell Viability Assay, Promega) were completed by the same protocol as previously described. Additionally, the impact of the JNK inhibitor to mitigate radiation-induced cell death and CONP-driven radiosensitization was also determined from the CellTiter-Glo Luminescent Cell Viability Assay (Promega) data, which was normalized to control levels.

**Orthotopic Injection of Pancreatic Cancer Cells in Athymic Nude Mice**

Female athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute Frederick Cancer Research and Development Center. The mice were housed and maintained in specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the National Institute of Health. The mice of 4 to 6 weeks of age were used in accordance under institutional guidelines with approved IACUC protocol. Human care of the mice was thoroughly considered. To develop tumors, L3.6pl cells, as well as L3.6pl cells stably expressing wild type TRX1, C32S mutant TRX1, and the empty pLVPZ vector, were harvested from culture dishes and injected as previously described (56-58).
Therapy of Established Human Pancreatic Carcinoma Tumors Grown in the Pancreas of Nude Mice

Mice were randomized immediately following injection of L3.6pl cells (1x10^6) into the pancreas into two groups (n = 20) as follows: (a) twice weekly intraperitoneal (i.p.) injections of saline in control groups; (b) twice weekly (Tuesday and Thursday) i.p injections of CONPs (15 µM; 0.01 mg/kg). Two weeks later each group was randomized into 2 sub-groups (n = 10) as follows: (c) continued with twice weekly i.p injections of saline (for control group) or twice weekly i.p. injections of CONPs; (d) twice weekly i.p. injections of saline or CONPs and thrice-weekly administration of 5 Gy radiation for 2 weeks (30 Gy total). All treatment groups continued to receive twice weekly i.p. injections of saline (control) or CONPs for 2 additional weeks (for a total of 6 week treatment). Mice were sacrificed at 8 weeks (day 56) and subjected to necropsy. Tissues were analyzed for caspase 3 cleavage and JNK phosphorylation to correlate with in vitro experiments.

Immunohistochemical Analysis

Paraffin-embedded tissues were used for p-JNK and cleaved caspase 3. Sections (4 to 6 µm thick) were mounted on positively charged Superfrost slides (Fischer Scientific, Co.) and dried overnight. Sections were de-paraffinized in xylene, subjected to a graded series of alcohol [100%, 95%, and 80% ethanol (v/v) in deionized H₂O], and rehydrated in deionized water followed by PBS (pH 7.5). Slides were placed at 97°C in 0.1 mol/L citrate buffer (pH 6.0) for 10 min to achieve antigen retrieval, followed by washing with PBS that contained 0.1% triton and 0.1% BSA. Endogenous
peroxidase was blocked with 3% hydrogen peroxide in PBS whereas nonspecific binding was blocked with 10% normal horse serum and 2% BSA in PBS. The slides were then incubated at 4°C in a moist chamber with either the p-JNK or cleaved caspase 3 antibody (overnight). Slides were washed with PBS that contained 0.1% triton and 0.1% BSA in PBS. The slides were then incubated with a peroxidase-conjugated mouse IgG secondary (Santa Cruz Biotechnology; 1:500 dilution, 1 hour), a positive reaction was visualized by incubating the slides with stable 3,3′-diaminobenzidine (Invitrogen Corporation) for 8 to 10 min. Counterstaining was achieved by rinsing the sections with two changes of tap water, placing them in Gill's filtered hematoxylin (EMD Chemicals) for 5 min, then successively dipping into tap water, acid alcohol (EMD Chemicals), tap water, lithium carbonate (EMD Chemicals), and tap water. Slides were dehydrated by incubations in 95% ethanol, 100% ethanol, and xylene, then mounted with Crystal Mount (Fischer Scientific, Co.). For each antibody, the corresponding normal mouse IgG and horseradish peroxidase were used as controls, and wash buffer was used as an additional control in immunohistochemistry studies. Immunohistological microscopy was performed using a 40X objective on a Nikon E400 microscope (Nikon Instruments, Melville, NY). Routine procedures were used to capture images.

Statistical Analysis

The in vitro experiments were completed in triplicate and the data are presented as mean ± SD. Statistical analysis was performed using Student's t test, assuming equal variance, and P value was calculated based on two-tailed test. P < 0.05 was
considered statistically significant. GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA) was used for all statistical analyses.

Results

**CONPs Drive RT-Induced JNK Activation in Cancer Cells**

To determine the pathway activated in response to RT in cells pre-treated with CONPs, a multi-analyte ELISA for activation of proteins in key pathways associated with human cancer was performed on L3.6pl human pancreatic cancer cells exposed to CONPs, RT, and the combination therapy. Of the screened targets, only JNK activation was significantly increased by the combination of CONPs followed by RT (Figure 8A); none of the other targets (AKT, Erk1/2, p53) displayed enhanced activation in response to the pre-treatment with CONPs compared to RT alone (Figure S6A-C).

The ability of CONPs to drive RT-induced JNK activation, as determined by JNK phosphorylation, was further assessed via western blot analysis of L3.6pl and Panc1 pancreatic cancer cell lines at various time points following RT (Figure 8A&B). The activation of JNK was significantly increased in both cancer cell lines by 72 hours following exposure to RT. In contrast, when JNK phosphorylation was determined in normal pancreatic epithelial cells (hTERT-HPNE, Figure 8A&B), the opposite impact was evident; pre-treatment with CONPs induced a slight decrease in JNK phosphorylation in normal cells compared to the impact of RT alone. CONPs alone did not induce significant JNK phosphorylation in either pancreatic cancer or normal pancreatic epithelial cells.
When basal expression and phosphorylation of JNK in untreated cells were determined, both L3.6pl and Panc1 pancreatic cancer cell lines had higher total JNK expression than the corresponding normal hTERT-HPNE cells (Figure 8D). Based on the ratio of phosphorylated JNK (p-JNK) to total JNK, nearly all of the JNK present in normal pancreatic epithelial cells was phosphorylated, i.e. activated. However, there was some difference in amount of phosphorylated and total JNK in pancreatic cancer cells, indicating that not all of the JNK present is activated in un-treated pancreatic cancer cells. Interestingly, ratio of active to total JNK correlated with cellular expression of thioredoxin 1 (TRX1) (Figure 8D), a redox sensing protein upstream of JNK. TRX1 overexpression by cancer cells compared to the corresponding normal cells was confirmed via western blot. Additionally, normal cells in which a greater percentage of the basal JNK is phosphorylated express lower levels of TRX1 compared to the pancreatic cancer cells which expressed higher levels of TRX1 and lesser degree of basal JNK phosphorylation.

**CONP-Driven JNK Activation Mediates RT-Induced Apoptosis in Pancreatic Cancer Cells**

As JNK activation can be both pro- and anti- apoptotic, we next analyzed caspase 3 and caspase 7 activation as markers of apoptotic cells *in vitro*. Caspase 3/7 activation increases over time in response to RT in both L3.6pl and Panc1 pancreatic cancer cells. In L3.6pl cells, which are inherently more sensitive to RT-induced cell death, CONP-enhanced caspase activation is not evident until 72 hours post RT (P<0.01, Figure 9A). However, in Panc1 cells which are inherently more resistant to RT-
induced cell death, the CONP-induced increase apoptotic signaling is discernible by 48 hours post RT (P<0.01) and persists through 72 hours post RT (P<0.05, Figure 9B). The data indicate enhanced, stable activation of apoptosis in both pancreatic cancer cell lines.

Conversely, CONPs appear to delay caspase activation in normal pancreatic hTERT-HPNE cells. At 48 hours post RT, caspase 3 and caspase 7 activation are significantly decreased in cells pre-treated with CONPs compared to cells treated with RT alone (P<0.01, Figure 9C). 24 hours later, at 72 hours post RT, there is no longer a difference between the caspase activation in cells treated with or without CONPs prior to RT, indicating that the CONP-induced protection of normal pancreatic epithelial cells from RT-induced apoptosis is a transient phenomenon.

**CONPs Drive RT-Induced ASK1 Activation in Pancreatic Cancer Cells**

Apoptosis signaling kinase 1 (ASK1), a protein responsible for activating apoptotic signaling through JNK, is directly downstream of the redox sensing protein TRX1. Upon TRX1 oxidation, ASK1 dissociates and auto-phosphorylates to become activated. Therefore, ASK1 phosphorylation provides the link between ROS induced TRX1 oxidation and the activation of JNK to induce apoptosis. ASK1 phosphorylation was determined 1 hour after RT exposure in each cell line (Figure 10A). L3.6pl cancer cells pre-treated with CONPs showed greater than a 1.5 fold (P<0.0001) increase in ASK1 phosphorylation over RT alone, while pre-treated Panc1 cancer cells exhibited a greater than 3 fold increase (P<0.001) in ASK1 activation compared to only RT. RT
alone was not able to induce significant ASK1 activation in either pancreatic cancer cell line. CONPs alone did induce mild ASK1 activation in the same cell. Conversely, normal hTERT-HPNE cells showed roughly a 4 fold decrease in ASK1 phosphorylation at the same time point when pre-treated with CONPs compared to RT alone (Figure 9A). Additionally, CONPs alone did not significantly alter ASK1 activation in normal pancreatic epithelial cells compared to control (untreated) levels. (Figure 9B) Results were quantified and graphed relative to control (untreated) p-ASK1 levels within each cell line.

Bright field images of the p-ASK1 stained slides were used to confirm that all slides contained approximately the same number of cells upon the completion of staining (Figure S7). Staining results were quantified and graphed to illustrate the differences in p-ASK1 positive cells (Figure 9B). Of note, none of the treatments significantly impacted TRX1 expression. TRX1 expression was consistent across all treatment groups within each cell line (Figure 9C).

**CONPs Increase JNK Activation and Apoptotic Signaling in Vivo**

To corroborate the apoptotic signaling observed *in vitro*, we next tested the combination therapy on established pancreatic tumors in nude mice. JNK activation (Figure 11 A-D) and caspase 3 cleavage (Figure 11 E-H) were determined in tumor sections from mice receiving only saline injections, radiation alone, CONPs alone, or a combination of CONPs and RT. Results clearly show increases in both JNK activation (brown staining) and caspase 3 activation, induced by cleavage (brown staining), in
each of the treatment groups compared to control (saline injected) mice. However, it is also evident that the combination of CONPs and RT yielded the most significant increase in both JNK activation and caspase 3 activation (Figure 11 D&H), showing a dramatic increase over tumors from mice that received RT (Figure 11 B&F) or CONPs (Figure 11 C&G) alone.

**JNK Inhibition Mitigates CONP-Induced Radiosensitization of Pancreatic Cancer Cells**

To determine if JNK activation was the molecular driver of CONP-induced sensitization, we next used JNK inhibitors to block JNK activity in cells exposed to RT, CONPs, or the combination therapy. Results showed that, in the presence of a JNK inhibitor, caspase 3/7 activation and sensitization to RT-induced cell death were completely mitigated in L3.6pl pancreatic cancer cells (Figure 12 A&C). Similarly, in Panc1 pancreatic cancer cells, caspase 3/7 activation was significantly reduced and sensitization to RT-induced cell death was completely alleviated when compared to cells treated without the inhibitor (Figure 12 B&D). Results were confirmed with a secondary inhibitor (Figure S8), demonstrating that JNK function is essential for CONP-induced radiosensitization in pancreatic cancer cells.

The ability of JNK to mediate CONP-driven RT-sensitization was further confirmed via JNK knockdown with JNK1/2 siRNA. Confirmed via western blot 7 days post transfection (to mimic the 96 hours post RT viability time point), knockdown of JNK expression was achieved in Panc1 cells, whereas siRNA treatment of L3.6pl cells was ineffective (Figure S9A). Following siRNA transfection, CONPs were only able to
moderately increase apoptotic signaling, as evidenced by minimal caspase 3/7 activation (Figure S9B). Further, pre-treatment with CONPs did not increase cell death induced by RT under the siRNA JNK knockdown conditions (Figure S9C), again confirming that JNK is a critical mediator of CONP-driven RT-sensitization in pancreatic cancer cells.

**TRX1 Oxidation is Required for CONP-Induced Radiosensitization of Pancreatic Cancer Cells**

To confirm if the TRX1-ASK1 pathway is the critical activator of JNK to mediate radiosensitization by CONPs, we next used L3.6pl and Panc1 cell lines that stably expressed wild type TRX1-HA, C32STRX1-HA, C35TRX1-HA, or the empty pLVPZ vector. Protein expression was confirmed via western blot for HA expression by each cell line (Figure S10). While wild type TRX1 should interact with ASK1 the same as endogenously expressed TRX1, both mutant proteins are unable to become oxidized, and therefore, constitutively bind to ASK1 preventing ASK1 activation and the subsequent apoptotic signaling.

Data shows that the mutation of TRX1 blocks the increased apoptosis in parental L3.6pl and Panc1 cancer cells pre-treated with CONPs (Figure 13). However, L3.6pl and Panc1 cells displayed different responses to the overexpression of wild type TRX1. In L3.6pl cells, increasing wild type TRX1 expression made the cells more sensitive to RT as indicated by caspase 3/7 activation (Figure 13A) and cell death (Figure 13C). Yet, in Panc1 cells, increased wild type TRX1 expression slightly reduced caspase
activation (Figure 13B) without altering overall cellular sensitivity to RT-induced cell death (Figure 13C).

Discussion

Once it was determined that CONPs did not alter the ability of RT to induce cell death via DNA damage (Figure 1), but instead induced significant increases in ROS exclusively in pancreatic cancer cells (Figure 5), apoptotic signaling was implicated as the mechanism of sensitization (Figure 6-7). There are many pro- and anti-apoptotic pathways within the cell, several of which have been determined to play key roles in cancer development and progression, such as the AKT, ERK, and p53 pathways. AKT activation has been linked to cancer cell growth, survival, and proliferation (70), as well as correlated to the invasiveness of pancreatic cancers (71). ERK1/2 has been shown to protect pancreatic cancer cells from apoptosis and promote progression through the cell cycle (72). AKT and ERK1/2 signaling become activated in cancers. P53, a canonical tumor suppressor gene which normally plays a key role in induced cell death in response to significant DNA damage, becomes inactive in more than 60% of pancreatic cancers (73). While AKT (74), ERK1/2 (62), and p53 (75) may all become active in response to ROS, none were involved in the cellular response to CONP-driven RT-induced ROS (Figure S6).

However, activation of the JNK pathway was significantly up-regulated in pancreatic cancer cells by the combination of CONPs and RT. JNK activation can be both pro- and anti-apoptotic, driving cell proliferation, survival, and differentiation,
well as apoptosis (76). It is generally accepted that the length and strength of JNK activation determines whether it promotes survival or apoptosis, with transient activation promoting survival and prolonged activation driving cell death (76). Unfortunately, the time point at which activation becomes classified as “prolonged activation” is not clearly defined. Yet, JNK activation generally induces apoptotic effects in cancer cells exposed to RT (6). Caspase 3/7 activation is recognized as a hallmark of JNK induced apoptosis; therefore, caspase 3/7 activation was used to determine if JNK activation was driving apoptosis or survival. Increased caspase activity directly correlated with increased JNK activation in both pancreatic cancer cells and pancreatic tumors treated with CONPs and RT, suggesting that CONP-driven JNK activation resulted in apoptosis under the co-treatment. In opposition, caspase activation was delayed in normal pancreatic epithelial cells, suggesting CONP-mediated protection from RT.

Further, JNK activation was a promising candidate as the driver of sensitization based on its redox responsive upstream regulators, mainly thioredoxin 1 (TRX1). When cellular ROS, such as hydrogen peroxide, oxidize TRX1, a protein responsible for converting oxidative stress into a cellular response, it dissociates from Apoptosis Signaling Kinase 1 (ASK1). To become active, ASK1 must dissociate from TRX1 and auto-phosphorylate. In turn, active ASK1 directly induces JNK activation leading to apoptosis.

Our previous work has shown that CONPs induce H₂O₂ and ROS production in pancreatic cancer cells (19). As such, we suggest that CONP-enhanced RT-induced
ROS production results in the increased oxidation of TRX1 in pancreatic cancer cells to mediate apoptosis. The overexpression of TRX1 by cancer cells (Figure 8D) increases the total pool of TRX1 in the cell and, hence, the pool of reduced TRX1 available to inhibit ASK1 activation. Increased ROS could further up-regulate the expression of ROS scavenging proteins, like TRX1, which would be radio-protective. However, the various RT and CONP treatments did not impact TRX1 expression in either pancreatic cancer cells or normal pancreatic epithelial cells. TRX1 oxidation is necessary, but not necessarily sufficient, to ensure ASK1 activation; another reduced TRX1 molecule could bind the inactive ASK1 before activation occurs and prevent activation. Consequently, ASK1 phosphorylation was chosen as the indicator of sufficient TRX1 oxidation to induce apoptotic signaling. Further, ASK1-mediated sustained activation of JNK has been shown to be required for ROS-induced apoptosis, with ASK1 knockout cells being substantially more resistant to H$_2$O$_2$ (77). With pre-treatment of pancreatic cancer cells with CONPs enabling RT to induce significantly more ASK1 phosphorylation than RT alone, it can be suggested the CONP-driven TRX1 oxidation initiates the radiosensitization of pancreatic cancer cells. Experiments to test this interesting possibility in vivo are in progress.

Additional support for the significance of the ASK1/JNK signaling axis can be found in the differential response of normal and cancer cells to the pre-treatment with CONPs prior to RT. In contrast to the increase in ASK1 activation and caspase activation in cancer cells, CONPs appear to confer transient protection to normal pancreatic epithelial cells. CONPs have demonstrated the ability to temporarily
decrease radical production in response to radiation (Figure 5) but were not able to protect normal cells from radiation induced cell death long term (Figure 6). Similarly, CONPs were able to mitigate initial ASK1 activation and the resulting downstream caspase activation; however, caspase activation eventually recovered to the level of cells treated with RT alone. The phenomenon of transient radical scavenging in normal cells was suggested to result from the production of ROS eventually exceeding the radical scavenging capacity of CONPs, which would correspond with the delay in caspase activation.

The ability of CONPs to protect normal cells or sensitize cancer cells to RT is, most likely, intimately related to the uptake and localization of CONPs in particular cell types. The concentration of CONPs in a cell may be a key determinant in the degree of radical generation or scavenging. Unlike other canonical radical modulators which require a 1:1 ratio between scavenging molecule and radical, CONP surface chemistry enables a single nanoparticle to interact with multiple radicals, potentially making activity an exponential function of concentration. Additionally, as pH has been shown to play an important role in what radical manipulation properties CONPs exhibit, the localization in acidic or neutral cellular compartments could significantly impact CONP intracellular activity. Currently, the means to determine uptake requires highly specialized machinery and the ability to determine localization of individual particles remains elusive, rendering these important questions difficult to answer at this time.
Conclusion

In conclusion, this work characterizes the mechanism by which CONPs enhance RT-induced cell death selectively in human pancreatic tumor cells by activating the redox sensing machinery in the cell. These findings provide further insight into the cellular implications of CONP-based therapies, especially in conjunction with RT or other potential radical inducing agents. Overall, this work supports the pursuit of CONPs as a novel tumor tissue sensitizer to increase the therapeutic index of RT to benefit pancreatic cancer patients.
Figure 8. CONPs drive RT-induced JNK activation in pancreatic cancer cells.

(A) L3.6pl pancreatic cancer cells were treated with 10 μM CONPs followed 24 hrs later by 0 or 5 Gy RT. Lysate was collected 72 hrs post RT for ELISA analysis, which showed significantly increased JNK activation. (B-C) Pancreatic cancer cells (L3.6pl or Panc1) and normal pancreatic epithelial cells (hTERT-HPNE) were treated in identical fashion with lysate collected 24-72 hrs post RT. Western blot analysis showed (B) p-JNK1 and (C) p-JNK2 levels increased in L3.6pl and Panc1 cells, but not hTERT-HPNE cells by 72 hrs post RT. (D) Basal expression of p-JNK, JNK, and TRX in untreated cells was determined.
Figure 9. CONP-enhanced JNK activation induces apoptosis in pancreatic cancer cells.

Cells were treated with or without 10 μM CONPs for 24 hours followed by 0 or 5 Gy RT. Caspase 3/7 activation was then determined 48 or 72 hours post RT by Caspase Glo assay, normalized to viability, and graphed relative to control (untreated) groups. (A) L3.6pl and (B) Panc1 cancer cells showed increased caspase 3/7 activation in response to the co-treatment. (C) Normal hTERT-HPNE cells displayed a transient protection from RT-induced caspase activation, but the protection was not lasting. *P<0.05, **P<0.01.
Figure 10. CONP pre-treatment increases RT-induced ASK1 activation in pancreatic cancer cells.

(A) Pancreatic cancer cells (L3.6pl and Panc1) and normal pancreatic epithelial cells (hTERT-HPNE) were pre-treated with 0 μM or 10 μM CONPs for 24 hours followed by exposure to 0 Gy or 5 Gy RT. 4 hours later, cells were fixed and stained for p-ASK1. Representative images are shown. (B) The number of fluorescent cells in 10 fields of view per condition, per cell line was determined and normalized to control (untreated) levels. (C) Lysate from cells treated in identical fashion was collected 96 hours post RT. TRX1 expression was determined via western blot. *P≤0.0001.
Figure 11. CONP-induced JNK activation results in apoptosis in pancreatic tumors.

Tumor tissues were collected at the time when mice were sacrificed. Formalin-fixed and paraffin embedded, immediately adjacent tissue sections were used for staining. (A-D) p-JNK staining (brown) and (E-H) cleaved caspase 3 staining (brown) show that RT or CONPs alone both increased apoptotic signaling compared to un-treated mice, while the combination of CONPs and RT (D, H) dramatically increased JNK activation and the corresponding apoptotic signaling when compared to any other group.
Figure 12. CONPs are unable to drive RT-induced apoptosis in the absence of JNK function.

L3.6pl or Panc1 pancreatic cancer cells were treated with 20 µM specific JNK inhibitor (SP600125) with or without 10 µM CONPs for 24 hours prior to RT. Caspase 3/7 activation and cell viability were determined 24-96 hours post RT. Caspase 3/7 activation was completely mitigated in response to treatment in (A) L3.6pl cells and dramatically reduced in (B) Panc1 cells. Sensitization was no longer observed in (C) L3.6pl or (D) Panc1 cells following JNK inhibition. *P≤0.005 compared to control.
Figure 13. CONPs are unable to drive sensitization in the absence of TRX1 oxidation.

Cell lines expressing wild type TRX1, C32S TRX1, C35S TRX1, or empty pLVPZ vector were treated with 10 μM CONPs for 24 hrs followed by 5 Gy RT. Caspase 3/7 activation and cell viability were determined 24-96 hours post RT. Caspase 3/7 activation was completely mitigated in response to treatment in (A) L3.6pl cells and dramatically reduced in (B) Panc1 cells. Radiosensitization (as determined by reduced viability) was no longer observed in (C) L3.6pl or (D) Panc1 cells pre-treated with CONPs following TRX mutation. *P≤0.05 compared to RT alone. TRX1 expression is shown in Fig. S10.
CHAPTER 5: CERIUM OXIDE NANOPARTICLES DOWNREGULATE ANGIogeneSIS IN PANcreATIC TUMORS

Introduction

Cancer cells are characterized by uncontrolled cell growth and division, resulting in tumor formation in solid human malignancies, such as pancreatic cancer. Both hematologic and solid cancers require the growth of new blood vessels, known as angiogenesis (78). Specifically, tumor angiogenesis includes the development of new irregular blood vessels from the established vascular network (66). Whereas the actual cancer cells were historically the targets of therapies, the last ten years has seen the addition of agents designed to target the vasculature required to support tumor growth (78).

Tumor blood vessel formation facilitates disease progression in many ways, including the deposition of oxygen and nutrients, as well as the removal of catabolites and carbon dioxide (78). Additionally, endothelial cells have been shown to produce growth and anti-apoptotic factors which they supply to tumors; one endothelial cell can control the growth of up to 100 tumor cells (78). Finally, angiogenesis enables tumor cells to penetrate vessel walls and be transported to distant metastatic sites in the body (2). The array of processes affected by angiogenesis makes it an interesting and important factor to consider, especially in the context of a radical modulating agent such as CONPs.
Vascular endothelial growth factor (VEGF) is one of the key drivers of angiogenesis in cancer (2). Though many types of cancer cells constitutively overexpress VEGF without any stimuli, hypoxia (low oxygen) and acidosis (low extracellular pH) are two conditions commonly found in expanding tumors that have been shown to drive angiogenesis by up regulating the expression of VEGF (3). Radiation therapy has also been shown to up regulate VEGF expression, by decreasing the available oxygen in the cell (6). It is thought to be through the up regulation of VEGF that many of the negative effects of radiation are mediated in the cells which survive radiation treatment, including increased aggressiveness, proliferation, migration, invasion, and angiogenic ability (6). Therefore, a radiation adjuvant that mitigated the RT-induced VEGF expression could have both immediate and long term effects on patients.

As angiogenesis plays an important role in tumor growth, progression, metastasis, and response to radiotherapy, therapies that modulate oxygen availability in cancer cells must consider the impact on angiogenesis. CONPs are one such compound that has been shown to modulate ROS levels in pancreatic cancer cells, especially in response to RT (19), which suggests that they may also impact angiogenesis in pancreatic tumors as ROS have recently been shown to regulate angiogenesis through the regulation of VEGF expression (66). Treatment with CONPs has been shown to inhibit angiogenesis in ovarian cancer, as observed by decreased CD31 staining (66). Despite the evidence suggesting therapeutic potential, this is the
first study to determine the role of CONPs in regulating angiogenesis in pancreatic tumors.

Methods and Materials

Antibodies and Reagents

Purified rat anti-mouse CD31 antibody (550274) was purchased from BD Pharmingen (San Jose, CA). VECTASTAIN Elite ABC system (pk-7200) and DAB Peroxidase Substrate Kit (SK-4100) were purchased from Vector Laboratories (Burlingame, CA).

Orthotopic Injection of Pancreatic Cancer Cells in Athymic Nude Mice

Female athymic nude mice (NCI-nu) were purchased from the Animal Production Area of the National Cancer Institute Frederick Cancer Research and Development Center. The mice were housed and maintained in specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, United States Department of Health and Human Services, and the National Institute of Health. The mice of 8 to 12 weeks of age were used in accordance under institutional guidelines with approved IACUC protocol. Human care of the mice was thoroughly considered. To develop tumors, L3.6pl cells were harvested from culture dishes and injected as previously described (56-58).
Therapy of Established Human Pancreatic Carcinoma Tumors Grown In the Pancreas of Nude Mice

Immediately following injection of cancer cells ($1 \times 10^6$) into the pancreas, the mice were randomized into two groups ($n = 20$) as follows: (a) twice weekly intraperitoneal (i.p.) injections of saline in control groups; (b) twice weekly (Tuesday and Thursday) i.p injections of CONPs ($15 \mu M; 0.01 \text{ mg/kg}$). Two weeks later each group was randomized into 2 sub-groups ($n = 10$) as follows: (c) continued with twice weekly i.p injections of saline (for control group) or twice weekly i.p. injections of CONPs; (d) twice weekly i.p. injections of saline or CONPs and thrice-weekly administration of 5 Gy radiation for 2 weeks (30 Gy total). All treatment groups continued to receive twice weekly i.p. injections of saline (control) or CONPs for 2 additional weeks (for a total of 6 week treatment). Mice were sacrificed at 8 weeks (day 56) and subjected to necropsy. Primary tumors in the pancreas were excised and weighed. Tumor volumes were determined by liquid displacement analysis.

Staining for Angiogenesis

Slides were baked overnight at 57°C, followed by deparaffinization and rehydration via subsequent washes (twice each) with xylene, 100% ethanol, 95% ethanol, and dH$_2$O. Citrate buffer (10 µM) at pH 6 brought to sub boiling temperature for 10 minutes was used as an antigen unmasking agent. Slides were subsequently washed in dH$_2$O and incubated in 3% H$_2$O$_2$ for 10 minutes to block endogenous peroxidase. Following additional washing in dH$_2$O, sections were blocked with a blocking solution containing horse serum at room temperature for 20 minutes. Sections
were then incubated in primary antibody against mouse CD-31 at a dilution of 1:100 at room temperature for 30 minutes. After washing with PBST, biotinylated “universal” secondary antibody was added and incubated for 30 minutes at room temperature. Slides were then washed with PBST before being incubated 30 minutes at room temperature with R.T.U. ABC reagent. Additional washes with PBST were then performed, followed by addition of DAB reagent to each slide for 8 minutes and then submersion in dH$_2$O. Slides were counterstained with hematoxylin per the manufacturer’s instructions. After subsequent washes, slides were dehydrated by incubation in 95% ethanol, 100% ethanol, and xylene. Sections were mounted using Permount Mounting Media (Fisher).

Results

*Combination Therapy With CONPs and RT Decreases Angiogenesis in Pancreatic Tumors*

Tumors from mice receiving each treatment all displayed angiogenesis as indicated by the positive brown CD-31 staining (Figure 14). (A-B) Tumors from control mice (saline injected) showed the greatest level of angiogenesis, followed by (C-D) mice that underwent only radiation therapy. (E-F) mice receiving CONPs only showed a similar amount of staining to RT treated mice, but at a lower intensity. (G-H) Mice that received CONPs in combination with RT showed the lowest level of angiogenesis of any group, with very few CD-31 positive areas visible.
Discussion

The degree of pancreatic tumor vascularization has been inversely linked to patient outcomes, with hyper-vascularization associated with decreased survival (2). Conversely, tumor hypo-vascularization has been linked to extended survival in patients and researchers have shown that inhibition of pancreatic cancer angiogenesis in vivo suppresses tumor growth (2). Further, the response of the tumor vasculature is a critical determinant in the success of radiation therapy (6). In exposed endothelial cells, RT can activate pro-survival pathways to support tumor vascularization, contributing to the failure of radiation therapy by increasing angiogenesis within tumors (6). It would appear that treatment with CONPs down regulates angiogenesis that would be present in the absence of CONPs. However, based on the current data, it is unclear whether RT is effectively killing more cells, thereby inhibiting angiogenesis, or if CONP inhibited angiogenesis is enabling RT to kill more cells. Likely, the reality is a mix of the two situations. Nevertheless, this study suggests that CONPs impact both cancer cells as well as tumor microenvironment to sensitize cancer cells to RT. It would be both interesting and important to determine if CONPs inhibited angiogenesis through the down regulation of VEGF, as suggested and shown in the ovarian model (66).

Of note, the study that determined CONPs inhibited angiogenesis in an ovarian cancer mouse model also noted that CONPs were able to inhibit metastasis of ovarian cancer cells (66). As it has been shown that the up regulation of VEGF increases the metastatic ability of cancer cells, it is suggested that the inhibition of angiogenesis by
CONPs is the driver behind the reduction of metastasis in the ovarian model (66). Despite the fact that L3.6pl is a highly metastatic cell line derived from liver metastasis, none of the mice in the control group of this study developed metastasis detectable by visual inspection at the time of termination. Therefore, we were unable to determine if CONPs reduced the metastatic potential of pancreatic tumor cells in the current study. However, more sophisticated analysis of the liver following an experiment with an extended duration may address this issue. Further, performing an experiment where L3.6pl cancer cells were injected through the tail vain of mice treated with CONPs would be an interesting study to address the impact of CONPs on angiogenesis and the subsequent metastasis based on the resulting metastasis to the liver, among other potential locations.

Conclusion

Currently, these results are only preliminary. However, the results appear very positive. CONPs displayed the clear ability to down regulate angiogenesis and, excitingly, an enhanced ability to down regulate angiogenesis in the presence of radiation. The results are in line with both the suggested outcome in pancreatic cancer and the outcomes demonstrated in ovarian cancer. Overall, the ability of CONPs to decrease angiogenesis in pancreatic tumors shows promise and should be further pursued as a potential mechanism of radiosensitization for pancreatic cancers.
Figure 14. CONP treatment decreases blood vessel formation in pancreatic tumors.

Tumor sections collected at the time of termination were stained with CD-31 as a marker for angiogenesis. Representative images are shown at 10x and 40x. Control, RT, and CONP treated groups all had comparable levels of angiogenesis. Co-treated tumors (G,H) showed significantly lower levels of angiogenesis compared to all other groups.
CHAPTER 6: CONCLUSION

Impact

This is the first report on the usage of cerium oxide nanoparticles to treat pancreatic cancer. CONPs demonstrated selective toxicity to human pancreatic cancer cells without inducing toxicity in the corresponding normal human pancreatic epithelial cells. Though it was suggested by several groups, this is also the first report to demonstrate the ability of CONPs to sensitize cancer cells to RT. By ruling out mitotic catastrophe and then systematically analyzing ROS production and apoptotic signaling, this work provided a specific, novel mechanism by which the sensitization of pancreatic cancer cells by CONPs occurs.

Many of the mechanisms and pathways discussed here in the context of pancreatic cancer are, by no means, unique to that specific disease site. Certain factors, like uptake, do need to be confirmed for each cell type, but some do not. For instance, most cancer cells are thought to exist at an acidic pH that would favor $\text{H}_2\text{O}_2$ production by CONPs through SOD activity; most normal cells are thought to exist at a neutral pH which would favor radical scavenging by CONPs. Solid tumors found in many different disease sites are widely accepted to experience hypoxia, acidosis, and angiogenesis. With CONP toxicity to several types of cancer already established, it would be interesting to investigate the ability of CONPs to sensitize other types of cancer to radiation treatment.
The interactions of CONPs in cells are still being elucidated. As such, we cannot rule out pathways or interactions not specifically addressed in this work. Apoptosis is a carefully regulated process in normal cells and becomes even more tightly controlled in cancer cells as they attempt to evade death. With the numerous regulatory proteins and feedback loops, the regulation of other apoptotic pathways through ROS production, direct oxidation by CONPs, or other unidentified mechanisms are real possibilities which still need to be considered in pancreatic cancer and other forms of the disease.

Moving Forward

Pancreatic Cancer

Despite advances in the diagnosis and treatment of many cancers, pancreatic cancer patients still face a bleak outlook upon diagnosis. The rapid progression of the disease commonly results in late stage diagnosis and the need to treat patients with advanced and unrespectable tumors. Unfortunately, current treatment options are either completely ineffective or unable to improve patient outcomes long term. Improvements in the therapies currently available and identification of novel therapeutic strategies remains an important task, especially in pancreatic cancer. The establishment of more effective treatments could have a dramatic impact on the amount of time patients survive after being diagnosed with pancreatic cancer. The use of CONPs as a therapy or an adjuvant therapy for pancreatic cancer shows potential in making that happen.
Cerium Oxide Nanoparticle Therapies

Despite some conflicting in vitro data regarding toxicity, in vivo data about the toxicity and application of CONPs for the treatment cancer are positive thus far. In contrast with currently available ROS modulators, which are characterized by a short half-life, necessitate extensive dosing, and usually require the introduction of one molecule for each radical to be modified, CONPs persist in the body with a single particle scavenging or generating many free radicals or inducing the oxidation of several targets through the auto-regenerative capacity of CONPs. As a compound that is selectively toxic to cancer cells and selectively sensitizes cancer cells to established therapies, without harming normal cells or, in some cases, actually protecting normal cells, the therapeutic potential for CONPs appears to be vast.

However, for CONP-based therapies to gain acceptance and approval, some questions must still be addressed. We need a better understanding of where CONPs go when introduced into a cell or biological system. Current dosing regiments may be wildly excessive or may be considerably below the level optimum for CONP-induced protection or sensitization. Determining what percentage of CONPs make their way to a tumor or at what concentration tumors become saturated would certainly shed light on the ideal dosing regiments. Understanding the nanokinetics and nanodynamics of CONPs would also facilitate better implementation of CONP-based therapies.

Finally, increased standardization and categorization of CONPs is essential. There is extensive diversity in particles synthesized by various individual group or
commercial players, from size to shape to 3+/4+ ratio, yet all are referred to as “cerium oxide nanoparticles.” Each of those characteristics can have a dramatic effect on the toxicity and activity associated with the specific particle. Therefore, for therapy to become standardized, the particles themselves must also become standardized. If the particles can be standardized and dosing can be optimized, CONPs have real potential as a therapeutic agent for the treatment of cancer.


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APPENDIX B: SUPPLEMENTAL FIGURES
Supplemental Figure 1. CONPs have a concentration and time dependent impact on \( H_2O_2 \) production.

Supplemental to Fig. 2 showing all the CONP concentrations and time points examined. CONPs were included in water 0.5 h prior to RT (A & B), 1 h post-RT or 24 hr post-RT. Time in hours, time point post-RT.
Supplemental Figure 2. RT-induced H\textsubscript{2}O\textsubscript{2} production is quantifiable and comparable in water or PBS.

H\textsubscript{2}O\textsubscript{2} production by CONPs in PBS at neutral pH (7.4) is comparable to that of CONPs suspended in water. When CONPs were added to PBS prior to RT, there was not a discernible difference between H\textsubscript{2}O\textsubscript{2} production by CONPs suspended in water and CONPs suspended in PBS.
Supplemental Figure 3. CONPs do not introduce artifact into the Amplex Red readings.

Supplemental to Figure 2 & 3 showing CONP presence does not disturb the recording of H$_2$O$_2$ value by Amplex Red assay. CONPs were either left in the reaction (Present) or removed by centrifugation (Removed) prior to fluorescent analysis of H$_2$O$_2$. 
Supplemental Figure 4. L3.6pl cancer cells are more acidic than normal hTERT-HPNE cells.

Supplemental to Fig. 4 showing that L3.6pl pancreatic cancer cells (A) are more acidic than hTERT-HPNE normal pancreatic epithelial cells (B). The acidity inside the cells was determined as described in Methods and presented as mean fluorescent intensity.
Supplemental Figure 5. Acidic conditions inhibit CONP radical scavenging.

Supplemental to Fig. 3 showing that under acidic conditions, CONPs lose the ability to scavenge radicals, but not enhance radical production. Graphs show the extended analysis of H2O2 production by various CONP concentrations at varying time points beyond what is described in figure 3. A. CONPs were added to acidic PBS (pH 3) 24 hours prior to 3 or 5 Gy RT. B. CONPs were added to acidic PBS (pH 5) 24 hours prior to 3 or 5 Gy RT.
Supplemental Figure 6. CONPs do not alter AKT, ERK 1/2, or p53 signaling in pancreatic cancer cells.

Supplemental to Figure 8. L3.6pl pancreatic cancer cells were treated with 0 or 10 μM CONPs followed by exposure to 0 or 5 Gy RT 24 hours later. Cell lysate was collected and analyzed via ELISA 72 hours later for (A) p-AKT, (B) p-ERK 1/2, or (C) p-p53.
Supplemental Figure 7. Bright field imaging of p-ASK1 stained slides.

Supplemental to Figure 10. The slides imaged with fluorescent microscopy to determine ASK1 phosphorylation were also imaged with bright field microscopy to confirm that slides contained comparable numbers of slides at the time of imaging.
Supplemental Figure 8. CONPs are unable to drive sensitization in the absence of JNK function.

Supplemental to Figure 12. Cells were treated with 20 μM JNK inhibitor (BI 78D3) with or without 10 μM CONPs 24 hours prior to RT. Caspase 3/7 activation and cell viability were determined 24-96 hours post RT. Caspase 3/7 activation was mitigated in response to treatment in (A) L3.6pl cells and dramatically reduced in (B) Panc1 cells. Sensitization was no longer observed in (C) L3.6pl or (D) Panc1 cells following JNK inhibition. *P≤0.005 compared to untreated.
Supplemental Figure 9. CONPs are unable to drive radiosensitization in the absence of JNK expression.

Supplemental to Figure 12. Cells were transfected with JNK specific or control siRNA followed 24 hours later by 0 or 10 μM CONPs followed by RT 24 hours later. (A) siRNA knockdown of JNK in L3.6pl and Panc1 cells was determined via western blot 96 hours post RT. (B) Caspase 3/7 activation and (C) sensitization were no longer observed in Panc1 cells after JNK kd. *P≤0.005 compared to Ctrl. +P≤0.05 compared to RT.
Supplemental Figure 10. Infected cell lines express wild type or mutant TRX1.

Supplemental to Figure 13. L3.6pl and Panc1 cells were infected with HA tagged wild type TRX1, C32S mutant TRX1, C35S mutant TRX1, or the empty pLVPZ vector. Western blot for HA shows expression only in the cell lines expressing wild type or mutant TRX1-HA.
APPENDIX C: PROTOCOLS
Protocol Figure 1 A

Protocol A/B

50 μL
H₂O or
PBS

RT

0 hr

Amplex Red

0.5 hr

Fluorescent Analysis
Protocol A/B

50 μL CONPs

RT

0.5 hr
or
23.5 hrs

Amplex Red

0.5 hr

Fluorescent Analysis

Protocol Figure 2 A-B
Protocol Figure 2 C-D

Protocol C/D

RT 48 μL H₂O ▼ 0 hr

2 μL CONPs ▼ 2 hr or 23.5 hrs

Amplex Red ▼ 0.5 hr

Fluorescent Analysis
Protocol Figure 2 E-F

Protocol E/F

RT
48 μL
H₂O

24 hr

2 μL
CONPs

2 hr
or
23.5 hrs

Amplex
Red

0.5 hr

Fluorescent
Analysis

Protocol Figure 2 E-F
Protocol A/B

50 μL CONPs

RT

0.5 hr or 23.5 hrs

Amplex Red

0.5 hr

Fluorescent Analysis

Protocol Figure 3 A-B
Protocol Figure 3 C-D

RT
48 μL
H₂O
↓
0 hr

2 μL
CONPs
↓
2 hr
or
23.5 hrs

Amplex
Red
↓
0.5 hr

Fluorescent
Analysis
Protocol Figure 3 E-F

Protocol

H₂O
0 hr

CONPs
0-30 min

WST or Amplex Red Reagent
0.5 hr

Determine Absorbance
Cells

10μM CONPs

5 Gy RT

0 hrs Or 24 hrs

Carboxy-H₂DCFDA

0.5 hrs

Fluorescent Imaging

Protocol A

Protocol Figure 4A
Protocol Figure 4C

Protocol C

Cells ↓ O/N

5 Gy RT ↓ 24 hrs

10μM CONPs ↓ 3 hrs

Or

Carboxy-\nH$_2$DCFDA ↓ 24 hrs

↓ 0.5 hrs

Fluorescent Imaging
Protocol Figure 5A

Protocol

Cells → Overnight

10 μM CONPs → 24 hrs

5 Gy RT → 96 hrs

Viability Assay
Protocol Figure 5B

1. Cells
   - Overnight
2. 10 μM CONPs
   - 24 hrs
3. 5 Gy RT
   - 0 hrs
4. Cells
   - 2 wks
5. Colonies
   - Stained and Counted
Animal Dosing Schematic Figure 6, 11, 14 and Table 1

NP: 15 μM
RT: 5 Gy/dose (30 Gy total)
Protocol Figure 7

Protocol

Cells

Overnight

10μM CONPs

24 hrs

5 Gy RT

0 hrs

Cells in Agarose

Comet Assay (Single Cell Electrophoresis)
Protocol Figure 8A

Protocol

Cells ↓ Overnight
10 µM CONPs ↓ 24 hrs
5 Gy RT ↓ 72 hrs
Cell Lysate ↓ ELISA
Protocol

Cells

10 μM CONPs

5 Gy RT

Overnight

24 hrs

24-72 hrs

Cell Lysate

Western Blot:
P-JNK
Total JNK

Protocol Figure 8 B-C
Protocol 9 A-C

Protocol

Cells \downarrow \quad \text{Overnight}

10 \mu M \text{ CONPs} \downarrow \quad 24 \text{ hrs}

5 \text{ Gy RT} \downarrow \quad 48-72 \text{ hrs}

\quad \downarrow 

\text{Caspase 3/7 Activation Assay}
Protocol Figure 10A

Protocol

Cells
↓
Overnight
10 μM CONPs
↓
24 hrs
5 Gy RT
↓
4 hrs
P-ASK1 Staining
Protocol

Cells

Overnight

10 μM CONPs

24 hrs

5 Gy RT

96 hrs

TRX1 Western Blot

Protocol Figure 10C
Cells → Overnight
10 μM CONPs + JNK Inhibitor → 24 hrs
5 Gy RT → 96 hrs
Caspase 3/7 Activation Assay

Protocol Figure 12 A-B
Protocol Figure 12 C-D
Protocol Figure 13 A-B
Protocol Figure 13 C-D
APPENDIX D: IACUC APPROVAL LETTERS
September 16, 2011

Dr. Cheryl Baker
6900 Lake Nona Blvd
Burnett School of Biomedical Sciences
Orlando, FL 32827

Subject: Institutional Animal Care Use Committee (IACUC) Addendum Submission.

Dear Dr. Baker,

This letter is to inform you that the following addendum submitted was approved by the IACUC.

**Animal Project:** 11-01

**Title:** Biology and Therapy of Human Cancers in Mouse Models. Subtitle: Nanoceria: A Novel Nanoparticle Adjuvant Therapy to Increase the Efficacy of Radiotherapy for Lung Cancer Patients (Addendum #2)

**Approval Date:** 9/15/2011

Please see the attached copy of the approved addendum and please keep a copy for your records. Should you have any questions, please do not hesitate to call me at (407) 822-1164.

Sincerely,

[Signature]

Cristina Caamaño
Assistant Director, Research Program Services
Office of Research & Commercialization
INSTRUCTIONS: Use this form to request IACUC review of a minor change in an approved IACUC protocol and submit the request via e-mail to IACUC@mail.ucf.edu or mailed to the IACUC Office: ATTN: Cristina Casanaño, IACUC Coordinator, 12201 Research Parkway, Suite 502, Orlando, FL 32826-3246 or campus mail 32816-0150. Phone: 407-882-1164, Fax: 407-823-3299.

All Addendums must be approved by the IACUC prior to implementation.

Protocol Number: 11-01

Principal Investigator: Cheryl H. Baker

Title of Protocol: Biology and Therapy of Human Cancers in Mouse Models

Type of Project: Category A ___  Category B ___  Category C ___  Category D ___  Category E ___

Changes to be Made: (provide sufficient detail to allow evaluation by the IACUC)
If new personnel will be added, training information for each procedure involved needs to be provided on this form.

Please add Dr. Jihe Zhao as he will serve as the new Co-PI.

Jihe Zhao, M.D., Ph.D. > 25 years of experimental animal handling for research purposes particularly cancer research. He has trained in animal restraint, tumor inoculation, injections (s.c., tail vein, i.p). He has also trained in tumor and tissue extraction, blood collection, euthanasia, and assessment of tumor development and animal survival/death.

Reasons for Addendum/Modification:

Dr. Turkson has left UCF and will therefore, no longer serve as Co-PI.

Signature of Principal Investigator  Cheryl Baker

Date 9/14/11

UCF IACUC Addendum Form

Version March 2008
APPENDIX E: DISSERTATION DEFENSE ANNOUNCEMENT
ABSTRACT

Side effects of radiation therapy (RT) remain the most challenging issue for pancreatic cancer treatment. In this report we determined whether and how cerium oxide nanoparticles (CONPs) sensitize pancreatic cancer cells to RT. CONP pretreatment enhanced radiation-induced reactive oxygen species (ROS) production preferentially in acidic cell-free solutions as well as acidic human pancreatic cancer cells. In acidic environments, CONPs favor the scavenging of superoxide radical over the hydroxyl peroxide resulting in accumulation of the latter whereas in neutral pH CONPs scavenge both. CONP treatment prior to RT markedly potentiated the cancer cell apoptosis both in culture and in tumors and the inhibition of the pancreatic tumor growth without harming the normal tissues or host mice.

Mechanistically, CONPs were not able to significantly impact RT-induced DNA damage in cancer cells, thereby ruling out sensitization through increased mitotic catastrophe. However, JNK activation, which is known to be a key driver of RT-induced apoptosis, was significantly upregulated by co-treatment with CONPs and RT in pancreatic cancer cells in vitro and human pancreatic tumors in nude mice in vivo compared to CONPs or RT treatment alone. Further, CONP-driven increase in RT-induced c-Jun terminal kinase (JNK) activation was associated with marked increases in Caspase 3/7 activation, indicative of apoptosis. We have shown CONPs increase ROS production in cancer cells; ROS has been shown to drive the oxidation of thioredoxin (TRX) 1 which results in the activation of Apoptosis Signaling Kinase (ASK) 1. The dramatic increase in ASK1 activation following the co-treatment of pancreatic cancer cells with CONPs followed by RT in vitro suggests that increased JNK activation is the result of increased TRX1 oxidation. The ability of CONPs to sensitize pancreatic cancer cells to RT was mitigated when the TRX1 oxidation was prevented by mutagenesis of a cysteine residue, or the JNK activation was blocked by an inhibitor. Additionally,
angiogenesis in pancreatic tumors treated with CONPs and RT was significantly reduced compared to other treatment options.

In summary, these data demonstrate an important role and mechanisms for CONPs in specifically killing cancer cells and provide novel insight into the utilization of CONPs as a radiosensitizer and therapeutic agent for pancreatic cancer.

Committee Members:
Dr. Jihe Zhao (Chair)
Dr. Deborah Altomare
Dr. William Self
Dr. Shadab Siddiqi

Publications:


Approved for Distribution by Dr. Jihe Zhao, Committee Chair

The public is welcome to attend.
REFERENCES

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