ABSTRACT

BOSS, MARY-KEARA. Modulation of Oxidative Stress Influences Tumor Response to Therapy. (Under the direction of Dr. Mark Dewhirst and Dr. Marlene Hauck)

High levels of intracellular oxidative stress is both a characteristic of cancer as well as a means of treating cancer. The studies presented here investigate how reduction-oxidation (redox) shifts within tumor cells alters therapeutic response.

Classic radiobiology assumes that tissue response to radiotherapy occurs because of effects on directly irradiated cells; however, there is growing interest in changes occurring in the regional, "bystander" cells. These cells can undergo genotypic and phenotypic changes secondary to exposure to oxidative stress from the nearby irradiated cells. Radiation-induced oxidative stress has been shown to increase transcriptional activity of a tumor promoting transcription factor, hypoxia-inducible factor-1 (HIF-1). Tumor cells with increased HIF-1 expression are associated with increased aggressive tumor characteristics, such as angiogenesis, cell motility, and metastasis. We hypothesized that oxidative stress from irradiated tumor cells could stimulate aggressive tumor changes in regional bystander tumor cells via overexpression of HIF-1. Such changes in the behavior of sublethally-irradiated bystander tumor cells would have serious clinical implications, particularly considering the movement to highly tumor conformal radiation therapy techniques and the risks of "marginal miss". Using microbeam radiation therapy (MRT) to administer partial 4T1 tumor radiation in a window chamber model to investigate local effects and simulation of a marginal miss with a 4T1 orthotopic dorsal mammary tumor model to study distant effects, it was found that radiation induced bystander effects can lead to aggressive tumor changes. Locally, increased angiogenesis, bystander HIF-1 expression, and regional metastasis was seen in the
MRT treated tumors compared to controls, and marginal miss treated tumors exhibited increased oxidative stress and distant metastasis compared to controls.

To disrupt these radiation bystander effects through shifting oxidative stress, we treated mice with a redox modulating compound: manganese porphyrin (MnP). MnPs are superoxide dismutase (SOD) mimics which have been shown to catalytically inactivate radiation-induced oxidative stress, downregulate HIF-1, and improve the radiation response in several tumor types. MnPs are redox modulators, and it is their effect on signaling pathways, particularly inhibition of those activated by HIF-1, that has led to improved tumor cell killing with radiation. Experiments were performed with 4T1 orthotopic dorsal mammary tumors to test how adjuvant MnP would influence the development of marginal miss-induced distant metastasis compared to saline-treated controls. Surprisingly, we found that MnP increased distant metastasis when used as both a single agent and when combined with radiation. MnP-treated tumors had significantly reduced oxidative stress compared to saline treated controls. Recent studies report that antioxidants can increase survival and metastatic efficiency of circulating breast cancer and melanoma tumor cells, so we hypothesize that the antioxidant effect of MnP in our experiment led to increased metastasis compared to saline-treated controls.

Finally, I present the results of a pharmacokinetic and toxicity study of the novel manganese porphyrin, MnTBUOE-2-PyP5+ (MnBuOE), in normal dogs as a precursor study to investigating the utility of MnBuOE as an adjuvant to canine lymphoma therapy in a comparative canine oncology clinical trial. While MnBuOE administration was associated with a number of toxicities for these dogs, it was found that the drug reaches very high plasma drug levels in this species. By reducing dose, it was possible to deliver the drug
relatively safely. Importantly, this study reported for the first time that MnBuOE accumulates in lymph nodes; an important result for a potential adjuvant cancer treatment. These results suggest that it may be possible to reduce MnBuOE dose and dosing frequency for canine patients to reduce toxicity while maintaining adjuvant clinical efficacy. The results from these studies underscores the importance of studying how modulation of tumor oxidative stress may lead to clinical benefits as well as risks for disease progression.
BIOGRAPHY

Mary-Keara (Keara) Boss was born in Worcester, MA on November 18, 1981. She grew up with her parents and five siblings in Springfield, MA. Keara attended Holy Name School for her elementary school education and then received her high school diploma in 1999 from Cathedral High School in Springfield, MA. Keara attended the University of Massachusetts-Amherst and received her Bachelor of Science degree in Animal Science with a focus in Pre-Veterinary Studies with summa cum laude honors. Keara moved to Raleigh, NC in 2004 to attend veterinary school at North Carolina Veterinary University. During her studies, she became fascinated with both radiation oncology and basic science research. Upon graduation from NCSU in 2008, Keara completed a small animal rotating internship at the University of Pennsylvania Veterinary Hospital. She returned to NCSU for her radiation oncology residency from 2009 to 2011. Keara successfully passed her radiation oncology board exam and became a Diplomate of the American College of Veterinary Radiology in 2011. After finishing her residency, Keara began her PhD studies under the mentorship of Dr. Mark Dewhirst and Dr. Marlene Hauck, and, since then, she has been working to complete the research featured in this dissertation. Keara lives in Cary, NC with her husband Brian. They have two beautiful, happy children, Harvey and Ellen. They are preparing to move to Fort Collins, CO, as Keara has accepted a position as an Assistant Professor at Colorado State University.
# TABLE OF CONTENTS

## LIST OF TABLES

- viii

## LIST OF FIGURES

- ix

## CHAPTER 1: Linking the History of Radiation Biology to the Hallmarks of Cancer

1. Abstract .......................................................................................................................... 1
2. Introduction .................................................................................................................... 1
3. Enabling Replicative Immortality .................................................................................. 3
4. Evading Growth Suppressors .......................................................................................... 5
5. Resisting Cell Death ......................................................................................................... 8
6. Genome Instability and Mutagenesis ............................................................................... 11
7. Avoiding Immune Destruction ....................................................................................... 16
8. Tumor Promoting Inflammation ..................................................................................... 19
9. Inducing Angiogenesis .................................................................................................... 21
10. Sustaining Proliferative Signaling .................................................................................. 25
11. Deregulating Cellular Metabolism ............................................................................... 28
12. Activating Invasion and Metastasis ............................................................................... 30
13. Moving Forward ............................................................................................................ 33
14. Conclusions ................................................................................................................... 33
15. Figures .......................................................................................................................... 35

## CHAPTER 2: Partial Tumor Radiation Drives Tumor Aggression

1. Introduction .................................................................................................................... 40
2. Materials and Methods ................................................................................................ 40
3. 4T1 cell lines ................................................................................................................... 44
4. Surgical procedures ....................................................................................................... 45
5. Biological timeline for development of orthotopic dorsal mammary tumors and metastasis .................................................................................................................. 47
6. Tumor growth monitoring ............................................................................................. 47
7. Window chamber irradiation and tumor microenvironment analysis ......................... 47
8. Orthotopic dorsal mammary tumor radiation and distant metastasis analysis ............ 50
9. Statistical analysis ........................................................................................................ 55
10. Animal welfare statement ............................................................................................ 58

## Results

- Window chamber results: partial tumor radiation by microbeam affects local tumor microenvironment .................................................................................................................. 59
- Orthotopic tumor radiation results: radiation marginal miss affects distant metastasis .................................................................................................................. 65

## Discussion

- Window chamber experiments: partial tumor radiation affects local tumor microenvironment .................................................................................................................. 69
Orthotopic tumor experiments: radiation marginal miss affects distant metastasis..........................................................................................................................77
Conclusions..................................................................................................................81
Figures..........................................................................................................................83
CHAPTER 3: Tumor Microenvironmental Redox State Drives Distant Response to Radiation........................................................................................................111
Introduction..................................................................................................................111
Materials and Methods.............................................................................................114
  4T1-Luc cell line.........................................................................................................114
  Surgical implantation...............................................................................................115
  Biological timeline for development of orthotopic dorsal mammary tumors and metastasis........................................................................................................116
  Tumor growth monitoring........................................................................................116
  Manganese porphyrin preparation and administration............................................116
  Surgical excision.......................................................................................................117
  Image guided radiation therapy................................................................................118
  Bioluminescence imaging of lung metastasis..........................................................119
  Blood collection........................................................................................................119
  Tumor dissection......................................................................................................120
  Lung preparation for histology................................................................................120
  Gross metastasis counts..........................................................................................120
  GSSG/GSH................................................................................................................120
  Histologic quantification of metastasis.....................................................................122
  Modeling of metastatic colonization and growth within lungs.................................122
  Statistics....................................................................................................................123
Results.........................................................................................................................125
  Influence of MnP on primary tumor growth and development of metastasis...........125
  Influence of MnP on tumor surgical recurrence and the development of metastasis..............................................................................................................125
  Influence of marginal miss and MnP on radiation tumor growth and the development of metastasis.................................................................126
Discussion....................................................................................................................128
Figures.........................................................................................................................136
CHAPTER 4: Potential for a novel manganese porphyrin compound as adjuvant canine lymphoma therapy.................................................................................151
Introduction..................................................................................................................151
Methods........................................................................................................................153
  Dogs............................................................................................................................154
  MnBuOE structure and preparation..........................................................................154
  Monitoring................................................................................................................154
  Single-dose pharmacokinetic study..........................................................................155
Defining maximally tolerated dose....................................................156
Multiple dose pharmacokinetic study................................................156
Histopathologic evaluation...............................................................156
Measurement of MnBuOE in plasma and organs..............................157

Results..............................................................................................158
Single-dose pharmacokinetic study...................................................159
Defining maximally tolerated dose...................................................159
Multiple dose pharmacokinetic studies..........................................162
Histopathologic evaluation...............................................................163
Tissue drug levels...........................................................................163

Discussion.......................................................................................164

Figures.............................................................................................173

CHAPTER 5: Future Directions.............................................................181
Partial tumor radiation and radiation marginal miss.......................181
Tumor microenvironmental redox state drives distant response to radiation.................................................................182
Potential for a novel manganese porphyrin compound as adjuvant canine lymphoma therapy..................................................184

REFERENCES..................................................................................186
LIST OF TABLES

TABLE 3.1: Summary of results from Experiments A, B, and C.....................................150
TABLE 4.1: PK Parameters.........................................................................................179
TABLE 4.2: Health Assessments....................................................................................180
LIST OF FIGURES

CHAPTER 1

Figure 1.1: Enabling replicative immortality..........................................................35
Figure 1.2: Genome instability and mutation..........................................................36
Figure 1.3: Inducing angiogenesis/Sustaining proliferative signaling.....................37
Figure 1.4: Deregulating cellular energetics............................................................38
Figure 1.5: Hallmarks of cancer with associated radiation biology concepts......39

CHAPTER 2

Figure 2.1: Graphical depiction of the central hypothesis.................................83
Figure 2.2: Graphical depiction of the clinical risks of radiation marginal miss..........................................................84
Figure 2.3: Orthotopic 4T1-luciferase dorsal mammary tumors and pulmonary metastases.............................................................................85
Figure 2.4: Microbeam irradiation.......................................................................86
Figure 2.5: Regional demarcation of tumor boundaries and vessel segmentation.........................................................................................87
Figure 2.6: Experimental design for radiation marginal miss experiments........88
Figure 2.7: Orthotopic dorsal mammary tumor radiation technique and focal dosimetry.........................................................................................89
Figure 2.8: Orthotopic dorsal mammary tumor radiation technique and regional dosimetry.........................................................................................90
Figure 2.9: Modeling of metastatic colonization and growth within lungs........91
Figure 2.10: Patterns of angiogenesis after irradiation..........................................................92
Figure 2.11: Changes in vascular length density and percentage vascular coverage over time..........................................................................................................................93
Figure 2.12: Microbeam radiation effects on vascular function over time..............94
Figure 2.13: Changes in fluorescent reporter expression over time..................95
Figure 2.14: Changes in vascular hemoglobin concentration over time............96
Figure 2.15: Rates of change in vascular hemoglobin concentration within tumors........................................................................................................................................97
Figure 2.16: Changes in hemoglobin O2 saturation within the tumor-associated vasculature........................................................................................................................................98
Figure 2.17: Regional metastasis formation following microbeam radiation.....99
Figure 2.18: Tumor cells form a bridge between areas of regional metastasis..100
Figure 2.19: Histological analysis of extracted tumors...........................................101
Figure 2.20: Radiation Marginal Miss Experiment A.................................102
Figure 2.21: Variation in tumor growth curves for Experiment A....................103
Figure 2.22: Radiation Marginal Miss Experiment B.................................104
Figure 2.23: Radiation Marginal Miss Experiment C.................................105
Figure 2.24: Quantification of pulmonary metastasis in Experiment C...........106
Figure 2.25: Variation in tumor growth curves for Experiment C...............107
Figure 2.26: Oxidative stress in primary 4T1-Luc orthotopic dorsal mammary tumors ..................................................................................................................108

Figure 2.27 A proposed model of tumor response to microbeam versus wide-field irradiation ........................................................................................................................................109

Figure 2.28 A proposed model of tumor response to radiation marginal miss..110

CHAPTER 3

Figure 3.1: Graphical depiction of the central hypothesis.................................136

Figure 3.2: Experimental design for MnP and radiation marginal miss experiments......................................................................................................................................................137

Figure 3.3: Single agent MnP increases distant metastasis...................................139

Figure 3.4: Surgery and adjuvant MnP reduces distant metastasis.................140

Figure 3.5: MnP and radiation marginal miss Experiment A............................141

Figure 3.6: Variation in tumor growth curves for Experiment A......................142

Figure 3.7: MnP and radiation marginal miss Experiment B.........................143

Figure 3.8: Variation in tumor growth curves for Experiment B......................144

Figure 3.9: MnP and radiation marginal miss Experiment C..........................145

Figure 3.10: Variation in tumor growth curves for Experiment C.................146

Figure 3.11: Quantification of pulmonary metastasis in Experiment C..........147

Figure 3.12: Oxidative stress in primary 4T1-Luc orthotopic dorsal mammary tumors...............................................................................................................................................................148
Figure 3.13: A proposed model of the influence of MnP and radiation to the primary tumor on the development of distant metastasis.................................149

CHAPTER 4

Figure 4.1: Pharmacokinetic profile of MnBuOE in dog after single subcutaneous injection..............................................................................................................173
Figure 4.2: Correlation of pulse measured by machine or manual counts........174
Figure 4.3: Pulse measured after single MnBuOE injection.............................175
Figure 4.4: Variations in indirect blood pressure following administration of single dose of MnBuOE..............................................................................................176
Figure 4.5: Variations in heart rate and blood pressure following administration of single dose of MnBuOE..................................................................................177
Figure 4.6: Tissues after 2-week multi-dose subcutaneous treatment...............178
CHAPTER 1
LINKING THE HISTORY OF RADIATION BIOLOGY TO THE HALLMARKS OF CANCER

ABSTRACT

Hanahan and Weinberg recently updated their conceptual framework of the “Hallmarks of Cancer”. The original paper, published in 2000, is among the most highly cited reviews in the field of oncology. The goal of this review is to highlight important discoveries in radiation biology that pertain to the Hallmarks. We identified early studies that exemplified how ionizing radiation affects the hallmarks or how radiation was used experimentally to advance the understanding of key hallmarks. A literature search was performed to obtain relevant primary research, and topics were assigned to a particular hallmark to allow an organized, chronological account of the radiobiological advancements. The hallmarks are reviewed in an order that flows from cellular to microenvironmental effects. Relevant hallmarks to this thesis project are: resisting cell death, inducing angiogenesis, activating invasion and metastasis, deregulating cellular energetics, and tumor promoting inflammation.

INTRODUCTION

Numerous influential scientists and clinicians have contributed to the field of radiation biology. Tracing the history of radiation biology could follow several focuses and developmental pathways. In this review, we have organized the history of radiation biology
according to Hanahan and Weinberg’s expanded Hallmarks of Cancer:  the original six hallmarks - 1. enabling replicative immortality, 2. evading growth suppressors, 3. resisting cell death, 4. sustaining proliferative signaling, 5. inducing angiogenesis, 6. activating invasion and metastasis; emerging hallmarks – 7. avoiding immune destruction, 8. deregulating cellular energetics; enabling characteristics – 9. genome instability and mutation, 10. tumor promoting inflammation. Our purpose was to chronologically highlight studies that exemplified how ionizing radiation contributes to each or how radiation was used experimentally to advance the understanding of the hallmark. This approach is intended to provide an alternative approach to discussing the history of radiation biology.

A literature search was performed using both Web of Science and PubMed to obtain primary research publications and review articles. Topics were assigned to a particular hallmark, and although we recognize that overlaps exist, this organization allowed a concise, but in no way comprehensive, account of the stepwise influences on radiation biological advancements. The hallmarks are organized in an order that allows the radiation biology topics to progress from cellular to microenvironmental effects. Because the field is very broad, we have focused on the first discoveries that relate to the Hallmarks and expand where needed to more current literature to emphasize certain points. Each section is concluded with a list of “Current Radiobiology Challenges” to indicate areas of active radiobiology research related to the hallmark. We cannot cover the whole field in this review and for those scientists who have contributed to this knowledge who are not highlighted here; this is not meant to reflect any lack of respect.
ENABLING REPLICATIVE IMMORTALITY

Cancer cells require the ability to replicate unlimitedly, uninhibited by normal space restrictions, to form a macroscopic tumor. Normal cells will progress through a tightly regulated, limited number of cell divisions; thereupon, they face either senescence, which is an irreversible, non-proliferative, viable state, or crisis, cell death. Cancer cells do not respect the bounds of senescence or crisis, and attain a state of immortality. The classic in vitro model of unlimited replication is the ability to continuously passage tumor cells in cell culture. Radiobiologists were the first to optimize replicative immortality of mammalian somatic cells in the laboratory; specifically, the ability to allow a single cell to grow into a clonal population. From there, many of the fundamental discoveries of radiation biology arose.

In 1954, Dr. Theodore Puck desired to study the genetics and metabolism of animal cells; however this was not possible at that time due to the lack of a simple, effective technique for large-scale colony production from single cells\(^2\). He was searching for a way to achieve this feat, with the same high plating efficiency achieved with bacteria by microbiologists, to quantify the number of cells in a population capable of reproduction\(^3\). At this time, Earle et al. had success with growing single mouse cells into colonies from within capillary tubes with conditioned nutrient media derived from mass cultures of growing cells; although, the plating efficiency for this complex process was only 4%\(^4\). Understanding that the key to the growth of single cells in culture was the production of diffusible factors from neighboring
cells, Philip Marcus, a graduate student of Puck, devised a unique design with fellow researcher Leo Szilard from the idea, “Since cells grow with high efficiency when they have many neighbors, you should not let the single cells know they are alone.” A colony of cervical cancer derived (HeLa) “feeder cells” was irradiated in culture to create a population of reproductively sterile, metabolically active cells, for the purpose of maintaining single cells inoculated on a microscope slide, positioned within the same culture media (Figure 1). They achieved a plating efficiency of 100% with this technique. Shortly after, it was realized that the low plating efficiency of single mammalian cells was secondary to a harsh trypsinization and washing process. Through development of a gentler technique, the necessity of the irradiated “feeder cell” population was eliminated.

The reproductive function of a single mammalian cell could now be assessed *in vitro* and the effects of various stressors could be quantified for cell populations by counting the formation of colonies. While it had been well established that irradiation interferes with the growth of microorganisms, it was Puck’s laboratory that demonstrated for the first time the nature in which radiation inhibits the ability of mammalian cells to undergo unlimited replication. Importantly, it was discovered that mammalian cells are exquisitely more sensitive to radiation than microorganisms, with a unique survival curve comprising an initial shoulder followed by exponential cell killing. Further, it was noted that the cells exhibited a mitotic lag following radiation, suspected to reflect repair. These basic characteristics of irradiated HeLa cells were determined to be shared by normal human cells, as well.

Although the “target” concept had been demonstrated previously in various experiments, it
was the development of techniques to visualize the karyotype of irradiated cells, showing that the rate of chromosomal aberrations corresponded with cell killing, which most clearly defined the main cellular target of radiation damage as DNA $^8$-$^10$.

What followed was a flurry of discoveries made by assessing the unlimited replicative ability of single cells under modified experimental conditions. In regard to irradiation of cells in vitro, split-dose experiments revealed sublethal damage repair $^{11}$; survival curves for varying types of ionizing radiation were developed $^{12}$; differences in survival throughout the cell cycle were discovered $^{13}$-$^{14}$; dose-rate effects were identified $^{15}$; the oxygen enhancement ratio was defined $^{16}$; and the concept of potentially lethal damage was realized $^{17}$.

*Current Radiobiology Challenges: Intrinsic radiosensitivity may enable translation of in vitro radiation results to preclinical and clinical scenarios; Application of historic radiobiology fundamentals to current radiation therapy techniques (hypofractionation, stereotactic radiosurgery)*

**EVADING GROWTH SUPPRESSORS**

The cell cycle is an organized balance of signals promoting and restricting replication and division; however, neoplastic cells exist with dysfunctional growth control mechanisms. Throughout the early experimental trials assessing the survival characteristics of irradiated mammalian cells, researchers were seeing evidence of intermittent variations in the cell growth cycle following irradiation. The basic phases of the cell cycle had been defined through the study of plants and microorganisms as S phase (DNA synthesis), M phase
(mitosis), and functionally undefined gaps, G_1 (between M and S), G_2 (between S and M), G_0 (senescence).

Autoradiography, utilizing tritiated thymidine to label DNA, allowed comparing labeled and unlabeled mitotic figures to the fraction of cells in mitosis to track the movements of cells through the phases of the cell cycle. Many researchers had documented similar findings that S phase was most resistant to irradiation and mitosis most sensitive, but it was Yamada and Puck\textsuperscript{18} that reported that a sublethal dose of radiation produced a temporary G_2 arrest in the HeLa cell line. This radiation-induced G_2 block was easily repeatable in HeLa cells and other transformed cell lines. Interestingly, it wasn’t until pure normal diploid human cells in culture were studied that a delayed entry into S phase following irradiation was revealed, characterizing a G_1 block\textsuperscript{19}. The G_1 and G_2 arrests were investigated using numerous \textit{in vitro} and \textit{in vivo} models, greatly facilitated by advancements in recording cell cycle radiation effects with flow cytometry\textsuperscript{13,20-23}.

It was intuitively suspected that the cause for the G_1 and G_2 blocks was to repair radiation damage done to fragile DNA prior to duplication of a faulty template, in synthesis, or distribution of defective genes to a daughter cell, in mitosis. These transient delays became known as checkpoints. Both checkpoints were regarded as a safety mechanism to limit the propagation of heritable errors and promote the survival of the cell.

A breakthrough in the basic understanding of the cell cycle arose from research as to the radiation-induced effects of the tumor suppressor protein, p53. Irradiation of a myeloid leukemia cell line caused a transient increase in p53 levels in coordination with decreased
DNA synthesis; further, cells with wild-type p53 displayed G₁ and G₂ blocks following radiation, but cells with absent or mutated p53 genes only were arrested in G₂. These results revealed that p53 played a critical role in the G₁ block. Additionally, transfection of wild-type p53 into malignant cells lacking p53 partially restored the post-irradiation G₁ arrest, while over-expression of mutated p53 in tumor cells with wild-type p53 abrogated the G₁ block. Ataxia-telangiectasia (AT) is a disease in humans that causes radiation hypersensitivity and genetic instability, putting patients at an increased risk for the development of cancer. In studying human AT cells, the normal cellular response of increased expression of p53 following radiation was absent; these findings signified a link between the AT defect and the p53 pathway in triggering the G₁ checkpoint. In addition to regulating the G₁ arrest, p53 was implicated in inducing apoptosis.

It had been determined that close to half of all human cancers are affected with mutated p53 or abrogated p53 signaling. This was the underlying reason why the G₁ checkpoint was not documented in HeLa or other transformed cell lines, and it was only revealed when freshly cultured normal fibroblasts were studied. Based on the knowledge that interfering with the G₂ checkpoint resulted in increased radiosensitivity, it was hypothesized that the G₁ arrest could have significant therapeutic implications. It was soon realized, though, that the response of mutated p53 to radiation had contradictory radiation effects according to cell type; lymphoid cells had reduced radiation sensitivity due to disruption of the apoptotic response, fibroblasts might not show an apparent change in radiation sensitivity if they arrest in G₁. Despite this variability, it was suspected that there
was a common initiating factor to the p53 pathway shared by all cell types. This was determined to be the mutated AT gene (ATM). Mice with disrupted ATM genes were phenotypically similar to humans with AT: following irradiation, they displayed lymphocytic resistance to apoptosis, fibroblastic inefficiency in G1 arrest, and absent increase in p53 levels. With these discoveries, it is known that ATM is responsible for signaling p53 to initiate the G1 checkpoint following DNA damage to protect the integrity of the cell; subsequent studies have implicated ATM in the S- and G2-phase checkpoints and DNA damage signaling and repair.

**Current Radiobiology Challenges:** Understanding the benefits and risks of radiation response following manipulation of cell cycle control; Development of novel therapeutic checkpoint modulators as radiosensitizers

**RESISTING CELL DEATH**

The fundamental discoveries of radiation biology were developed due to the fact that ionizing radiation causes cell death. Because the formation of chromosomal aberrations correlated with cell killing, it was established early that DNA was the lethal target of radiation; however, with time, the mechanisms by which various types of cellular damage progress to lethality were defined, and it became apparent that radiation-induced cell death is a very complex process. It is thought that clonogenic cell killing represents cell kill due to multiple mechanisms which culminate in the prevention of clonogen propagation following ionizing radiation.
Ionizing radiation efficiently induces lesions in DNA, the most lethal being double-strand breaks, by direct hit or indirectly through damage by free radicals. Mitotic death may follow such chromosomal damage if left unrepaired due to failure of the cell to divide successfully. This may occur at the first attempted mitosis or the cell may divide several times before the inappropriate separation of DNA is fatal. As this had been witnessed microscopically, it was considered the major mechanism of radiation-induced cell death, inducing immediate tissue effects by predominantly eradicating the rapidly dividing stem cell populations.

In 1972, Dr. John Kerr defined a programmed, active method of controlled cell deletion that could be initiated or inhibited by a variety of stimuli. He credited James Cormak, a Greek professor, for suggesting the term “apoptosis”, which used in Greek describes the “dropping off” of petals from flowers or leaves from trees. As Dr. Kerr reported the presence of spontaneous apoptotic cells within tumors, the challenge of understanding the regulation of apoptosis attracted a great deal of attention, particularly due to the potential implications for cancer therapy.

Shortly after the recognition that p53 controlled the G1 checkpoint, it was shown experimentally that increased p53 expression could activate an apoptotic response following irradiation in vitro and in vivo in various tissues. The fact that cells could die by way of apoptosis, instead of mitotic death, following irradiation was novel and exciting; however, there was a great degree of variability between cell types in regard to apoptotic susceptibility. For example, lymphoid cells, salivary gland parenchyma, and crypt cells of the small
intestine were the first defined as cells that are very susceptible to radiation-induced apoptosis, while fibrous tissue was deemed resistant.

It was soon discovered that DNA damage within the nucleus, by way of activating the p53 pathway, was not the sole site to stimulate apoptosis. Haimovitz-Friedman et. al performed a series of experiments in bovine aortic endothelial cells and demonstrated that ionizing radiation triggered a rapid hydrolysis of sphingomyelin from the plasma membrane to produce cytoplasmic ceramide as the second messenger which activated the apoptotic pathway in these cells. This sphingomyelin/ceramide pathway occurred in cell preparations devoid of nuclei which revealed that this form of apoptosis was independent of direct DNA damage. It was determined that radiation is capable of activating two independent apoptotic signaling mechanisms secondary to nuclear and/or membrane-based damage.

In addition to the variability in cell type susceptibility and the origin of signaling of the apoptotic response, it became apparent that there was a temporal difference in regard to p53-dependent apoptosis subsequent to radiation. Acute apoptosis, occurring within 4-6 hours following radiation, was induced by p53 expression, while a delayed, 24 hours or more, p53-independent apoptosis exists. Interestingly, this delayed p53-independent apoptosis, sometimes subsequent to several mitotic cycles, is mediated by de novo synthesis of ceramide, without the activation of the sphingomyelin pathway.

Multiple anti-apoptotic mechanisms exist which are intended to maintain the natural homeostasis of cellular proliferation and deletion within tissues. The most widely studied,
Bel-2, which is an inner mitochondrial membrane protein, has been shown extensively to inhibit apoptosis. However, over expression of the Bel-2 gene (or its pro-apoptotic gene partner, Bax), does not alter final clonogenic survival in cells in which apoptosis is not the main mode of cell death. Further, a novel response to radiation, autophagy, or “self-eating” has been described. Autophagy, which results in the development of acidic vesicular organelles to degrade cellular proteins, has been shown to contribute to cell death, as well as serve as a protective survival mechanism, in response to radiation.

As it is apparent that cell death can result from both mitotic and apoptotic killing induced by radiation, it is imperative to understand and exploit the differences that exist between, not only neoplastic and normal tissue, but cell type and cellular target, to maximize the efficacy of cancer therapy.

*Current Radiobiology Challenges: Deciphering and targeting radiation DNA damage-induced signal transduction pathways; modulation of p53-induction after radiation stress*

**GENOME INSTABILITY AND MUTATION**

Abnormal chromosomal arrangements in cells following exposure to ionizing radiation have been described since the beginning of experimental radiobiology. The study of these chromosomal aberrations, both acute (immediately following irradiation) and heritable (in progeny several generations from the parental cell exposure) initially led to identifying components and function of genes involved in the DNA damage response (DDR).
and DNA repair. Additional DDR and DNA repair information came from complementing radiosensitive Chinese Hamster Ovary (CHO) cells with exogenous DNA that led to the cloning of DSB repair genes. Ionizing radiation causes a variety of DNA damage as discrete lesions within multiply-damaged localized sites. DNA double-strand breaks (DSB) are important lesions as if not repaired correctly, unrepaired DSBs can lead to cell death or the propagation of chromosomal deletions and translocations. DNA mutations within the genome during radiation can lead to carcinogenesis in cells that have an abnormal DDR.

Dr. Barbara McClintock began in her studies of the cytogenetics of radiation-induced chromosomal aberrations in the 1940s. She examined the behavior of broken ends of chromosomes in maize and described the formation of ring chromosomes and chromatid bridges at mitosis. In her words, “The conclusion seems inescapable that cells are able to sense the presence in their nuclei of ruptured ends of chromosomes and then to activate a mechanism that will bring together and then unite these ends, one with another.”

Repair of DSBs occurs by at least two processes: homologous recombination (HR) and non-homologous end-joining (NHEJ). These pathways are distinct in the proteins used within each pathway, the cell cycle phases in which they act, their chromatin context and their relative fidelity of repair; and yet, they function in complementary ways. HR involves repair of a DSB by producing a copy of the damaged DNA from an intact homologous DNA strand. This repair process functions in late S/G2 phase when sister chromatids are available in close proximity. While this is the main form of DSB repair in yeast, non-homologous end-joining (NHEJ) is the predominant mode of irradiation repair in
NHEJ does not require an undamaged homologous molecule for repair and involves ligation of broken chromosome ends. Therefore, although this process occurs in all phases of the cell cycle, it is prone to error and the creation of deletions or genetic mutations. It has been recently proposed that the NHEJ process is still the first choice of rapid DSB repair in the G2 phase and the HR pathway facilitates repair of complex lesions that persist in heterochromatin linking chromatin biology to choice of DSB repair processes.

Building upon the understanding that ATM and p53 control cell cycle checkpoints and influence cell death, studies utilizing ionizing radiation contributed to the identification of the molecular components of DSB repair. It was found that ATM is central to initiating both HR and NHEJ through the direct phosphorylation of repair substrates or other kinases. In HR, this involves activating Rad51 and BRCA1/2, while the DNA-PK/Ku complex facilitates NHEJ. However, after the discovery of the MRE11-NBS1-RAD50 complex (MRN) as a key sensor of DSBs, it was determined that phosphorylation of the histone H2AX by ATM is one of the first processes following DSB induction and sensing. Activated H2AX is associated with chromatin signaling at the site of damage which then allows the recruitment of the repair factors to the injured DNA. It is now appreciated that initiation of a DSB leads to a series of protein post-translational modifications such as phosphorylation, ubiquitylation and acetylation in the immediate vicinity of the DSB to confine repair to specifically-affected parts of the genome. This is exemplified by the ATM-dependent phosphorylation event coming together with RNF8-mediated chromatin ubiquitylation leading to an ordered accumulation of DNA repair factors (e.g. RNF168, the
BRCA1-interacting RAP80 protein, 53BP1) at the break site using an ubiquitin-binding domain.\textsuperscript{54}

There are numerous spontaneous and experimentally-induced mutation syndromes caused by the dysfunction of various components of the DSB repair pathways that manifest as certain radiation hypersensitivity and chromosomal instability phenotypes. For example, mutation of the NHEJ process which is critical for the formation of T- and B-lymphocyte receptors was performed to create the \textit{scid} mouse phenotype.\textsuperscript{55,56} When \textit{scid} mice were irradiated, it was discovered that their myeloid cells and fibroblasts were markedly more radiosensitive when compared to control mice.\textsuperscript{55}

Beginning with initial work published in 1980, the laboratory of Dr. John B. Little performed experiments over several decades which support the hypothesis that radiation can induce a genome-wide process of instability in mammalian cells that is transmitted over many generations of cell replication leading to an enhanced frequency of genetic changes occurring among the progeny of the original irradiated cell.\textsuperscript{57,58} A large series of experiments investigating mutagenesis was performed by studying the induction of \textit{HPRT} mutations in CHO cells treated with X-rays or \(\alpha\)-particles (\textbf{Figure 1.2})\textsuperscript{59}. The instability phenotype was induced in approximately 10-20% of cell populations irradiated \textit{in vitro} with doses of 4-12Gy, and the spectrum of DNA structural damage in mutations arising in such unstable populations differed significantly from that for direct radiation-induced mutations.\textsuperscript{58}

In addition, genetic instability can be induced in irradiated cells or develop spontaneously in cancer cells through defective or absent telomeres, the protective
nucleoprotein caps at the end of chromosomes. One of the roles of telomeres is to prevent abnormal chromosomal fusion which can result in various gene mutations\textsuperscript{60}. Irradiation can induce telomeric damage at terminal and interstitial chromosomal sites, leading to abnormal chromosomal rearrangements\textsuperscript{61}.

The DDR response is enacted by accidental or chronic low dose exposure of normal tissue. Abnormalities in these pathways can result in increased cell death due to the inability to repair DNA damage or allow the inappropriate proliferation of transformed, mutant cells. Research that elucidates the mechanisms that enable the cell to respond to and repair radiation-induced DNA damage has great implications for cancer therapy. For example, advances in radiation therapy may be possible by taking advantage of synthetic lethality. Synthetic lethality refers to a genetic interaction where the combination of mutations in two or more genes leads to cell death, while a mutation in just one of those genes would allow cell viability. Understanding the germline and somatic genetics of the DDR in cancer cells can lead to the exploitation of genetic synthetic lethality in which DNA repair-deficient cells are sensitive to ionizing radiation and/or agents that knock out remaining DDR pathways (e.g. PARP, ATM and DNA-PKcs inhibitors)\textsuperscript{62,63}.

Current Radiobiology Challenges: Understanding radiation-induced genetic instability for radiation protection purposes; Targeting the DNA-damage response to improve tumor radiosensitivity, identification of tumor specific pathways to increase synthetic lethality.
AVOIDING IMMUNE DESTRUCTION

Cancer develops in the host through deception of the immune system. Functional immune surveillance involves highly integrated cell and tissue pathways utilizing local and systemic signaling with inflammatory and immune molecules to eradicate pathogens and abnormal or damaged cells from the body. Initially, it was accepted that radiation causes immunosuppression exclusively; however, as the complexities of tumor immunity are elucidated, radiation should be considered “immunomodulatory” due to the varying effects that may follow exposure.64

The roles of the immune cells are defined by their involvement in either innate or adaptive immunity. Cells of the innate pathway include phagocytic, antigen presenting cells (APC), such as macrophages and dendritic cells, and those of the adaptive pathway consist of cells with randomly generated antigen receptors, like B and T lymphocytes. Generally, APCs are activated by immunogenic stimuli, migrate to lymphoid organs to present antigen to T cells, and stimulate the adaptive immune response.

Early experimental radiation studies demonstrated that, of the pathological changes that occurred following whole body irradiation, the rapid death of lymphocytes was most striking. While lymphocytes were regarded as the most radiosensitive cell in the body, this finding was puzzling due to their non-mitotic state when compared to other notably radiosensitive cells.65 Ultimately, it was determined that lymphocytes undergo apoptosis post-radiation, and the profound immunosuppression that follows whole body irradiation was credited to this effect.
In time, more details regarding additional immune effects of irradiation were discovered. Aside from the radiosensitivity of the immune cells, details were uncovered as to how radiation affects the functionality of the cells actively participating in the tumor immune response. For example, while radiation induces increased leukocyte rolling and adhesion to the endothelial cells of the vascular wall in normal blood vessels \(^{66,67}\), irradiated tumor vasculature demonstrated changes in their endothelial surface properties such that they became less adhesive to leukocytes \(^{66}\). This effect, in particular, would be a case of a defect in the effector phase of tumor immunity through inadequate homing and infiltration of activated T cells. More recent work, however, demonstrates that 10Gy irradiation can upregulate certain adhesion molecules in tumor microvasculature that can be used to increase immune cell trafficking and/or targeted therapeutics \(^{68}\).

Different types of cancer cells exhibit variable sensitivity to radiation-induced death, but the understanding that radiation causes a wide range of cellular and stromal effects, in addition to tumor cell death, has prompted investigations as to the use of radiation in an immunomodulator. The term Radiation Hormesis refers to the hypothesis that low doses of ionizing radiation are beneficial by a number of mechanisms, including stimulation of the immune response \(^{69}\). Many investigators have tested the potential for cancer cells killed \textit{in vivo} by radiation therapy to serve as a good source of antigens for APCs to present to T cells \(^{70}\). Additionally, modification of tumor immunity through signal simulation has been shown to serve as a radiation sensitizer. Mason et al. utilized an injectable Toll-like receptor agonist coupled with radiation therapy in a mouse tumor model to demonstrate that the combination
resulted in a decreased effective dose of radiation needed to induce tumor regression. Further, the animals cured of tumors with this protocol were more resistant months after curative therapy to autologous tumor challenge than mice that were cured with radiation therapy only.

An intriguing, although rare, phenomenon known as the Abscopal Effect has been reported for a number of malignancies, whereby local radiotherapy is associated with the regression of metastatic cancer at a distance from the irradiated site. While the mechanisms of this effect remain undefined, it is suggested that the immune system plays an important role. A recent case report described an observed abscopal effect for a patient with metastatic melanoma treated with radiotherapy and ipilimumab, a monoclonal antibody that inhibits an immunologic checkpoint on T cells, CTLA-4.

As with many cellular and tissue effects, alterations in tumor immunity vary with radiation dose, tumor type, and microenvironmental conditions, and many questions remain. Perhaps through adaptation of radiation dosing, with or without additional immunological manipulating agents, it could be possible to utilize the immune system to improve cancer treatment strategies.

Current Radiobiology Challenges: Understanding the influence of the immune system on the irradiated tissue microenvironment, distant neoplastic disease, and systemic health; Developing targeted immunomodulation as an adjuvant to radiation therapy to improve radioresponse and enhance systemic anti-tumor immunity.
TUMOR PROMOTING INFLAMMATION

While it is important to understand the cellular response to ionizing radiation, we must consider that cells exist as components of a complex microenvironment. Tumor cells are intimately associated with supporting stromal cells, immune cells, and microvasculature. This tissue reacts as a unit to acute radiation damage. However, response to radiation is not limited to what is in the beam path; there are unique localized and systemic responses to radiation exposure that can result in perpetual effects.

Solid tumors exist in a state of active inflammation, and treatment with radiation therapy can both promote and reduce inflammation by modulation of various inflammatory pathways. The factors of the radiation-induced inflammatory response may induce beneficial anti-tumor effects, but of considerable concern are the modifications to the microenvironment that can occur that enhance tumor growth and damage normal tissues.

Immune cells play a significant role in inflammation, with macrophages considered a key contributing cell type. In the 1970s-80s, radiation was found to be a useful tool for studying macrophages, as exposure to radiation resulted in primed macrophage activation in vitro. It was found that activated macrophages release reactive oxygen species (ROS) in a time- and dose-dependent manner. In 1987, Gallin and Green considered “it is possible that the stimulatory action of radiation on the oxidative burst of macrophages is partly responsible for the tissue damage that occurs post irradiation.”

It had been established that activated macrophages were the primary cell type to produce the cytokine, tumor necrosis factor (TNF), a principal mediator of inflammation and
the immune response. Researchers found that TNF induces tumor cell death \textit{in vitro} and \textit{in vivo}. From there, Hallahan et al. demonstrated that human sarcoma cells were able to generate increased TNF levels independently following irradiation \textit{in vitro}, and that the addition of exogenous TNF enhanced the killing of these tumor cells by radiation. These synergistic effects of TNF and radiotherapy were exhibited for the first time \textit{in vivo} by Sersa et al. in a mouse tumor model.

The free radical, nitric oxide (NO), is another important cellular signaling molecule involved in inflammation. Ibuki et al. determined that both irradiation of macrophages and low doses of ROS increased the NO production of macrophages \textit{in vitro}. Tying the responses of NO and macrophages together, McKinney established that the increased NO from macrophages is mediated by the induction of TNF. Each of these interrelated signaling factors contributes to the complex tumor inflammatory microenvironment in which tumor cells themselves contribute to the inflammatory process.

As these connections were made, ROS were suspected to be involved in a “bystander” response described by Nagasawa and Little in 1992. Chinese hamster ovary cells were exposed to \(\alpha\)-particles \textit{in vitro}. It was estimated that less than 1\% of the nuclei were traversed by the particle track, however, there was an increased number of sister-chromatid exchanges in the nonirradiated cells. Building upon this observation, studies were performed that consistently showed radiation damage in cells that were not directly irradiated; further, the transfer of media from irradiated cells to nonirradiated cells induced the same type of bystander damage. Also, demonstration of \textit{in vivo} bystander injury was
shown in clonal descendants of hematopoietic stem cells after bone marrow irradiation in mice. These experiments have defined the phenomenon known as the Bystander Effect. While the mechanism of these effects remained undefined, it is strongly suspected that a chemical signaling process, involving ROS, extracellular factors, and cellular gap-junctions, exists.

Mothersill emphasizes that, although characteristics of the Bystander Effect, described above, are suggestive of inflammatory responses, it is important to note the effects seen occur at radiation doses below those necessary to induce inflammation of the kind associated with tissue injury. Regardless, many biologists suspect that perpetual radiation effects following exposure to high therapeutic or accidental radiation doses could be indicative of a continual, systemic inflammatory tissue reaction to radiation injury; for example, the classic, and curious, phenomenon of clastogenic factors isolated from the peripheral blood of long-ago irradiated individuals capable of inducing damage in non-irradiated cells. Inflammation is intimately involved with many of the integrated “Hallmarks of Cancer” pathways that influence tumor phenotypes and responses to therapy.

*Current Radiobiology Challenges: Balancing the pro-inflammatory and anti-inflammatory effects of radiation exposure to optimize tumor radiosensitivity and protect normal tissue; Determining the mechanisms and biologic consequences of the radiation bystander effect to improve radiation therapy practices and radiation protection standards.*
INDUCING ANGIOGENESIS

It has been recognized for over 100 years that tumor vasculature is unique and abnormal. *In vivo* imaging in the early twentieth century led to the understanding that, in order for a developing solid tumor to grow into a macroscopic mass, it must induce the surrounding normal tissue to either share its oxygen and nutrient supply or create its own vascular network \(^{93-95}\). The process by which a tumor forms new blood vessels and remolds existing vasculature involves highly integrated signaling and communication between cells of the microenvironment. The interplay of ionizing radiation effects with tumor and vascular cell death or stimulation may be exploited for improved therapeutic outcomes.

By the 1950s, it was well known that cells deficient in oxygen supply, or hypoxic, at the time of irradiation are much less damaged by radiation than those which are oxygenated \(^{96}\). Thomlinson and Gray revealed in their histological studies of bronchogenic carcinomas that these tumors *in vivo* contain cells that were predicted to vary in degree of oxygenation; specifically, that the peripheral tumor cells, closest to vessels, were more aerobic than those at the centers of nodules because of increasing distance from a blood supply \(^{97}\). They presumed that the peripheral cells would be more easily destroyed by irradiation, while the central cells would be radioresistant. By the 1960s, Rubin and Casarett provided some of the first data suggesting that tumor stromal cells (endothelial cells) might contribute to the primary effect of radiation in the destruction of tumors. Using transplanted tumors subjected to fractionated radiation in rats, the microcirculation did not involute as rapidly as the parenchymal tumor cells, leading to a relative state of supervascularization, and presumed
increase of tumor tissue oxygenation. Thus, their theory was that radiation fractionation could increase the radiosensitivity of the mass as a result of reoxygenation. More recent studies have shown that the underlying mechanism for supervascularization is more complex.

Several noteworthy studies demonstrate the importance of inducing vascular death for effective tumor eradication by radiation therapy. Tumors stimulate persistent angiogenesis through expression of many factors, one of the most important being the family of vascular endothelial growth factor (VEGF) proteins. Building upon the observation that serum VEGF levels are increased in patients with malignant tumors after radiation therapy, Gorski et al. evaluated this paracrine relationship between the tumor and its vasculature as a potential target for radiation-induced antitumor activity. Treatment of tumor-bearing mice with a neutralizing antibody to VEGF before irradiation was associated with a greater than additive antitumor effect; supporting the idea that induction of VEGF by ionizing radiation contributes to the protection of tumor blood vessels from radiation-mediated cytotoxicity and tumor radioresistance (Figure 1.3). Further, in a series of experiments investigating high dose radiation-induced apoptosis of endothelial cells in tumor vessels, Garcia-Barros et al. demonstrated that microvascular death was a key mechanism in tumor responses to radiation.

As numerous cytokines were discovered to be involved in vascular radioresistance, Moeller et al. posited that blockade of the cytokine production at the source of the common pathway, hypoxia inducible factor-1 (HIF-1), might prove effective as an antitumor treatment with radiotherapy. HIF-1 is capable of stimulating the expression of downstream genes
involved in tumor metabolism, growth, and angiogenesis, including VEGF. Moeller found that HIF-1 accumulated in tumors after radiation exposure; this occurred concomitantly with reoxygenation. The increase in HIF-1 was attributable to increased production of reactive oxygen species, as well as enhanced translation of HIF-1 regulated transcripts. The resulting increase in HIF-1 regulated cytokines enhanced endothelial radioresistance, and through inhibition of HIF-1 activation, tumor radiosensitivity was significantly increased as a result of enhanced vascular destruction.

In more recent studies, it has been shown that bone marrow-derived myeloid cells play an important role in modification of the tumor microenvironment following irradiation. Ahn and Brown demonstrated that bone marrow-derived myeloid cells enter irradiated tumors to promote restoration of tumor vasculature through the process of vasculogenesis, thereby allowing growth of the tumor following irradiation.

An interesting therapeutic paradox exists whereby antiangiogenic agents can improve tumor oxygenation by “normalizing” the abnormal tumor vasculature. Preclinical and clinical evidence to support this concept has been reviewed recently by Goelet. al. This vascular normalization may exist within the tumor microenvironment for a limited time. Treatment of solid tumors with antiangiogenic agents can create an observed “normalization window”, typically within 1-2 days of starting treatment. Irradiation of a solid tumor during this period of optimal perfusion and oxygenation could translate to improved tumor radiosensitivity and therapeutic response.
It is clear that there is a fine line that challenges the fundamental therapeutic goals of radiation therapy in regard to the importance of tumor vasculature in influencing tumor reoxygenation: 1) it increases radiosensitivity for the next dose of radiotherapy, however 2) it contributes to enhanced endothelial cell survival because of HIF-1 mediated increases in VEGF production. Which of these two phenomena contribute to improved overall tumor control remains controversial.

**Current Radiobiology Challenges:** Manipulating angiogenesis and the tumor-associated vasculature to facilitate perfusion and oxygenation of tumor cells for optimal radiosensitivity; Targeting the tumor-promoting mediators associated with radiation-induced tumor reoxygenation.

**SUSTAINING PROLIFERATIVE SIGNALING**

In normal tissues, growth factors regulate cellular proliferation and differentiation. Cancer cells, however, develop a reduced requirement for exogenous growth factors as they become capable of autonomous activation of autocrine and paracrine growth pathways. As the goals for radiation therapy include tumor regression and eradication, this aberrant proliferation must be inhibited to prevent resistance and recurrence.

The growth of a solid tumor is determined by cell production and cell loss. Studies performed in the 1960s using autoradiographic methods determined that the rate of cell production is a function of the fraction of proliferating cells and the cell cycle time\(^{107}\). Through investigation as to how irradiation induces changes in tumor growth rate, Hermens...
and Barendsen revealed that during the later stages of regression of irradiated rhabdomyosarcomas in rats, there is a rapid, but subclinical, increase in proliferation\textsuperscript{108}. The first clinical evidence for tumor cell repopulation came from Withers et al.’s comprehensive retrospective analysis of the relationship between radiation dose and overall irradiation time in head and neck cancer\textsuperscript{109}. The findings strongly suggested that increasing the overall duration of radiotherapy results in decreased local tumor control through accelerated repopulation\textsuperscript{109}.

Some of the key proteins regulating cell survival and proliferation are the transmembrane epidermal growth factor receptors (EGFR) and ligands epidermal growth factor (EGF) and transforming growth factor-\(\alpha\) (TGF\(\alpha\)). EGFR is overexpressed in many tumor types and is associated with aggressiveness and resistance to therapies. It has been shown \textit{in vitro} and \textit{in vivo} that tumors with high expression of EGFR are more radioresistant (Figure 1.3)\textsuperscript{110-112}. Studies have reported that cell survival and repopulation are regulated by the activation and expression of EGRF/TGF\(\alpha\) which is induced following radiation\textsuperscript{113,114}. Further, treatment of neoplastic cell lines \textit{in vitro} with EGF is protective against ionizing radiation, while this effect is abrogated when the cells are exposed to monoclonal antibodies against EGFR\textsuperscript{115,116}. With these findings, Balaban et al. suggested that radiation may mimic the action of a natural ligand on EGFR and trigger resistance to radiation by receptor activation\textsuperscript{116}.

Strategies to counteract tumor proliferation with radiation therapy have included modifications to treatment protocols and biological inhibitors. Alternative radiotherapy
regimens, particularly used for treatment of head and neck squamous cell carcinomas (HNSCC) have been assessed. Such schedules as hyperfractionation and/or acceleration have been designed to increase the dose-intensity, and reducing the total treatment time is meant to reduce the repopulation of tumor cells between treatments, resulting in improved locoregional control. In meta-analysis of HNSCC, alternative fractionation showed a significant benefit for locoregional control when compared to conventional protocols.

Biological antiproliferative agents have been shown to alter tumor radiosensitivity in experimental models and clinically. Blockade of EGFR through anti-EGFR monoclonal antibodies or EGFR-selective tyrosine kinase inhibitors have been proven to be effective adjunct treatments to radiotherapy in vitro and in vivo, as well as in multinational, randomized, phase 3 trials.

Despite the success of these new treatment strategies, at most 15% of patients with HNSCC will have increased locoregional control or survival. Alternative treatment modalities, such as adjuvant therapies targeting proliferative pathways downstream to EGFR, are being intensely investigated to counter radioresistance more effectively and improve patient outcomes.

New information as to the initial driving events responsible for tumor repopulation after radiotherapy has been provided by Huang et al. They describe that dying, irradiated tumor cells use the apoptotic process to generate potent growth-stimulating signals through activated caspase-3 to stimulate the repopulation of tumors. This recent discovery may
lead to enhanced tumor control and curability through the development of novel caspase-3 inhibiting agents.

*Current Radiobiology Challenges: Interfering with tumor proliferation and repopulation through manipulation of radiation treatment protocols and novel targeted therapeutics.*

**DEREGULATING CELLULAR ENERGETICS**

It has been discussed with the preceding hallmarks that tumors adapt and proliferate despite harsh conditions of inflammatory stress and disorganized, ineffective microcirculation. The tumor microenvironment is not only characterized by varying degrees of hypoxia, but also nutrient deprivation and low extracellular pH. The unusual metabolism of cancer cells is intimately involved in creating such an abnormal physiologic state, and the intricacy of how ionizing radiation alters this cancer hallmark is an active area of research.

Normal tissues are able to sustain routine cellular functions through the oxidation of nutrients to provide energy in the form of adenosine triphosphate (ATP). Glucose metabolism is regulated in response to changes in cellular oxygen levels. Initially, glucose is converted to pyruvate by glycolytic enzymes. Under well-oxygenated conditions, pyruvate is oxidized in the tricarboxylic acid cycle to form water and a substantial amount of ATP (oxidative phosphorylation); however, under hypoxic conditions, lactate dehydrogenase A converts pyruvate to lactate and generates very little ATP (glycolysis).
German biochemist and physiologist, Dr. Otto Warburg, was the first to define the difference in glucose metabolism between normal and neoplastic cells. In 1924, Warburg analyzed the ratio of oxidative phosphorylation to glycolysis in different tissues of cancer cells and normal cells. He found that glycolysis, under aerobic conditions, was particularly high in aggressive tumors when compared with benign tumors and normal tissues. This discovery of tumor-specific, oxygen-independent glycolysis was labeled the Warburg Effect. This peculiar metabolic preference of tumor cells drives a significantly inefficient breakdown of glucose for ATP and creates an acidic microenvironment through the build-up of the byproduct lactic acid.

As has been mentioned in previous sections, HIF expression drives many neoplastic pathways. In the case of tumor metabolism, crosstalk between HIF and the proto-oncogene c-MYC drives the exceptionally high rate of glycolytic activity of cancer cells to maintain sufficient ATP for tumor growth and progression. HIF-1 has been shown to regulate glycolysis, as well as expression of the glucose transporters, GLUT1 and GLUT3, which mediate cellular glucose uptake. Determined to be upregulated following irradiation, HIF-1 can enhance tumor radiosensitivity by increasing ATP metabolism and proliferation rates; however, this is countered as HIF-1 also promotes radioresistance through vascular protection. Alternatively, activation of oncogenes, such as c-Myc, or inactivation of p53 can contribute to the Warburg effect in a HIF-1 independent fashion.

The increased dependence of cancer cells on the glycolytic pathway provides a biochemical basis for the design of therapeutic strategies to preferentially kill cancer cells by
pharmacological inhibition of glycolysis. Inhibitors of the glycolytic pathway have been tested for their ability to cause radiosensitization, such as 2-deoxyglucose (2-DG). 2-DG is a glucose analog that acts as a competitive inhibitor of glucose metabolism. In 1996, Mohanti et al. reported the results of their clinical trial combining 2-DG with radiotherapy in cerebral glioma patients. Their study showed that administration of 2-DG in combination with radiation is safe and could be tolerated without acute toxicity and late radiation damage to the brain. Further clinical studies combining radiation therapy with glycolytic inhibitors will determine if this is an effective approach to tumor control.

While there remains a gap in our understanding of how energy inefficient cancer metabolism benefits the progressing neoplasm, a key to enhanced radiosensitivity may lie in exploiting this tumor-specific state.

**Current Radiobiology Challenges:** Determining the influence of tumor cell metabolism on the radiation response; Investigations of glycolytic inhibitors as an adjuvant to radiotherapy

**ACTIVATING INVASION AND METASTASIS**

The final Hallmark to be reviewed is Invasion and Metastasis, the processes of which are particularly convoluted and intricate. The properties required to attain the phenotype of an invasive and metastatic neoplasm involve the multistep, coordinated foundation of the previously discussed hallmarks. As such, this concluding section will highlight the influence
of several cancer-promoting genes, proteins, and molecules, unique to or complimentary to invasion and metastasis, and how they pertain to radiation biology.

Radiation therapy is a principal treatment modality in clinical oncology because it is an effective means of local tumor control and can be highly curative for many cancer types. However, there were observations in the earliest stages of radiation oncology that ineffective irradiation of solid tumors could ultimately result in the enhancement of metastasis. The first major experimental investigation of the effects of local tumor irradiation on metastasis was reported by Drs. Kaplan and Murphy in 1949\textsuperscript{135}, and then the first prospective, randomized trial performed by Strong et al. in 1978 provided significant evidence that there is a correlation between radiation exposure of a primary tumor and the development of metastasis\textsuperscript{136}. Additional clinical studies revealed that patients with local failure after radiation therapy have a higher incidence of distant metastases than those who maintain local control\textsuperscript{137,138}.

It has now been realized that interactions of the complex tumor microenvironment can drive the development of an invasive, metastatic tumor. Several noteworthy clinical trials were performed in the 1990s which investigated the influence of various tumor parameters on outcome following radiation therapy. Significant positive correlations between low tumor oxygenation or high lactate concentration and poor radiation treatment outcomes, particularly the development of regional or distant metastasis, have been repeatedly reported for numerous tumor types\textsuperscript{139-145}.

Experimental molecular oncology studies suggest that the progression of a solid tumor to a metastatic phenotype is not simply the result of dysregulated signal transduction
pathways, but instead is achieved through a stepwise selection process driven by hypoxia\textsuperscript{146}. The adaptation of populations of neoplastic cells to hypoxia facilitates cancer cell dissemination through the up- or down-regulation of critical metastasis-associated genes, such as E-/N-cadherin for epithelial-mesenchymal transition\textsuperscript{147,148}, uPAR for degradation of the basement membrane and extracellular matrix\textsuperscript{149}, HGF/MET for cell motility\textsuperscript{150,151}, and VEGF for stromal interactions, intra/extravasation, and angiogenesis\textsuperscript{152}. The systematic alteration of these phenotypic regulators allows cells to escape the hostile microenvironment of the primary tumor and colonize a secondary location.

Considered separately from the influence of hypoxia on tumor aggression, radiation exposure may promote invasion and metastasis, as well. It has been shown that ionizing radiation can cause significant tumor reoxygenation and elicit a stress response in cells\textsuperscript{102,129}. Further, this response induces the expression of HIF-1, known to activate many genes known to be important in cancer metastasis\textsuperscript{153}.

The standard radiotherapeutic goals are effective and efficient eradication of the primary tumor for long-term control or curability. Certain physiologic conditions are known to be associated with a more advanced, malignant tumor, likely to be resistant to therapy and to metastasize. Repeated exposure of such tumors to radiation may exacerbate these traits if not countered by novel, targeted therapies against the mechanisms of neoplastic adaptation and progression.

\textit{Current Radiobiology Challenges: Understanding the local and systemic mechanisms of radiation-induced or radiation-influenced tumor cell invasion and metastasis;}
Identification of targeted inhibitors of tumor cell invasion, colonization, and/or metastatic development as adjuvants to radiotherapy.

MOVING FORWARD

The field of radiation biology continues to evolve. While the Hallmarks of Cancer describe the intricacies of the development and progression of neoplasms, we have reviewed the original discoveries which have led to our understanding of how the tumor cells and microenvironment react and change following exposure to ionizing radiation. Exciting new radiobiology research featured at the 59th annual meeting of the Radiation Research Society included presentations in the topics of radiation DNA damage and repair, the roles of miRNA in radiation response, effects of oxidative stress, radiation immune responses, inflammatory responses, vascular effects, the radiation responses of stem cells, radiation effects of normal tissue, and microenvironmental radiation responses. While the laboratories of established, expert radiobiologists continue to make late-breaking discoveries, the next generation of radiation researchers is active, enthusiastic, and eager to contribute meaningfully to the field.

CONCLUSION

Hanahan and Weinberg’s Hallmarks of Cancer provides a useful conceptual framework for understanding the complex biology of cancer, and it is evident that intimate associations link each. The categorical focus of these cancer principles allows the
specialized refinement of molecular mechanisms; however, the appreciation that each hallmark operates as part of a greater circuitry will promote discoveries that further advance radiation biology and therapy. The achievements of radiation biologists and clinicians featured in this review article mark particular eras in interpretation of oncologic concepts (Figure 1.5). It is stimulating and motivating to envision what current breakthroughs and innovations will be the radiation biology history of tomorrow.
Figure 1.1: Enabling replicative immortality. Schematic diagram of the arrangement used to grow clones of HeLa cells. A - support for microscope slides; B – microscope slide(s) on which the clones develop; C – clone; D – Petri dish; E – level of liquid nutrient medium; F – layer of feeder cells. (Reproduced from Puck and Marcus 1955\textsuperscript{2} with permission from Marcus)
Figure 1.2: Genome instability and mutation. Frequencies of HPRT mutations among clonal populations of CHO cells examined approximately 23 population doublings after they were derived from non-treated cells (controls) or single cells surviving irradiation with X-rays (4-8Gy) or α-particles (2Gy). The number of clones examined is shown at the bottom of the x-axis. Numbers in the shaded area represent the number (%) of clones showing no mutations. Increased frequencies of mutations were observed in a significant of the clonal populations derived from irradiated cells. (Reproduced from Little et al. 1997 with permission of the Radiation Research Society)
Figure 1.3: Inducing angiogenesis/Sustaining proliferative signaling. (A) Effect of VEGF blockade before treatment with IR in human tumor xenograft (Seg-1 esophageal adenocarcinoma). Tumor-bearing mice were treated with neutralizing anti-VEGF antibody, IR, or a combination of the two, and tumor volumes were measured. Square, control; diamond, IR alone; triangle, anti-VEGF antibody alone; circle, IR combined with anti-VEGF antibody. Tumor-bearing mice were treated with 20Gy in four 5Gy fractions on days 0, 1, 2, and 3. There was a statistically significant difference between the IR alone and combined treatment groups for all days after day 6 ($p < 0.02$ for all days). (B&C) Relationship between EGFR expression and radiocurability of murine carcinomas. (B) both wild-type (open circles) and mutant p53 (closed circles) tumors. (C) wild-type p53 tumors only. The levels of EGFR are inversely correlated with radiocurability. (Reproduced from Gorski et al. 1999\textsuperscript{100} (A), and Akimoto et al. 1999\textsuperscript{111} (B&C) with permission from AACR)
Figure 1.4: Deregulating cellular energetics. HIF-1 maintains tumor bioenergetics. (A) Ex vivo ATP bioluminescence. ATP is significantly depleted in 4T1 and HCT116 tumors in response to HIF-1 blockade ($p < 0.05$). (B) PC-3 cells were cultured under hypoxia with high glucose (HG; 5g/l)- or low glucose (LG; 0.1g/l)-containing media, with or without doxycycline stimulation. Low glucose concentrations caused a significant right-shift in the survival curve and HIF-1 inhibition further enhanced this effect ($p < 0.05$). (Reproduced from Moeller et al. 2005\textsuperscript{129} with permission from Elsevier)
Figure 1.5: Hallmarks of cancer with associated radiation biology concepts. The featured examples of biological molecules, mechanisms, and effects reviewed as they pertain to radiation biology and therapy are summarized according to the expanded categorization of the hallmarks. (Modified from Hanahan and Weinberg2011 with permission from Elsevier).
CHAPTER 2

PARTIAL TUMOR RADIATION DRIVES TUMOR AGGRESSION

INTRODUCTION

Classical radiation biology considers what happens to tissues within a targeted radiation field; but, there is growing interest in what is occurring within cells and tissues located outside of the radiation field. These are known as non-targeted, or "bystander" cells. It has been demonstrated that these non-targeted cells can undergo genotypic and/or phenotypic changes, chromosomal instability, and even cell death through what is known as the "bystander effect"\(^\text{92}\). The bystander effect can occur directly adjacent to directly irradiated tissue or at an extended distance within the body\(^\text{92}\). While the specific processes of the bystander effect are still being elucidated, two generally well-accepted mechanisms are 1) soluble mediators are released by irradiated cells; such as reactive oxygen or reactive nitrogen species; 2) mediator molecules are passed via gap-junctions for intercellular communication\(^\text{92}\). For the focus of this study, we consider radiation-induced reactive oxygen species. Reactive oxygen species are responsible for what is known as "oxidative stress" within cells and tissues. Oxidative stress is the imbalance of between the production of reactive oxygen species and the body's ability to readily counteract or detoxify their harmful effects.
In addition to their role in the radiation bystander effect, reactive oxygen species can also influence the response of a directly irradiated tumor. In fact, the Dewhirst laboratory was the first to show that radiation can actually induce a protective tumor response via the generation of reactive oxygen species and overexpression of the tumor-promoting transcription factor, hypoxia inducible factor-1 (HIF-1)\textsuperscript{102}. HIF-1 is a heterodimer, consisting of an inducibly expressed HIF-1α subunit and a constitutively expressed HIF-1β subunit\textsuperscript{154}. HIF-1α is normally kept at low levels in the presence of oxygen via proteosomal degradation mediated by the VHL complex\textsuperscript{154}. As the name suggests, under hypoxic conditions, the HIF-1α subunit accumulates due to a decrease in the rate of proteolytic degradation; the resulting HIF-1α/HIF-1β heterodimers bind to DNA at hypoxia response elements of numerous genes to upregulate their transcription\textsuperscript{154}. HIF-1 regulates not only hypoxia-related gene expression, but also pathways which support tumor growth and progression, such as angiogenesis, epithelial-mesenchymal transition, tumor cell migration and invasion, and metastasis\textsuperscript{154}. In addition to the regulation of HIF-1α by fluctuations in oxygenation, the study by Moeller et. al demonstrated that HIF-1α stabilization was increased in tumors after radiation\textsuperscript{102}. This occurred as radiation exposure induced hypoxia-reoxygenation injury in the tumor, leading to reactive oxygen species, which stabilized HIF-1α\textsuperscript{102,155}. Ultimately, this increased HIF-1 expression resulted in tumor endothelial cell/vascular protection and radioresistance\textsuperscript{102}. High levels of intratumoral reactive oxygen species can remain over time following radiation, leading to a persistent increase in HIF-1. Radiation-induced reactive oxygen species are created by mitochondrial damage\textsuperscript{156} and hypoxia/reoxygenation injury\textsuperscript{102}. 
in the days following radiation, but oxidative stress also continues via infiltration of macrophages into the tumor\textsuperscript{157}. These infiltrating macrophages produce excessive amounts of nitric oxide (NO). NO prevents HIF-1\textalpha binding to the VHL complex for degradation\textsuperscript{158} so increased HIF-1 expression is maintained.

Knowing that HIF-1 expression is increased due to oxidative stress following global tumor radiation, we performed a series of studies to understand whether the radiation bystander effect can increase HIF-1 in sublethally-irradiated tumor regions via the generation of oxidative stress. Further, we aimed to understand how these intratumoral radiation bystander effects impact tumor behavior locally and distantly. Since HIF-1 is associated with a number of tumor promoting pathways, we hypothesized that bystander cells, bombarded with reactive oxygen species, would undergo aggressive tumor changes as a result of increased HIF-1 expression (Figure 2.1).

There are clinical implications of the increase in HIF-1 transcriptional activity after radiotherapy, particularly if these changes occur in sublethally irradiated cells. One scenario occurs with conformal radiation therapy methods. Conformal radiation therapy, such as image guided radiation therapy (IGRT), permits precise delivery of radiation to a target volume while minimizing dose to surrounding tissue by tightly conforming to the tumor margin\textsuperscript{159}. Thus, margins around gross tumor volumes have become narrower. As margins
shrink, however, there is increasing risk that some tumor cells may be missed from the target field and sublethally irradiated; this is referred to as marginal miss (Figure 2.2).

There is a clinical precedence for believing that marginal miss can occur and that it can lead to a worse outcome. For example, there are clinical data showing that conformal radiation yields worse outcomes than traditional methods. Partial orbital radiation for patients with orbital lymphoma showed a higher local recurrence rate compared with whole orbital treatment\textsuperscript{160}. A higher biochemical failure rate was observed in prostate cancer patients whose treatment plan was based on implanted fiducial markers compared to those planned with traditional bony landmarks\textsuperscript{161}. In this case, the fiducial markers reduced field size, increasing the risk for marginal miss. Marginal miss has been reported after conformal radiation in breast and head and neck cancers\textsuperscript{162-165}. To note, we use the term “sublethally-irradiated”, since cells outside the target dose volume will receive some low radiation dose. Indeed, a small percentage of cells will succumb to this low dose, but the majority of the cells will survive. Also, we recognize that sublethal irradiation can also occur within a tumor because of hypoxia\textsuperscript{96}. Here, we focus on sublethally-irradiated cells associated with marginal miss, but the consequences of sublethal irradiation in hypoxia are also relevant.

In this study, we investigate intratumoral radiation bystander effects. Bystander effects were examined in the context of the consequences of partial tumor radiation for the local tumor microenvironment and distant metastasis formation. The roles of oxidative stress and HIF-1
expression are examined as critical factors in the mechanism of intratumoral radiation bystander effects. To study the local tumor effects, we administered partial tumor radiation using microbeam radiation (MRT) in our dorsal skin fold window chamber model and evaluated several tumor microenvironmental parameters. Then, to study distant effects, we utilized advanced, small animal IGRT to simulate marginal miss in an orthotopic dorsal mammary tumor model and evaluated the development of pulmonary metastasis.

MATERIALS AND METHODS

4T1 Cell Lines

4T1-RFP-GFP cell line

A genetically engineered 4T1 murine mammary carcinoma cell line was used for this study. This line was stably transfected with a plasmid vector encoding a green fluorescent protein (GFP) reporter. Expression of GFP in the cell is driven by a HIF-1-modulated hypoxia responsive element (HRE). An HRE is a DNA sequence in the promoter region of a gene that HIF-1 binds to in order to induce enhanced gene transcription. Thus HIF-1 expression within a tumor grown from this line can be directly visualized and quantified through the intensity of the GFP signal. This line was also stably transfected with a red fluorescent protein (RFP) gene under control of the CMV (cytomegalovirus) promoter. Expression of RFP is constitutive (always active) and is used to visualize the spatial location and extent of the tumor.
4T1-Luciferase Cell Line

A modified 4T1 murine mammary carcinoma cell line was used for this study (a gift from Dr. Michael Wendt, Case Western Reserve University). This stably transfected cell line constitutively expresses Firefly Luciferase-2 under a CMV promoter with Zeocin resistance (500ug/mL) for selection in vitro. These 4T1-luciferase-expressing cells (4T1-Luc) were cultured in Dulbecco's modified eagle medium (DMEM, high glucose with sodium pyruvate and glutamine) with 10% fetal bovine serum and Zeocin (500ug/mL) and incubated at 37°C in 5% CO₂. Cells were passaged 1:10 when 75-90% confluent. These cells were routinely tested and confirmed to be free from Mycoplasma contamination.

Surgical Procedures

Dorsal window chamber preparation

A thorough explanation of the dorsal window chamber surgical procedure is provided in prior publications. Briefly, nude mice were anesthetized using a ketamine/xylazine preparation and the surgical area was disinfected using exidine and ethanol. In each mouse, a circular incision, 12mm in diameter, was made in the loose skin of the dorsal surface. The skin and underlying connective tissue were removed completely. The dorsal skin was pinched up to form a skin fold which exposed the dermal tissue on the opposite side of the area of excised skin. The incision was aligned with a hole cut into a specially constructed titanium frame, which was then sutured onto the animal to form a stable window into the
underlying tissue. Approximately $2 \times 10^4$ 4T1-GFP-RFP cells suspended in a serum-free media were injected beneath the exposed fascial plane. A sterile glass coverslip was then inserted into the titanium frame to form an air-tight, optically-accessible window into the inoculated tumor and surrounding tissue. Irradiation was carried out one week after tumor cell transplant. At this time they had formed solid, vascularized, tumors 3-5mm in diameter. We have shown previously that these tumors are hypoxic and express HIF-1 at this size range.

102 169 170

Orthotopic Dorsal Mammary Tumor Implantation

4T1-Luc orthotopic mammary tumors were surgically implanted into the dorsal mammary fat pad of BALB/c mice. Mice were anesthetized using intraperitoneal injections of ketamine (85 mg/kg) and xylazine (8.5 mg/kg), fur overlying the dorsal thorax was removed using a chemical depilatory (Nair, potassium thioglycolate), and the surgical field was prepared using three alternating applications of chlorhexidine gluconate scrub solution and 70% ethanol. A small skin incision was made overlying the dorsal mammary fat pad. The fat pad was visualized, and approximately $5 \times 10^5$ 4T1-Luc cells suspended in 100uL of serum-free DMEM were injected into the site. The incision was closed using 4-0 silk suture and a single cruciate suture pattern. Triple antibiotic was applied to the incision, and buprenorphine (0.1 mg/kg) was administered subcutaneously as analgesia for the recovering mice.
Biological Timeline for Development of Orthotopic Dorsal Mammary Tumors and Metastasis

Following the surgical implantation of tumor cells, mammary tumors grew reliably and reached treatment size (approximately 250mm$^3$) within six to ten days. Orthotopic dorsal mammary tumors were visible via bioluminescence imaging (Figure 2.3A). Metastatic lesions could be identified within the lungs via bioluminescence imaging as early as six days following tumor implantation (Figure 2.3B). Later, it was determined via histologic analysis of mouse lung sections, that metastatic cells could be identified as early as four days following tumor implantation with this technique.

Tumor Growth Monitoring

Palpable tumors were identified between 6 to 10 days following surgical implantation.

Tumors were measured daily with calipers, and volumes were calculated using the formula $V = (A^2 \times B \times \pi)/6$, where $A$ is the shortest diameter and $B$ is the longest diameter.

WINDOW CHAMBER IRRADIATION AND TUMOR MICROENVIRONMENT ANALYSIS

Optical Data Acquisition and Image Processing

Hyperspectral image series were used to spectrally separate oxy/deoxy hemoglobin components for analysis of hemoglobin O$_2$saturation$^{171}$. Total hemoglobin data (oxy + deoxy hemoglobin) were used to measure vascular hemoglobin concentrations. We also used the
hyperspectral system to separate fluorescent reporters of interest (RFP and GFP) from tissue autofluorescence to more accurately quantify levels of expression.

Total hemoglobin images were used to quantify vascular structural features (i.e. vessel centerlines, diameters, branch points) using the described vessel segmentation/skeletonization algorithm. Video images of blood flow within the tumor were used to map and quantify flow speed and direction. The algorithm used for this procedure is presented in a prior publication\textsuperscript{172}.

\textit{Window Chamber Tumor Irradiation and Dosimetry}

A nanotechnology-based x-ray experimental irradiator specifically designed to produce microbeam radiation was used for this study\textsuperscript{173-175}. The novel irradiation system utilizes a line source design (instead of the conventional point source) that is optimized to produce the highest beam intensity for the microbeam. Anesthetized mice were placed in the system with their titanium frames immobilized on a heated stage. Animals treated in the microbeam tumor irradiation group were exposed to 50Gy at a dose rate of 1Gy/min as 160 kVp photons passed through a collimator to produce a single full-width half-max (FWHM) 300µm wide microbeam through the middle of the tumor (Figure 2.4A). Radiation intensity fell off sharply, with areas greater than 200µm from the beam centerline receiving less than 10% of the maximum dose. This beam spanned the width of the window along its centerline, and its track was visualized using a radiation sensitive film glued to the front of the glass coverslip.
(Figure 2.4B). For the whole tumor irradiation group, the microbeam collimator was removed, and the entire window was irradiated while sparing the rest of the animal’s body. In both irradiation procedures, the delivered dose was 50Gy\textsuperscript{174,176}. The dose of 50Gy was selected based on preliminary experiments whereby a single microbeam of radiation induced a tumor vascular response and the corresponding wide field exposure allowed sufficient tissue viability for biologic comparisons. A mock irradiated group was also used as a control.

Window Chamber Imaging Schedules

The methods outlined above were used to optically observe and quantify changes in HIF-1 and vascular response after irradiation. Animals were imaged approximately two hours prior to irradiation, two hours after irradiation, and each day following through seven days post-treatment. For each time point, the windows were imaged using the hyperspectral system described above, with illumination, filtering, and exposure parameters adjusted to capture total hemoglobin/saturation, GFP fluorescence emission, and RFP fluorescence emission. The tumor and surrounding area were imaged at 2.5x magnification for these optical components, and it was from these images that the regions of tumor core, rim, and tumor-associated normal tissue were identified (Figure 2.5). Video images were also collected in a 24 Hz, 128 frame series at 5x magnification in order to capture the movement of discrete red blood cells as they moved through the vasculature.
Local Tumor Microenvironment Histologic Evaluation

After the completion of imaging on Day 7, animals were euthanized and their window chamber tumors were immediately extracted and flash frozen. The frozen tumors were then processed by routine methods and multiple (at least 4) successive 4 um sections were stained with hematoxylin and eosin (HE). After histological preparation, cellular features were evaluated by a pathologist who was blinded to the treatment groups at the time of assessment.

ORTHOTOPIC DORSAL MAMMARY TUMOR RADIATION AND DISTANT METASTASIS ANALYSIS

Image-Guided Radiation Therapy for Orthotopically Transplanted Tumors

Depending on the experiment, when the longest diameter of tumors was greater than 7mm or on the defined treatment day (Figure 2.6), mice were anesthetized with 1.5% isoflurane gas mixed with oxygen and placed in an X-RAD 225Cx (Precision X-ray Inc) small animal micro-CT irradiator. A collimating cone that produced a 10 x 10-mm radiation field was used to target the radiation beam to the dorsal mammary tumor. The field was rotated 45 degrees to create a diamond shape over the irradiated area (Figure 2.7). Fluoroscopy at 40 kVp and 2.5 mA with a 2-mm Al filter allowed proper alignment of the radiation field. The cranial and caudal aspects of the tumor were identified with small barium markers which had been applied to the tumors via visualization and palpation of anesthetized mice. A lead alloy shield was fixed to collimator to create a treatment field whereby the longest axis was 50% blocked or 90% open and 10% blocked for the marginal miss experimental treatment groups.
The treatment groups will be referred to by the volume of tumor radiation administered; for example, 100% tumor volume radiation (100% RT), 90% marginal miss (90% RT), non-irradiated control (0% RT). Mice were treated with one fraction of 15Gy administered as two exposures of 7.5Gy from parallel-opposed fields with a dose rate of 257 cGy/min at target depth with 225 kVp and 13 mA and a 0.3-mm Cu filter. Control mice were anesthetized but not irradiated. Depending on the experiment (Figure 2.6), mice were euthanized when individual tumors reached 1500mm$^3$, a defined time point following radiation had been reached, mice developed neurologic deficits, or mice had lost greater than 20% of their initial body weight.

Radiation Dosimetry for Orthotopic Tumor Irradiation

Focal Dosimetry: Using a nano-crystalline scintillator fiber optical detector (nanoFOD)$^{177}$, focal dosimetry data was obtained for regions of interest within and around the radiation treatment field (Figure 2.7) in order to determine the radiation dose which would be administered to directly irradiated tissue, blocked ("bystander") tissue, and regional normal tissue (lungs, spine) in the treated mice.

Regional Dosimetry: Additional regional dosimetry data was obtained using a rodentmorphic 3D dosimetry system and SmART-Plan small animal radiation treatment planning software in order to generate dose volume histograms (DVH) for tumor, lung, and spinal cord volumes$^{178}$ (Figure 2.8).
Bioluminescence Imaging of Lung Metastases

Mice were injected intraperitoneally with D-Luciferin (Potassium salt, GoldBio, 150mg/kg), and after 10 minutes, euthanized with an intraperitoneal injection of Euthasol (pentobarbital sodium and phenytoin sodium, 0.5uL per mouse). Lungs were dissected and individually imaged using an IVIS Kinetic system within 15 to 20 minutes of luciferin injection in order to measure total bioluminescence signal emitted. Total signal was measured over 60 seconds at a height of 5cm.

Tumor Dissection

Tumors were dissected following euthanasia, divided into two portions along the long axis of the tumor, flash frozen in liquid nitrogen, and stored at -80°C. The two tumor portions were saved in order to 1) analyze global oxidative stress and HIF-1 protein levels and 2) spatial HIF-1 and oxidative stress via immunohistochemistry.

Lung Preparation for Histology

Lungs were gently perfused with 1mL of phosphate buffered saline (PBS) followed by 1mL of 4% paraformaldehyde (PFA) in PBS via the intact attached trachea. The lungs were placed in 5mL of 4% PFA and stored at 4°C for 48 hours. The lungs were transferred to 5mL of PBS and stored at 4°C for 48 hours, then held within 5mL of 70% ethanol at 4°C until processing for histology.
**Gross Metastasis Counts**

When lungs had completed the lung histology preparation protocol, they were briefly removed from the ethanol solution and gross metastatic lesions were visualized and counted manually using a Leica StereoZoom 4 microsurgery magnification system.

**GSH and GSSG Analysis**

The ratio of oxidized to reduced glutathione (GSSG/GSH) was used as a measurement of the degree of oxidative stress within the treated tumors. The assay for tissue samples was inspired by the published assay for analysis of GSH and GSSG in blood\(^1\). The assay was adapted for analysis of tissue samples and to the LC/MS/MS equipment available.

**Tissue Processing:** Partially thawed tumor tissue was cut (~20 mg) and homogenized immediately in 500-μL conical polypropylene vial with: 4 parts (g/vol) of GSH-trapping solution (20 mM-ethylemaleimide (NEM; Sigma), 2% SSA and 2 mM EDTA in 15% methanol), 10 parts (g/vol) of 1% formic acid (Fluka) and two 2.5mm Zr-silica beads (Biospec Products Inc.) in FastPrep (Thermo-Savant) homogenizer at speed 5 for 40 s. After 30 min incubation at room temperature, 150 μL of the homogenate was transferred into a 2-mL conical polypropylene vial, 500 μL of chloroform (B&D) was added, agitated in FastPrep at speed 5 for 40 s, followed by centrifugation at 16,000 g for 5 min. GSH analysis: 5 μL of supernatant, 1 mL of deionized water, and 100 μL of 1 μM GSH-NEM-d3 (internal standard) was combined and placed into autosampler at 4 °C. GSSG analysis: 20μL of
supernatant and 20 μL of 1 μM GSSG-d6 (internal standard) in mobile phase A (see below) was combined and placed into autosampler at 4 °C.

**LC-MS/MS Analysis**

The measurement of GSH (as GSH-NEM derivative) and GSSG was performed on Shimadzu 20A series liquid chromatography (LC) and Applied Biosystems/SCIEX API5500 QTrap tandem-mass spectrometer(MS/MS). LC conditions: Column Agilent ZORBAX Eclipse Plus, C18 4.6x50 mm 1.8 μm particle size (P/N 959941-902) analytical column and Phenomenex, C18 4x3 mm guard cartridge (P/N AJ0-4287) at 45 °C. Mobile phase solvents (all MS-grade): A - 0.1% formic acid in water, 2% acetonitrile; B – acetonitrile. Elution gradient at 1 mL/min: 0-3 min 0-50% B, 3-3.1 min 50- 95% B, 3.1-3.5 min 95%B, 3.5-3.6 min 95-0%B. Run time: 7min. MS/MS conditions: MRM transitions (m/z) followed for GSH-NEM, GSH-NEM-d3, GSSG, and GSSG-d6 were 433-304, 436.2-307.1, 613.1-355, and 619.1-361, respectively. Calibration samples (n=6) in mobile phase A were prepared in the following appropriate ranges: 0.375-6.00mM for GSH and 1.88-30 μM for GSSG analysis and analyzed alongside the study samples. Quantification was performed by Analyst 1.6.2 software.

**Histologic Quantification of Metastasis**

Lungs were paraffin-embedded and sectioned with 5μM thickness in six 200μM steps through the coronal plane of the lungs. The tissue was stained with hematoxylin and eosin. All sections were scanned with a 5X objective using a Zeiss Axio Imager 2 microscopy
system, and images of all observable metastatic lesions were obtained. Using ImageJ, the area of individual metastatic lesions of the lungs was measured.

**Modeling of Metastatic Colonization and Growth within Lungs**

A mathematical model was derived to be used for assessment of tumor metastases (Figure 2.9). The model includes cells needed to establish metastatic colonization and secondary tumor growth rate within the lung. Histologic data of the number and size distribution of tumor nodules were used as input variables for this model. Using the growth rate of the primary tumor and the total number and area of metastatic pulmonary lesions from a pilot experiment it is possible to estimate the time that metastases form, as well as the growth rate in the lung:

\[
t_{ij} = (T - b \cdot (\log V_{ij}(t) - \log V_{ij}(t_{ij})))
\]

where \( T = \) the time of euthanasia, \( V_{ij} = \) initial tumor volume within the lung, \( t_{ij} = \) time a metastatic fragment of size \( V_{ij} \) colonized the lungs, and \( b = \) growth rate. This formula was validated using results from a pilot experiment that had growth rate data from the primary tumor and the physical measurements of tumor within lungs.

**STATISTICAL ANALYSIS**

*Window Chamber - Local Tumor Microenvironment Analysis*

After processing the acquired data for the extraction of micro-environmental parameters, median values were calculated within each spatial region, at each time point, for each animal.
We then calculated the linear fit of the change in median values for each region within each animal. The slope of this line represented the rate of change of any particular parameter over the treatment time course. Within each region, slopes were grouped according to treatment type (i.e. microbeam, whole, or mock irradiation, with N=6, 6, and 5, respectively), and statistical analysis using one-way ANOVA was used to detect significant treatment effects. The Tukey-Kramer method was then applied to these results to detect pair-wise statistical significance between treatment groups. We also calculated the time-averaged GFP expression in the tumor rim as a percentage of overall expression, and applied the same statistical tests for significant differences between treatment groups. In all figures, error bars represent the standard error of the mean.

Orthotopic Dorsal Mammary Tumor and Distant Metastasis Analysis

These formulas were used to analyze results from the experiments in Chapter 3, as well, so some listed variables (saline vs porphyrin) do not apply here.

Bioluminescence Quantification of Metastasis: Single comparisons were conducted using Student's T-test.
Counts of Gross Pulmonary Metastasis:

Because these are counts, we modeled the number of metastases as a Poisson random variable, with a mean $\lambda(d,p)$ that depended on radiation dose $d$ and whether Saline ($p = 0$) or porphyrin ($p = 1$) was given, as follows:

$$
\log \lambda(d, p) = \mu + \alpha_d + \beta_p + \alpha \beta_{dp}
$$

Where $\mu$ is the baseline mean, representing 90% RT dose and Saline, $\alpha_d$ is the effect of other dose, $\beta_p$ is the effect of Porphyrin and $\alpha \beta_{dp}$ is the interaction between radiation and porphyrin. Model was fit by maximum likelihood in the R computing package (www.r-project.org).

Counts of Gross Pulmonary Metastasis with respect to Time:

Because these counts were measured on mice observed over different lengths of time, we modeled the incidence rate of metastasis, assuming the incidence rate to be constant over the observed life of the mouse.

The observed number of metastases was modeled as a Poisson random variable, with a mean $\lambda(d,p) \ast T$, where $\lambda(d,p)$ is the incidence rate, that depended on radiation dose $d$ and whether Saline ($p = 0$) or porphyrin ($p = 1$) was given. The dependence of the incidence on treatments was modeled as follows:

$$
\log \left( \lambda(\cdot, \cdot) \right) = \mu + \alpha_d + \beta_p + \alpha \beta_{dp}
$$
Where $\mu$ is the baseline mean, representing 90% RT dose and Saline, $\alpha_d$ is the effect of other dose, $\beta_p$ is the effect of Porphyrin and $\alpha \beta_{dp}$ is the interaction between radiation and porphyrin. Model was fit by maximum likelihood in the R computing package (www.r-project.org).

**GSSG/GSH:**

We analyzed the ratio using a model of the form:

$$r_{dpkt} = \mu + \alpha_d + \beta_p + \gamma_t + \alpha \beta_{dp} + \alpha \gamma_{dt} + \beta \gamma_{pt} + \epsilon_{dpkt}$$

(2.4)

Where $r_{dpkt}$ is the GSS/GSSH ratio for the $d$-th RT dose, $p$-th dose of porphyrin (saline or porphyrin), $t$-th time of observation (abbreviated or extended) and $k$-th replicate. Model (2.4) explains variation in this ratio in terms of $\mu$, the baseline mean, representing 90% RT dose and Saline, $\alpha_d$ is the main effect of other dose, $\beta_p$ is the effect of Porphyrin, $\gamma_t$ is the effect of extended observation time and the other terms are interactions between each of the factors. The model was fit using ordinary least squares.

**ANIMAL WELFARE STATEMENT**

All procedures described in this study were performed in accordance with animal welfare protocols approved by Duke University’s Institutional Animal Care and Use Committee.
RESULTS

WINDOW CHAMBER RESULTS: PARTIAL TUMOR RADIATION BY MICROBEAM AFFECTS LOCAL TUMOR MICROENVIRONMENT

Vascular Structure

Qualitative observation of tumors irradiated with the single microbeam showed a consistent pattern of angiogenesis and reorientation after radiation. Angiogenic vessels were preferentially directed into the post-irradiation beam path (Figure 2.10). In comparison, neither the wide field nor the mock treated tumors showed a similar effect; in these cases, angiogenesis did occur, but it was generally less profound and showed no particular directional preference.

Using one-way ANOVA/Tukey-Kramer analysis as described above, we observed no significant differences in the rate of change of intratumoral vascular length density or percent vascular area over time among any of the treatment groups (Figure 2.11A&C). However, within the tumor associated normal tissue, the wide field group displayed a significantly decreased vascular length density by the end of the time course relative to the mock treated group (p = 0.0147), and a near significant decrease relative to the microbeam group (p = 0.054) (Figure 2.11B). By day 7, the wide field group also showed a significantly greater decrease in vascular area within the tumor-associated normal tissue, compared to either the
mock or microbeam groups (p = 0.0124 & p = 0.0222) (Figure 2.11D). These data indicate a radiation-induced vascular regression within the tumor-associated normal tissue region of the wide field group. The lack of effect within this region in the mock and microbeam groups may be due to the fact that most of the tissue in these regions received virtually no radiation dose (microbeam), or no radiation at all in the mock controls.

**Microvessel perfusion and flow direction**

We next analyzed hemodynamics from videos to determine microvessel blood flow patterns using our previously described mapping algorithm. Blood flow was maintained within the vascular network after treatment. We did observe patterns of vascular reorganization within the microbeam group, however. Consistent with structural data presented above, vessels tended to branch out into the region of radiation exposure in the microbeam group. This was particularly notable in one tumor, where vessels initially running largely parallel to the beam path on the day of irradiation changed their orientation to a more perpendicular orientation in just a few days (Figure 2.12 Error! Reference source not found.).

**Spatial and Temporal Patterns of HIF-1 Expression**

Median HIF-1-GFP signal consistently increased at each successive time point in the mock and single microbeam groups (Figure 2.13A). This constant increase was also observed in the wide field group through day 5, however at days 6 and 7, the signal decreased from the preceding day. To determine whether this effect was due to cell death we also measured
changes in RFP expression (Figure 2.13B). Since RFP serves as a constitutive reporter, the expression of this protein should serve as a concurrent indicator of the number of viable cells in the tumor. We therefore defined the ratio of GFP:RFP expression, as GFP expression per viable tumor cell. Tumor-wide GFP:RFP expression, showed no statistically significant trends (Figure 2.13C). However, the GFP:RFP expression in the tumor rim (integrated over all time points) was significantly higher in the microbeam group than in either the mock or widefield groups (p = 0.0323 & p = 0.0425, respectively) (Figure 2.13D).

Alterations in Vascular Hemoglobin Concentration

The overall change in vascular hemoglobin concentration within the tumor was highest in the microbeam treated group (Figure 2.14A). The most notable rate of change began on the third day post-treatment. The effect was not noted in the tumor-associated normal tissue (Figure 2.14B), but was prominent in the tumor itself, both within the rim (Figure 2.14C) and core (Figure 2.14D).

The rate of change in hemoglobin concentration within the tumor was significantly different for the wide field group compared to microbeam (p = 0.0243) (Figure 2.15A). When the time course was split into two segments (Pretreatment - Day 3 & Day 3 - Day 7), the rate of change in hemoglobin concentration was not significantly different among any groups within the first timeframe (Figure 2.15B). Within the second time frame, however, the rate of change within the microbeam group was significantly higher than either the mock or wide
field (p = 0.0285 & p = 0.0270, respectively) (Figure 2.15C). Only the microbeam group showed a significant difference in rates between the two time frames (p = 0.0215) (Figure 2.15D).

Alterations in Hemoglobin Oxygen Saturation (Wide field Group)
Hemoglobin saturation was tracked in the three different treatment groups (Figure 2.16A). Concurrent with the drop in HIF-1 reporter expression after wide field irradiation, we observed an increase in hemoglobin oxygen saturation relative to the mock and microbeam groups persisting through days 6 and 7 (p = 0.0039 and p = 0.0035, respectively) (Figure 2.16B).

Evidence of Tumor Cell Migration (Microbeam Treatment)
Formation of secondary clusters of tumor cell growth away from the primary tumor microbeam treatment was observed in the microbeam group (Figure 2.17). This was observed as early as three days post-treatment. Interestingly, areas of angiogenesis would begin to form at locations within the window remote from the primary tumor. RFP images showed that this angiogenic effect was associated with the presence of tumor cells at this location. This effect was not observed in the mock treatment group (Figure 2.17) Error! Reference source not found.. Neither was it observed in the widefield group, but since the widefield treatments were completed prior to the first observation of this effect (due to the time and complexity of installing the beam collimator and recalibrating the system), whole
window images were not collected. In order to demonstrate that these clusters were the result of tumor migration (rather than the accidental seeding of cells at these locations during surgery), we imaged the tumor at high resolution using confocal microscopy. At 10x magnification, we observed bridges of tumor cells that spanned the gap between two clusters (Figure 2.18). Furthermore, during a 90 minute imaging session, these bridge cells displayed coordinated mobility.

Histological Analysis of Tumors

Representative images of the tumors are shown in the supporting data (Figure 2.19). Histologic findings varied between the three treatment groups. In the mock treatment group, the neoplastic nodules were composed of haphazardly arranged pleomorphic polygonal to spindle-shaped cells with moderate anisocytosis and anisokaryosis. The number of mitotic figures in the mock treated group ranged from 30 to 55 per 10 high power fields (40x). Also observed were randomly scattered, patchy areas of lytic necrosis (affecting anywhere from less than 5% of the mass to up to 20% of the mass in different animals and in different planes of section).

Relative to the mock treatment group, the neoplastic cells of the wide field-treated group were extremely pleomorphic and very large. Individual cells were up to 50 um in diameter. There were frequent multi-nucleated cells with up to 8 nuclei in a single cell. Severe anisocytosis and anisokaryosis were noted, with some nuclei up to 8 times larger than others.
The mitotic index ranged from 12 to 25 mitotic figures per 10 high power fields (40x), and greater than half of the mitotic figures were bizarre. Patchy foci of lytic necrosis ranged from less than 5% up to 25% of the mass.

Within the microbeam irradiated tumors, two distinct phenotypes were observed. In most of the tissue sections approximately 80% of the neoplastic mass resembled the mock group of tumors. However, the remaining 20% of the tissue was composed of a central or slightly off-center, roughly linear band of neoplastic tissue that resembled the wide field group of irradiated masses with larger, more pleomorphic cells. This band of tissue ranged from about 0.2 up to 0.5 mm in diameter. Foci of lytic necrosis in both of the neoplastic populations ranged from less than 5% up to 20% of the mass.

In summary, mock treated tumors showed a cellular phenotype typical for this particular cell line. Patchy areas of necrosis were observed, presumably in areas of severe hypoxia. The number of mitotic cells present in the tumor indicated viability and continued patterns of growth. In contrast, tumor cells in the whole-field group showed indications of radiation-induced damage; giant multinucleated cells and aberrant mitosis were commonly observed. The microbeam treated tumors showed a linear band through the tumor with the same phenotype as the wide field group. The width of this zone was consistent with the width and orientation of the microbeam. Cells outside of the irradiated region were identical to the mock treated tumors.
ORTHOTOPIC TUMOR RADIATION RESULTS: RADIATION MARGINAL MISS AFFECTS DISTANT METASTASIS

Influence of Marginal Miss on the Development of Metastasis

Three variations of this experiment were performed. Of note, the proportion of tumor in the beam varied between 50 and 100%, as well as the defined endpoints (Figure 2.6). Mice (n=5-6/group) were treated with radiation (0Gy, 15Gy) administered to different volumes (0%, 50%, 90%, 100%) of primary dorsal mammary tumors in order to ascertain whether pulmonary metastasis was affected by varying extents percent of tumor volume irradiated.

Focal dosimetry data obtained using the nanoFOD system defined the radiation dose at the center of the open field to be 14.91Gy, the center of the open portion of the 50% tumor radiation field to be 14.58Gy, and the center of the blocked portion of the 50% tumor radiation field to be 0.48Gy (Figure 2.7). Point measurements of the radiation dose delivered 1mm from either side of the blocked region revealed a dose gradient of 11.8Gy on the open side of the field to 0.75Gy on the blocked side of the field. Regional dosimetry data obtained using the 3D rodentomorphic system and SmART-Plan small animal radiation treatment planning software defined the highest 20% of the dose delivered to the tumor volume (D20) was 20.5Gy, 20.3Gy, and 18.9Gy for the 100%, 90%, and 50% irradiated
tumors, respectively (Figure 2.8). With each of these treatment fields, lungs and spinal cord received negligible dose (Lungs 0.19-0.44Gy, Spinal cord 0.54-0.68Gy).

In Experiment A, the marginal miss group was treated with 50% tumor irradiation and compared with 0% and 100% tumor irradiation groups. Mice were euthanized when primary tumors reached a volume of greater than 1500mm$^3$ or the animals developed neurologic deficits secondary to spinal invasion of the primary dorsal mammary tumor (Figure 2.6). Metastatic burden quantified via bioluminescence imaging was increased in the mice treated with radiation, an unexpected result (Figure 2.20A). A more careful look at the data, however, showed that mice with irradiated tumors lived longer than the unirradiated controls. In this experiment, mice with 100% irradiated tumors lived longer than mice with 50% and 0% irradiated tumors (Figure 2.20B). By allowing mice to survive until their endpoint, these animals were at risk for developing metastases for a longer period than the unirradiated control mice. As validation of this point, we examined the relationship between total luminescence signal from lungs and cumulative tumor volume as well as time of euthanasia (Figure 2.20C). There was a linear increase in metastatic burden in lungs vs. either parameter. Individual tumor growth curves per treatment group for Experiment A are presented (Figure 2.21).

Experiment B was designed with a shorter experimental timeline, in an attempt to minimize the confounding effects of time to observation of metastases (Figure 2.6). In this
experiment, a five day window was set as a defined endpoint according to when the first mouse with a tumor that reached 1500mm$^3$. During this five day window, mice were euthanized when: 1) their tumor reached 1500mm$^3$, 2) they developed neurologic deficits, or 3) they were euthanized at the end of the five day window, regardless of the size of their primary tumor. Another variation implemented in Experiment B was that the tumor volume which simulated marginal miss was altered from 50% irradiated volume to 90% irradiated volume in order to mimic a more clinically relevant scenario. While this abbreviated study reduced variation in bioluminescence data compared to Experiment A, the pattern of the metastasis results was unexpected and difficult to interpret biologically; specifically, the group that received marginal miss exhibited lower metastatic burden than either of the other two groups (Figure 2.22).

After obtaining the results from Experiment B, we decided it would be wise to include another level of analysis for the metastasis data via direct quantification of metastatic burden. A selection of lung samples from Experiment B were processed and examined histologically to measure total number and total area of metastatic lesions per mouse. A formula was generated to model the rates of metastatic colonization and growth of 4T1-Luc cells within the lungs of mice. This formula was tested against the histologic data (Figure 2.9). The formula assumes that metastatic colonization of the lung occurs with multi-cellular clusters of cells. This has been shown to be the case for this tumor type$^{181}$. Using this formula, the
rate of metastatic colonization of the lungs, as well as the growth rate of these metastatic tumors, can be calculated and compared across treatment groups for future experiments.

Based on the results of Experiment B, Experiment C was designed to keep the time between tumor implantation and irradiation consistent (Figure 2.6). As in Experiment A, all animals were followed until tumors reached 1500mm3 or until they experienced paralysis from local tumor invasion. Here, the radiation treatment was performed nine days following tumor implantation. Animals were eligible to be irradiated if their tumors were 7-12mm diameter. Mice with tumors that were not the appropriate target volume on the defined radiation treatment day were excluded from the study.

Unfortunately, in Experiment C, it was observed that insufficient, and therefore, most likely inaccurate, bioluminescence signal was measured from the primary 4T1-Luc tumors. (Figure 2.23). Instead, manual counts of grossly visible metastatic lesions on the surface of the lungs were used for analysis (Figure 2.24). In this experiment, based on the counts of the gross lesions, the metastatic burden of mice in the marginally missed group was significantly greater than in the 0% and 100% groups (p= <0.0001, p=0.0091, respectively) (Figure 2.24); however, mice with marginally-missed tumors (90%) lived longer than completely irradiated (100%) and non-irradiated (0%) controls so the influence of survival time is contributing to the pattern of these metastasis results. Individual tumor growth curves per treatment group for Experiment C are presented (Figure 2.25)
Within Experiment C, two separate cohorts of mice were studied in order to analyze tumor oxidative stress and HIF-1 expression at different time points: 1) 24 hours following radiation and 2) at time of euthanasia. This was done so that the effects of radiation marginal miss could be interpreted according to both early and late changes in oxidative stress and HIF-1α expression within the primary tumor microenvironment. The ratio of oxidized to reduced glutathione (GSSG:GSH) was used as a means to characterize the degree of oxidative stress within each tumor. The GSSG:GSH ratio was significantly increased in the marginal miss group, compared with the non-irradiated group at the 24hr time point (p=0.0393) (Figure 2.26A). There was a significant difference in oxidative stress measured in tumors analyzed at 24 hours following radiation compared to endpoint across all treatment groups (p=0.0079) (Figure 2.26B). Analyses of HIF-1 expression in the primary tumors at these time points are pending.

**DISCUSSION**

**WINDOW CHAMBER EXPERIMENTS: PARTIAL TUMOR RADIATION AFFECTS LOCAL TUMOR MICROENVIRONMENT**

The data presented here compare the effects of 50 Gy-irradiation, using either wide field or microbeam techniques (with mock treated controls), on tumors grown in the skin-fold window chamber. In order to evaluate the effects of the various treatments on the tumor
microenvironment, we examined the windows daily over the pre- and post-treatment time course. Data on vessel structure and function indicate to what extent radiation caused vascular disruption within the tumor, as well as determine the capacity of the microvascular network to recover. Measurements of hemoglobin concentration and hemoglobin saturation provide complementary information on the perfusion and oxygen transport capabilities of the affected vasculature. Furthermore, regulation of HIF-1 expression was tracked and quantified through the observation of HIF-1 GFP expression. Partially irradiated tumors developed a robust angiogenic response post-MRT, resulting in the formation of unique patterns of tumor vasculature remodeling; these remodeled tumor-associated blood vessels infiltrated the irradiated region of the MRT-treated tumors in a fashion similar to what would be expected in a wound healing response. The Dewhirst lab has previously shown that 4T1 cells migrate toward, attach to, and utilize tumor-associated vessels to move; in fact, in that study, inhibition of tumor vascular growth via blocking VEGF signaling prevented this tumor cell migration (ref). In our window chamber study, tumor cells from partially-irradiated tumors were seen to migrate along the tumor-associated vessels. The combination of increased angiogenic activity and increased cell motility post-MRT creates a scenario whereby tumor cells are provided the means via the vasculature to migrate away from the primary tumor and form regional metastatic clusters, as was witnessed in the window chamber. This regional metastasis activity was not observed in the mock irradiated or wide field irradiated tumors.
Histology was performed after tumors were extracted on the final day of imaging, and these results confirmed that the differing irradiation techniques produced distinctive patterns of cell death within the tumor: diffuse cellular damage and death in the wide field irradiated tumors vs. linearized, focal cell damage and death in the microbeam irradiated tumors. These comprehensive data provide multi-parametric assessment of physiologic perturbation and recovery after mock, microbeam, and wide field tumor irradiation. Through automated demarcation of the pre-defined tumor regions, the dynamics of these microenvironmental effects can be spatially characterized. Since we see minimal cell death outside of the irradiated tumor region in the MRT group, the bystander effects described in these window chamber experiments are occurring in viable cells capable of undergoing aggressive tumor changes.

Over the eight-day time course, we found that 50 Gy-exposure causes: 1) unique physiologic changes in different tumor/normal tissue regions and 2) differential effects between wide field and microbeam irradiations. We propose that microbeam irradiation causes an upregulation in HIF-1 in the non-irradiated portions of the tumor through bystander mechanisms. HIF-1, in turn, potentiates vascular recovery and the development of regional metastasis. In contrast, wide field tumor irradiation results in widespread cell death. Cell death contributes to reoxygenation because a lowered cell density will reduce oxygen consumption. A visual representation of this proposed model is presented (Figure 2.27).
Wide field Irradiation

A decrease in vascular length density and percent vascular coverage was observed after treatment in the tumor-associated normal tissue. In contrast, vascular density and percent vascular coverage were unchanged in the tumor. The lack of change in hemoglobin concentration was in contrast with the other treatment groups, where concentrations increased over time. These results were interpreted as an inhibition of vascular growth due to the radiation treatment. The vessels which remained after treatment showed no apparent lack of functionality, however, as perfusion persisted and there was no visible vascular stasis.

Hemoglobin oxygen saturation increased dramatically in the last days of the study. This effect suggested oxygen consumption had decreased. The pathologic data showed that there was widespread cell death, which would reduce oxygen consumption rate. These results are consistent with the prevailing theory that oxygenated cells are preferentially killed by radiation, freeing up oxygen for diffusion into formerly hypoxic tumor regions.\textsuperscript{182}

Previously, we had shown that HIF-1 induction occurs coincidentally with reoxygenation after 3 daily fractions of 5Gy in this tumor model.\textsuperscript{155} We observed a dissimilar trend here, however. As tumor oxygenation increased, HIF-1 decreased. The lack of HIF-1 induction was likely due to the very high radiation dose the tumor received. A substantially reduced number of viable cells would reduce HIF-1-driven GFP production. The concurrent drop in RFP expression supports this explanation, as well as the observation of widespread tumor cell death in histological samples.
Single Microbeam Irradiation

Radiation was deposited by a 300µm wide beam which cut through the tumor, leaving non-irradiated tumor volumes on either side. The geometry of this scheme resulted in relatively smaller fractions of the tumor rim and tumor-associated normal tissue receiving radiation. Histological analysis of the microbeam-treated tumors confirmed this pattern of radiation exposure, with a central linear region displaying radiation damage similar to the wide field treated group, and an outer, non-irradiated mass with histological features similar to the mock treated tumors.

No direct evidence of vessel depletion or loss of functionality was observed in the microbeam irradiated group. In fact, changes in vascular length density and percent vascular coverage after microbeam irradiation largely followed the same trend as mock irradiated controls. A visual assessment of the pattern of vascular response reveals a pronounced angiogenic effect originating within the tumor-associated normal tissue along the radiation path.

Vascular function remained unaltered in the microbeam group, as assessed through maps of blood flow speed. Furthermore, we observed no significant alteration in hemoglobin oxygen saturation after treatment, suggesting that tumor oxygenation remained stable throughout the time course. Unique patterns of vascular remodeling were highlighted in the flow direction maps, however. Here we observed that blood flow was redirected into the irradiated beam
area. Thus, both functional and structural analyses of the vasculature indicate that microbeam irradiation leads to an increase angiogenesis to enhance the blood supply to irradiated tissue.

This vascular adaptation to localized radiation may be facilitated through HIF-1 expression, since HIF-1 expression has been shown to be increased after radiation treatment. Spatial analysis showed that the primary area of HIF-1 expression was not within the irradiated tumor core. A relatively high amount of HIF-1 activity was found in the tumor rim and this increase was circumferential, rather than following the path of the microbeam treatment. These data provide indirect evidence for bystander signaling to increase HIF-1 expression, perhaps emanating from the irradiated area, into unirradiated tumor tissues. The radiation bystander process is thought to be at least partially mediated through the radiation-induced production of reactive oxygen species. Damage to mitochondria may further potentiate this oxidative stress. The production of the diffusible and long-lived reactive species, hydrogen peroxide and nitric oxide, have been prominently implicated in the bystander response. Bystander-affected cells have been shown to increase the production of both of these reactive species. Both hydrogen peroxide and nitric oxide, in turn, stabilize HIF-1 in the presence of oxygen. Additionally, it has been shown that reactive oxygen species-mediated HIF-1 induction occurs on the transcriptional level and is dependent on NF-κB. Thus, the observation of increased HIF-1 expression in the rim, despite a lack of any direct damage from radiation, suggests that bystander signaling is active here. The increase in HIF-
1 expression after microbeam treatment likely contributes to the angiogenic response because it upregulates expression of two proangiogenic cytokines, VEGF and angiopoietin-2. The sharp increase in hemoglobin concentration beginning on the third day post treatment further implicates bystander-induced HIF-1 upregulation in the post-microbeam irradiation response. A primary effect of VEGF upregulation is an increase in vascular permeability. As a result of this change, plasma leakage into the interstitial space will cause microvascular hematocrit to rise. This is consistent with the post-irradiation increase in hemoglobin concentration within the tumor.

One of the unanticipated effects of microbeam treatment was the formation of regional metastasis. As early as three days post-treatment, regions of increased angiogenesis within the window were identified, remote from the primary tumor. RFP images showed that this angiogenic effect was associated with the presence of a tumor cells at this location. Regional metastasis was not observed in the mock treatment group nor the widefield group. Despite the frequent use of the 4T1 reporter cell line within our lab, this phenomenon was not noted in prior studies, leading us to speculate that the formation of these clusters was a microbeam-induced effect. In order to demonstrate that these clusters were the result of tumor migration, we imaged the tumor at high resolution using time-lapse confocal microscopy. Bridges of tumor cells spanned the gap between these two clusters, and displayed coordinated mobility. These results imply that partial tumor radiation via MRT increased tumor HIF-1 expression.
and had the effect of activating cellular motility mechanics and stimulating the development of regional metastasis.

*Interpretation of Treatment Effects*

Within the microbeam group the structural and functional characteristics of the vasculature as a whole did not change, relative to the pre-treatment data. Post treatment initiation of a HIF-1 response in the tumor rim may have protected the tumor against vascular depletion through the angiogenic recruitment of blood vessels from the tumor-associated normal tissue, as well as stimulated the development of regional metastasis along this neovasculature.

The driving factor in microvessel survival and/or recovery after radiation and the magnitude of this effect may be dependent upon the particular radiation scheme employed. Our results clearly indicate that vascular expansion is potentiated when the tumor is partially irradiated and that tumor cells can utilize these vessels to migrate and colonize secondary sites. We propose that this phenomenon is linked to the vascular protective, angiogenic, and pro-metastatic effects of HIF-1, as well as the unique pattern of intratumoral HIF-1 expression observed in this scenario. HIF-1 was shown to be upregulated primarily in the tumor rim, which received relatively less radiation exposure than the tumor core. Thus, the data presented here provide evidence that tumor-mediated intercellular signals initiated in the lethally irradiated portion of the tumor core enable vascular recovery through the induced expression of HIF-1 in sublethally-irradiated portions of the tumor. Following this indirect
upregulation of HIF-1, a profound vascular response is observed and associated with regional metastasis.

**ORTHOTOPIC TUMOR EXPERIMENTS: RADIATION MARGINAL MISS AFFECTS DISTANT METASTASIS**

Using an orthotopic dorsal mammary carcinoma model and advanced, image-guided small animal radiation, this study investigated how radiation marginal miss affects the development of pulmonary metastasis when compared to non-irradiated and completely irradiated tumors. Again, we hypothesized that oxidative stress would increase HIF-1 in the sublethally-irradiated region of marginally-(missed) tumors, and this would lead to increased tumor cell metastasis compared to completely irradiated tumors. Indeed, it was found that radiation marginal miss was associated with increased oxidative stress within the primary tumor 24 hours after radiation and these mice developed more pulmonary metastases at their endpoint compared to controls.

**Radiation Dosimetry**

In order to correlate the marginal miss results to changes within the irradiated primary tumor, it was necessary to characterize the radiation dose distribution across the treatment fields. We employed novel focal and regional dosimetry methods to define the radiation dose administered to portions of the mammary tumors, as well as the critical, regional normal
tissues (lungs, spinal cord). It was determined that there was a steep drop-off of the radiation
dose in the regions of marginal miss, so it was accurate to say that the majority of tumor cells
in this region of the radiation field would be sublethally irradiated. Additionally, the
radiation dose to the lungs and spinal cord was negligible. This is important to note because
radiation exposure to the lungs can influence the colonization of metastatic tumor cells\textsuperscript{188},
which could have skewed the results of this study, and radiation exposure to the spinal cord
can induce neurologic deficits over time. We did see that the mice developed paraparesis and
paralysis, but based on the very low radiation dose that could have been administered to the
spinal cord, as well as the very short timeline of this experiment, we can confidently say that
these neurologic deficits were due to direct tumor spinal invasion instead of radiation-
induced damage. Radiation damage to the spinal cord would manifest as late tissue effects
months following radiation exposure.

\textit{Optimization of Experimental Design}

Interpreting the results of the series of radiation experiments was very challenging. In
Experiment A, all mice were allowed to reach their natural endpoint, but with this approach,
mice whose tumors were completely irradiated lived longer, so the total bioluminescence
signal from the lung samples was much higher than, and therefore could not be compared to,
either the non-irradiated controls nor the marginal miss treatment groups. Attempts were
made to alter the experimental design in order to minimize the sources of variation in the
metastasis data. The thought was that by evaluating the metastatic burden of mice in a more
abbreviated experiment, within a narrow time frame following radiation, there would be less variability and a better chance for direct comparison across the treatment groups. Unfortunately, with that approach in Experiment B, variability in the bioluminescence data was reduced, but the resulting patterns of metastasis from the treatment groups did not seem sensible - specifically, that radiation marginal miss would suppress metastasis compared with 100%RT. Likely, in this study, the mice were euthanized before the full extent of the metastatic process could be completed to reveal differences between the treatment groups.

A second method of evaluating the metastasis data was developed. By way of analyzing the number and size of metastatic lesions in the lungs histologically and coupling this information to time and the growth rate of the 4T1-Luc primary tumor, a formula was derived which allowed for modeling of the metastatic colonization rate and growth rate of secondary tumors within the lungs. Knowing that distant metastasis results could now be analyzed in another way, and that this method required the factor of time, Experiment C was performed with an extended timeline. Also, Experiment C integrated a cohort of mice which were treated and euthanized 24 hours following radiation in order to evaluate peri-treatment changes within the primary tumor that could influence later metastasis results.

Radiation Marginal Miss Increases Distant Metastasis

As Experiment C was underway, it was observed that the bioluminescence signal from the 4T1-Luc tumor cells was no longer functioning properly in vivo. Once again, the final
metastasis results according to the bioluminescence imaging seemed nonsensical, because we observed lungs with obvious gross metastases that did not show up with bioluminescence imaging. Based on the observed poor correlation with tumor burden in vivo, we considered these results invalid likely due to failure of the constitutive luciferase reporter cell line over time in storage and in culture. As an alternative, metastasis quantification was performed via manual counts of gross metastatic lesions on the lungs of mice. With this approach, it was seen that mice with tumors that were irradiated with a simulated marginal miss had significantly increased metastatic burden compared to non-irradiated and completely irradiated controls; however, when survival time was factored into the analysis, this difference was not found to be significant. These results suggest that the simulated radiation marginal miss increased distant metastasis in our orthotopic tumor model; however, additional levels of analysis of the metastasis data will be performed to better characterize the effects of radiation marginal miss. This will involve quantifying histologic data from microscopic metastases, as well as modeling of the metastatic kinetics, using the formulation created by our collaborative biostatistician, Dr. Choudhury.

Radiation Marginal Miss Increases Oxidative Stress

In order to understand how oxidative stress within the primary tumor microenvironment influences the development of metastasis, the ratio of oxidized to reduced glutathione (GSSG:GSH) was measured. Marginal miss tumors analyzed at 24 hours following radiation had significantly increased oxidative stress compared to the non-irradiated controls; when
compared to the completely irradiated controls, the oxidative stress in the marginal miss group was increased, but not statistically significant. Regardless, the finding that the marginal miss group is associated with increased oxidative stress is important as it links increased oxidative stress following radiation marginal miss to the pattern of the development of increased metastasis at endpoint. A visual depiction of this proposed model is presented (Figure 2. 28)

*HIF-1 Expression*

Results are pending regarding differences in HIF-1 expression across the treatment groups. Again, HIF-1 is known to activate a number of tumor promoting pathways. Radiation-induced oxidative stress has been shown to increase HIF-1 expression, leading to tumor progression. HIF-1 protein levels from the tumors will be analyzed globally via western blot and regionally via immunohistochemistry. As there was increased oxidative stress in the marginal miss-treated tumors compared to controls at 24 hours following radiation, and this group had comparably increased metastasis, it is possible that that HIF-1 expression will be increased in this marginal miss group, supporting our hypothesis.

**CONCLUSIONS**

We provide evidence that partial tumor radiation can stimulate intratumoral radiation bystander effects. These bystander effects can drive aggressive tumor changes, resulting in
tumor progression locally and distantly. Our studies show that 1) overexpression of HIF-1 in bystander cells is associated with an induction of tumor vascularization and regional metastasis that follow along the path of these neovascular networks, and 2) marginally missed tumors have increased levels of oxidative stress compared to non-irradiated tumors, and mice with marginally missed tumors go on to develop a greater metastatic burden of tumor cells in their lungs. While the levels of significance of some of these results are still being established, additional analyses are underway to evaluate the characteristics of pulmonary metastases across treatment groups, as well as HIF-1 expression of the orthotopic 4T1-Luc tumors. This will involve quantifying histologic data from microscopic metastases, as well as modeling of the metastatic kinetics. However, these results provide more support for the concern that, through oxidative stress and HIF-1, radiation marginal miss can promote aggressive primary tumor changes locally and may lead to increased metastasis distantly.
Figure 2.1 Graphical depiction of the central hypothesis. Partial tumor radiation will generate reactive oxygen species in the irradiated tumor which will increase HIF-1 expression in the sublethally irradiated tumor region via bystander effects. This overexpression of HIF-1 will lead to aggressive tumor changes, such as angiogenesis, epithelial-mesenchymal transition, tumor cell migration and invasion, and metastasis.
Figure 2.2 Graphical depiction of the clinical risks of radiation marginal miss. Compared to conventional radiation techniques, conformal radiation therapy reduces normal tissue damage from radiation by minimizing treatment margins around a targeted tumor; however, these narrow margins increase the risk for radiation marginal miss of unidentified tumor cells. Through radiation bystander effects signaled via reactive oxygen species generated within the irradiated tumor, we hypothesize that the marginally-missed tumor cells will undergo aggressive tumor changes due to overexpression of HIF-1, leading to local and distant effects.
Figure 2.3 Orthotopic 4T1-luciferase dorsal mammary tumors and pulmonary metastases. (A) Mice were surgically implanted with 4T1 dorsal mammary carcinoma cells with a constitutive luciferase reporter gene to allow for bioluminescence imaging of the tumor cells. (B) Metastatic lesions could be identified within the lungs via bioluminescence imaging as early as six days following tumor implantation.
Figure 2.4 Microbeam irradiation. (A): The radiation intensity profile had a full-width half-max of approximately 300 um. (B): Window chamber-grown tumors were irradiated with the source situated above the window. The radiation penetrated the tumor orthogonally to the tissue plane. The length of the beam spanned the 13 mm diameter window.
Figure 2.5 Regional demarcation of tumor boundaries and vessel segmentation. (A): Fluorescence reporter images were used to identify the tumor boundary (red line). (B) From this, regions of tumor-associated normal tissue, tumor rim and tumor core were defined according to their distance from the tumor boundary. Within each of these regions, fluorescence signal was quantified. Additionally, vessels were segmented, and spatially localized vascular parameters were calculated.
Figure 2.6 Experimental design for radiation marginal miss experiments. Experiment A investigated the effects of radiation marginal miss with 0%, 50%, and 100% tumor volume radiation as tumors reached treatment size (Longest diameter >7mm). Mice reached their endpoint when their tumors were >1500mm$^3$, they developed neurologic deficits, or demonstrated >20% body weight loss. Experiment B was modified so radiation marginal miss was simulated with 90% instead of 50% tumor volume radiation. Tumors were irradiated as they reached treatment size (Longest diameter >7mm). Also, a five day window was set as the defined endpoint according to when the first mouse with a tumor that reached 1500mm$^3$. During this five day window, mice were euthanized when: 1) their tumor reached 1500mm$^3$, 2) they developed neurologic deficits, 3) >20% body weight loss or 4) they were euthanized at the end of the five day window, regardless of the size of their primary tumor. Experiment C was designed to keep the time between tumor implantation and irradiation consistent. Radiation treatment was performed nine days following tumor implantation. Animals were eligible to be irradiated if their tumors were 7-12mm diameter. Mice with tumors that were not the appropriate target volume on the defined radiation treatment day were excluded from the study. All animals were followed until tumors reached 1500mm$^3$, until they experienced paralysis from local tumor invasion, or developed >20% body weight loss.
Figure 2.7 Orthotopic dorsal mammary tumor radiation technique and focal dosimetry. (A) A diamond shaped radiation field was used to target the radiation beam to the dorsal mammary tumor while avoiding radiation exposure to the lungs and the spine. (B) Focal dosimetry data obtained using the nanoFOD system defined the radiation dose at the center of the open field to be 14.91 Gy, the center of the open portion of the 50% tumor radiation field to be 14.58 Gy, and the center of the blocked portion of the 50% tumor radiation field to be 0.48 Gy.
Figure 2.8 Orthotopic dorsal mammary tumor radiation technique and regional dosimetry. (A – C) Regional dosimetry data was obtained using a rodentmorphic 3D dosimetry system and SmART-Plan small animal radiation treatment planning software in order to generate dose volume histograms (DVH) (B) for tumor, lung, and spinal cord volumes (A). The highest 20% of the dose delivered to the tumor volume (D20) was 20.5Gy, 20.3Gy, and 18.9Gy for the 100%, 90%, and 50% irradiated tumors, respectively (C).
Figure 2.9 Modeling of metastatic colonization and growth within lungs. (A) Representative images of microscopic 4T1-Luc pulmonary metastases identified histologically. (B) The growth rate of the primary tumor and the total number and area of metastatic pulmonary lesions are used to estimate the time that metastases form in the lungs using the formula listed in (C). The difference in this metastasis time can be compared across treatments groups.
Figure 2.10 Patterns of angiogenesis after irradiation. Representative images of tumor-associated vasculature overlaid with hemoglobin oxygen saturation values. Three different microbeam, two different mock, and two different wide-field windows are shown. Longitudinal imaging at time points prior to irradiation through day 7 allowed visualization of the changes in tumor vascularization patterns. Over time, the patterns of vascular expansion in the microbeam group indicated a preferential infiltration of the irradiated portion of the tumor (arrows, with irradiated portion highlighted in red). Compared to the microbeam group, the angiogenic response within the mock and wide-field groups showed no apparent localized patterns of expansion or changes in vessel orientation. Vessels in these groups generally radiate inward from the tumor edge without notable variations in vessel density.
Figure 2.11 Changes in vascular length density and percentage vascular coverage over time. No significant differences in the rate of change in either parameter were observed among the treatment groups within the tumor itself (panels A and C). Within the tumor-associated normal tissue, however, the wide-field group showed a significant drop in vascular length density relative to the mock group (panel B) (p=0.0147). A significant drop in the wide-field group’s percentage vascular coverage was observed on the final day relative to both mock and microbeam groups (panel D) (p = 0.0124 and p= 0.0222, respectively)
Figure 2.12 Microbeam radiation effects on vascular function over time. In this single-representative-window chamber, the middle, left and right columns show bright field images, mapped blood flow direction and mapped blood flow speed, respectively. A notable change in vascular orientation was observed. Here vessels initially oriented parallel to the beam length were replaced by vessels running perpendicular to the irradiation portion of the tumor. Blood flow analysis showed no apparent loss of vascular function within the window after microbeam irradiation, as indicated by the detection of normal microvessel blood velocities.
Figure 2.13 Changes in fluorescent reporter expression over time. Both the microbeam and mock group showed a consistent increase in GFP (indicating HIF-1) over time, whereas the wide-field group saw a decay in expression around day 5 (panel A). A similar decay was observed in the expression of RFP (constitutively expressed) (panel B). When RFP was used to control for total protein expression, the ratio of median GFP to median RFP showed that the microbeam group was the only one to show a notable increase in relative expression of GFP (panel C). Within the tumor rim, GFP expression was proportionally higher in the microbeam group than in the mock or wide-field groups (panel D). (p=0.0323 and p=0.0425, respectively)
Figure 2.14 Changes in vascular hemoglobin concentration over time. Microbeam irradiation elicited a biphasic effect on vascular hemoglobin concentration within the tumor (panel A). Over the first four days of the time course, hemoglobin concentration remained relatively constant, compared to the marked increase later seen over the last four days. In comparison, the feeding vessels of the tumor-associated normal tissue maintained a more constant rate of change that did not seem to be affected by microbeam treatment (panel B). These vessels may have helped initiate the vascular response seen the tumor rim, and to a larger extent, the tumor core (panels C and D).
Figure 2.15 Rates of change in vascular hemoglobin concentration within tumors. Both mock- and microbeam treated tumors showed a consistent increase in vascular hemoglobin concentration over the entire time course. In contrast, concentrations within wide-field-treated tumors tended to remain static (panel A). Between the day of treatment and the third day post, no significant differences in the rate of change were observed among the treatment groups (panel B). From day 3–7, however, the microbeam group had a rate of change significantly greater than either the mock or wide-field groups (panel C) (p= 0.0285 and p=0.0270, respectively). When the rates of change were compared over the two timeframes, only the microbeam-treated tumors showed a significant change (panel D) (p=0.0215).
Figure 2.16 Changes in hemoglobin O2 saturation within the tumor-associated vasculature. Within the wide-field group, hemoglobin oxygen saturation increased abruptly over the last few days of the time course (panel A). The average rate of change was significant within the wide-field group when compared to either mock or microbeam treatments (panel B) (p=0.0039 and p=0.0035, respectively).
Figure 2.17: Regional metastasis formation following microbeam radiation: Whole-window pretreatment and day 7 images are shown for each animal. The red arrows indicate areas of secondary cluster formation.
Figure 2.18: Tumor cells form a bridge between areas of regional metastasis: A whole-window image of a microbeam-treated tumor and surrounding tissue appears in the upper left. When this image is magnified and RFP expression is overlaid, areas of angiogenesis and secondary tumor growth can be observed in the two nodules upwards of the primary tumor. The high resolution detection of RFP expressing tumor cells using confocal microscopy showed that within the region spanning the gap between these two secondary clusters, a bridge of semi-motile tumor cells could be observed.
Figure 2.19 Histological analysis of extracted tumors. After tumor extraction on day 7, the tumors were preserved and histologically assessed using H&E staining (panels A, B and C photomicrographs at 53 magnification, 400 um scale bar; panel D photomicrograph at 203 magnification, 100um scale bar). Panel A: In the mock group, central, patchy necrosis was observed within the tumor core (arrows). Panel B: The wide-field group showed more widespread damage, with a large number of pleomorphic irregular cells, bizarre mitotic figures and multinucleated cells. Panel C: The microbeam treated tumors showed two distinct neoplastic phenotypes. Radiation-damaged cells were primarily observed within the linear regions corresponding to the microbeam-treated volume (dashed lines). Panel D: Magnified images of the irradiated region showed an abundance of markedly enlarged, pleomorphic cells with bizarre mitotic figures and multinucleation (arrows).
Figure 2.20 Radiation Marginal Miss Experiment A. (A) Metastatic burden was quantified via total bioluminescence signal recorded from dissected lungs. Pulmonary metastasis was increased in the mice treated with marginal miss (50%) and complete (100%) tumor radiation compared to non-irradiated controls (0%). There was radiation treatment volume effect whereby the greater the tumor volume irradiated, the higher the measured bioluminescence signal (B) This radiation treatment volume effect translated to survival time. Mice with tumors treated with 100% RT lived longer than mice with 50% irradiated tumors or non-irradiated (0%) controls, respectively. (C) These metastasis results were associated with the influence of survival time. A relationship between total luminescence signal from lungs and both maximum time and cumulative tumor volume was reported. There was a linear increase in metastatic burden in lungs vs. either parameter.
Figure 2.21 Variation in tumor growth curves for Experiment A. Mice with tumors treated with 100% RT lived longer than mice with 50% irradiated tumors or non-irradiated (0%) controls.
Figure 2.22 Radiation Marginal Miss Experiment B. (A) Metastatic burden was quantified via bioluminescence imaging of dissected lungs. Pulmonary metastasis was decreased in the mice treated with marginal miss (90%) compared to complete (100%) tumor radiation and non-irradiated controls (0%). (B) The variability in the distribution of pulmonary metastasis results across treatment groups was improved compared to Experiment A. (C) Mice reached their endpoint within a window between Day 3 and Day 8 after radiation treatment. Individual tumor growth curves per treatment group are presented.
Figure 2.23 Radiation Marginal Miss Experiment C. (A) Metastatic burden was quantified via bioluminescence imaging of dissected lungs. Pulmonary metastasis was increased in the mice treated with marginal miss (90%) compared to complete (100%) tumor radiation and non-irradiated controls (0%). (B) Mice with marginally-missed tumors (90%) lived longer than those with completely irradiated (100%) or non-irradiated (0%) controls. (C) No bioluminescence signal was detected from this representative primary tumor, therefore the lack of signal from the corresponding lung tissue could not reliably be used to indicate the presence or absence of pulmonary metastasis. (D) Mice with dorsal mammary tumors at the beginning of the project, prior to Experiment A, have strong bioluminescent signal. (D) No bioluminescence signal was detected from this representative primary tumor. Quantification of the signal registered from the corresponding lung tissue could not reliably be used to indicate the degree of pulmonary metastasis in this experiment due to this evidence of a faulty reporter gene.
Figure 2.24 Quantification of pulmonary metastasis in Experiment C. (A) Gross metastatic nodules on the surface of the lungs were manually counted. Mice with marginally-missed tumors (90%) had increased pulmonary metastasis compared to completely irradiated (100%) and non-irradiated (0%) controls (p= <0.0001, p=0.0091, respectively). (B) Mice with marginally-missed tumors (90%) lived longer than completely irradiated (100%) and non-irradiated (0%) controls so the influence of survival time is contributing to the pattern of these metastasis results.
Figure 2.25 Variation in tumor growth curves for Experiment C. Mice with marginally-missed tumors (90% RT) lived longer than mice with 100% irradiated tumors or non-irradiated (0%) controls.
Figure 2.26 Oxidative stress in primary 4T1-Luc orthotopic dorsal mammary tumors. (A) Mice with partially irradiated tumors (90%) developed significantly increased oxidative stress compared to non-irradiated (0%) controls at 24 hours following radiation. (p=0.0393) (B) There was no difference in oxidative stress of the primary tumors across the treatment groups at endpoint. (C) There was a significant difference in oxidative stress measured in tumors analyzed at 24 hours following radiation compared to endpoint across all treatment groups (p=0.0079).
Figure 2.27 A proposed model of tumor response to microbeam versus wide-field irradiation. Microbeam irradiation causes an upregulation in HIF-1 in the sublethally-irradiated portions of the tumor through bystander mechanisms. HIF-1, in turn, potentiates vascular recovery. In contrast, widefield tumor irradiation results in widespread cell death, which eventually leads to reoxygenation due to decreased oxygen consumptions. Tumor cells originating from microbeam irradiated tumors are stimulated to move and utilize the tumor-associated vasculature to form regional metastatic foci.
Figure 2.28 A proposed model of tumor response to radiation marginal miss. Radiation-induced oxidative stress is generated in irradiated tumor tissue and increases throughout the regional, sublethally irradiated “bystander” tumor tissue. The increase in oxidative stress in the viable, sublethally irradiated tumor tissue results in overexpression of HIF-1, leading to increased tumor cell migration, invasion, and distant metastasis.
CHAPTER 3
TUMOR MICROENVIRONMENTAL REDOX STATE DRIVES DISTANT RESPONSE TO RADIATION

INTRODUCTION
Cells and tissues of the body are continuously trying to balance levels of free radicals. Free radicals are oxygen-containing molecules that have one or more unpaired electron. These free radical molecules are very reactive with other molecules, including cell components, such as DNA, protein, or lipids\(^{189}\). When free radicals react with these cellular structures, it can be damaging\(^{189}\). Cells utilize antioxidants to counter the free radicals; antioxidants readily donate an electron to free radicals without becoming destabilized themselves\(^{189}\). The health of the cell shifts according to these reducing and oxidizing reactions (the gain or loss of an electron, respectively) and this is known as the reduction-oxidation ("redox") status. When the amount of free radicals overwhelms the antioxidant capacity of the cell, an imbalance occurs and results in what is known as "oxidative stress". Oxidative stress can lead to many conditions in the body, including cancer, as oxidative stress is known to be associated with cancer initiation and progression\(^{189}\). Interestingly, increasing oxidative stress is also a means by which cancer cells can be killed via standard therapies, such as radiation and certain chemotherapy agents\(^{190-193}\). While this oxidative stress is intended to kill cancer cells, it can also damage sensitive normal tissues\(^{194}\). Ideal therapeutic approaches for treating cancer should balance the damaging oxidative stress to the cancer cells with antioxidant protection to normal tissues.
An example of a prominent antioxidant is superoxide dismutase (SOD). SOD is an enzyme that catalyzes the dismutation of the free radical superoxide into molecular oxygen and hydrogen peroxide\textsuperscript{189}. Hydrogen peroxide, a product of this reaction, is also reactive and damaging to cells, however, under normal conditions, it can be degraded quickly and efficiently by additional antioxidant enzymes, such as catalase and glutathione\textsuperscript{189}. Ultimately, SOD is considered an important antioxidant defense in countering oxidative stress.

Once again, radiation therapy kills cells through the generation of highly reactive free radicals. These free radicals damage critical cellular structures, such as DNA or cell membranes\textsuperscript{195} \textsuperscript{196}. Advanced radiation therapy techniques allow for precise targeting of irradiated tumor tissue, however, normal regional tissue is commonly exposed and damaged by radiation, as well, which can result in significant, and sometimes dose-limiting, acute and chronic side effects\textsuperscript{197} \textsuperscript{198}. Attempts have been made with adjuvant agents, such as amifostine, to scavenge the free radicals in normal tissues to reduce radiation injury\textsuperscript{199}, but there is always a very valid concern in using such compounds that, in protecting the normal tissues, the tumor may be inadvertently protected, as well.

Novel compounds, known as manganese porphyrins (MnPs), have been designed to shift the redox status of both normal cells and cancer cells\textsuperscript{200}. MnPs are SOD mimics, so, like endogenous SOD, they catalyze the dismutation of superoxide into molecular oxygen and
hydrogen peroxide; however, it has been shown that they are also capable of undergoing a variety of additional reactions, so instead of classification as simply antioxidants, they may be more appropriately described as modulators of the cellular redox environment\textsuperscript{200}. When MnPs are coupled with cancer therapies, such as radiation, they have been shown to sensitize tumor cells to treatment and protect normal tissues from damage through modulation of the redox status of these very different cell types\textsuperscript{197 198 201 202}.

As presented previously in Chapter 2, our group has shown that radiation results in acute tumor reoxygenation which leads to the generation of oxidative stress\textsuperscript{102}. This oxidative stress leads to overexpression of the tumor-promoting transcription factor hypoxia-inducible factor-1 (HIF-1), protection of tumor vasculature, and ultimately radioresistance\textsuperscript{102}. MnPs have been shown to catalytically inactivate this radiation-induced oxidative stress, downregulate HIF-1, sensitize the tumor vasculature to radiation, and improve the radiation response in several tumor types\textsuperscript{102 202 203}. While it seems counterintuitive that reducing oxidative stress following radiation would improve tumor response, it is important to recognize that MnPs are redox modulators, and it is their effect on signaling pathways, particularly inhibition of those activated by HIF-1, that has led to the improved tumor cell killing with radiation\textsuperscript{200}. Also very noteworthy, MnPs have been shown repeatedly to be able to protect a variety of highly sensitive normal tissues from radiation damage\textsuperscript{197 198 201} by modulating the redox status of the normal tissue microenvironment. The difference in the activity of MnPs in tumor vs. normal tissue is partly related to relative differences in baseline
levels of oxidative stress in tumor vs. normal tissue\textsuperscript{200}. MnPs have also been recently shown to accumulate in tumor tissues to a much greater extent than normal tissues, which may contribute to their effect in enhancing efficacy of radiation\textsuperscript{204}.

While developing our hypothesis that radiation marginal miss increases tumor aggression, we recognized that radiation-induced oxidative stress could lead to overexpression of HIF-1 in bystander tumor cells; stemming from this, adjuvant treatment with MnPs could be a means to inhibit these effects. This led to our second hypothesis that adjuvant MnP would reduce radiation-induced oxidative stress, downregulate HIF-1 in the tumor bystander cells, and abrogate or inhibit the aggressive tumor changes associated with radiation marginal miss (Figure 3.1). Minimizing risks of tumor aggression originating from radiation marginal miss by concurrent treatment with MnPs could impact many patients treated with highly conformal radiation.

In this study, we investigate how MnPs influence the tumor response to radiation marginal miss using an orthotopic dorsal mammary tumor model. Specifically, we study how adjuvant MnP shifts the redox status in the primary tumor and how this alters the development of distant metastasis.
MATERIALS AND METHODS

4T1-Luciferase Cell Line

A modified 4T1 murine mammary carcinoma cell line was used for this study (a gift from Dr. Michael Wendt, Case Western Reserve University). This stably transfected cell line constitutively expresses Firefly Luciferase-2 under a CMV promoter with Zeocin resistance (500ug/mL) for selection in vitro.

These 4T1-luciferase-expressing cells (4T1-Luc) were cultured in Dulbecco's modified eagle medium (DMEM, high glucose with sodium pyruvate and glutamine) with 10% fetal bovine serum and Zeocin(500ug/mL) and incubated at 37°C in 5% CO₂. Cells were passaged 1:10 when 75-90% confluent. These cells were routinely tested and confirmed to be free from Mycoplasma contamination.

Surgical Implantation

4T1-Luc orthotopic mammary tumors were surgically implanted into the dorsal mammary fat pad of BALB/c mice. Mice were anesthetized using intraperitoneal injections of ketamine (85 mg/kg) and xylazine (8.5 mg/kg), fur overlying the dorsal thorax was removed using a chemical depilatory (Nair, potassium thioglycolate), and the surgical field was prepared using three alternating applications of chlorhexidine gluconate scrub solution and 70% ethanol. A small skin incision was made overlying the dorsal mammary fat pad. The fat pad was visualized, and approximately $5 \times 10^5$ 4T1-Luc cells suspended in 100uL of serum-free
DMEM were injected into the site. The incision was closed using 4-0 silk suture and a single cruciate suture pattern. Triple antibiotic was applied to the incision, and buprenorphine (0.1 mg/kg) was administered subcutaneously as analgesia for the recovering mice.

*Biological Timeline for Development of Orthotopic Dorsal Mammary Tumors and Metastasis*

Following the surgical implantation of tumor cells, mammary tumors grew reliably and reached treatment size (approximately 250mm$^3$) within six to ten days. Orthotopic dorsal mammary tumors were visible via bioluminescence imaging (see Figure 2.3A). Metastatic lesions could be identified within the lungs via bioluminescence imaging as early as six days following tumor implantation (see Figure 2.3B). Metastatic lesions could be identified within the lungs via bioluminescence imaging as early as six days following tumor implantation. Later, it was determined via histologic analysis of mouse lung sections, that metastatic cells could be identified as early as 4 days following tumor implantation with this technique.

*Tumor Growth Monitoring*

Palpable tumors were identified between 6 to 10 days following surgical implantation. Tumors were measured daily with calipers, and volumes were calculated using the formula $V = (A^2 \times B \times \pi)/6$, where $A$ is the shortest diameter and $B$ is the longest diameter.
**Manganese Porphyrin Preparation and Administration**

For these experiments, the manganese porphyrin MnTBuOE-2-PyP\(^{5+}\) (MnBuOE) was used. The chemical structure of MnBuOE is shown in Figure 3.1. MnBuOE was synthesized and purified according to the procedures described elsewhere\(^{205,206}\). A loading dose of 0.2mg/kg MnBuOE was administered subcutaneously in the left hind leg three days after tumor cell implantation in the MnBuOE alone study, or 24 hours prior to surgery or radiation, respectively, in subsequent experiments. Then, a dose of 0.1mg/kg MnBuOE was administered in the same manner on a Monday, Wednesday, Friday schedule beginning at least 48 hours following the loading dose. This dosing protocol was selected as it was determined to yield pharmacologically active concentrations in tissues\(^{197,204}\). For consistency in discussions, MnBuOE will be referred to as MnP in this chapter, the general abbreviation for manganese porphyrin compounds.

**Surgical Excision**

When the longest tumor diameter was greater than 7mm, mice were treated with a loading dose of MnP (0.2mg/kg) or equivalent volume of saline. Dorsal mammary tumors were surgically excised 24 hours later. Mice were anesthetized using intraperitoneal injections of ketamine (85 mg/kg) and xylazine (8.5 mg/kg), residual fur overlying the dorsal thorax was removed using a chemical depilatory (Nair, potassium thioglycolate), and the surgical field was prepared using three alternating applications of chlorhexidine gluconate scrub solution and 70% ethanol. A small skin incision was made overlying the dorsal mammary tumor.
The gross tumor was excised via sharp dissection using a size 10 surgical blade. The incision was closed using 4-0 silk suture and a single cruciate suture pattern. Triple antibiotic was applied to the incision, and buprenorphine (0.1 mg/kg) was administered subcutaneously as analgesia for the recovering mice. Mice were euthanized (pentobarbital sodium and phenytoin sodium, 0.5uL per mouse) when the recurring tumor reached $1500 \text{mm}^3$.

**Image-Guided Radiation Therapy**

Depending on the experiment, when the longest diameter of tumors was greater than 7mm or on the defined treatment day, mice were given a loading dose of MnBuOE (0.2mg/kg) or equivalent volume of saline (*Figure 3. 2*). Twenty-four hours later, mice were anesthetized with 1.5% isoflurane gas mixed with oxygen and placed in an X-RAD 225Cx (Precision X-ray Inc) small animal micro-CT irradiator. A collimating cone that produced a 10 x 10-mm radiation field was used to target the radiation beam to the dorsal mammary tumor. The field was rotated 45 degrees to create a diamond shape over the irradiated area (See Figure 2.7). The cranial and caudal aspects of the tumor were identified with small barium markers which had been applied to the tumors of anesthetized mice. Fluoroscopy at 40 kVp and 2.5 mA with a 2-mm Al filter allowed proper alignment of the radiation field. A lead alloy shield was fixed to collimator to create a treatment field whereby the longest axis was 50% blocked or 90% open and 10% blocked for the marginal miss experimental treatment groups (See Figure 2.7). The treatment groups will be referred to by the volume of tumor radiation administered; for example, 100% tumor volume radiation (100% RT), 90% marginal miss
(90% RT), non-irradiated control (0% RT). Mice were treated with one fraction of 15Gy administered as two exposures of 7.5Gy from parallel-opposed fields with a dose rate of 257cGy/min at target depth with 225 kVp and 13 mA and a 0.3-mm Cu filter. Control mice were anesthetized but not irradiated. Depending on the experiment, mice were euthanized when the first tumor of the study reached 1500mm$^3$, individual tumors reached 1500mm$^3$, the defined time point following radiation had been reached, mice developed neurologic deficits, or mice had lost greater than 20% of starting body weight (Figure 3.2).

**Bioluminescence Imaging of Lung Metastases**

Mice were injected intraperitoneally with D-Luciferin (Potassium salt, GoldBio, 150mg/kg), and after 10 minutes, euthanized with an intraperitoneal injection of Euthasol (pentobarbital sodium and phenytoin sodium, 0.5uL per mouse). Lungs were dissected and individually imaged within 15 to 20 minutes of luciferin injection using an IVIS Kinetic system in order to measure total bioluminescence signal emitted over 60 seconds at a height of 5cm.

**Blood Collection**

After injection of euthanasia agent, but prior to death, 50uL of fresh blood was collected from an incision made in the region of the large vessels in the lateral neck of the mice. The blood was added to stabilization buffer. The blood was mixed gently and kept at room temperature for 45 minutes. The sample was centrifuged at 2000 x g for two to five minutes, and the supernatant and blood cells were stored separately at -80°C.
Tumor Dissection

Tumors were dissected following euthanasia, divided into two portions along the long axis of the tumor, flash frozen in liquid nitrogen, and stored at -80°C. The two tumor portions were saved in order to 1) analyze global oxidative stress and HIF-1 protein levels and 2) spatial HIF-1 and oxidative stress via immunohistochemistry.

Lung Preparation for Histology

Lungs were gently perfused with 1mL of phosphate buffered saline (PBS) followed by 1mL of 4% paraformaldehyde (PFA) in PBS via the intact attached trachea. The lungs were placed in 5mL of 4% PFA and stored at 4°C for 48 hours. The lungs were transferred to 5mL of PBS and stored at 4°C for 48 hours, then held within 5mL of 70% ethanol at 4°C until processing for histology.

Gross Metastasis Counts

When lungs had completed the lung histology preparation protocol, they were briefly removed from the ethanol solution and gross metastatic lesions were visualized and counted manually using a Leica StereoZoom 4microsurgery magnification system.
**GSSG/GSH**

The ratio of oxidized to reduced glutathione (GSSG/GSH) was used as a measurement of the degree of oxidative stress within the blood and treated tumors. Mice were euthanized and samples were obtained 24 hours after the last dose of MnP. The assay for tissue samples was inspired by the published assay for analysis of GSH and GSSG in blood\(^{179}\). The assay was adapted for analysis of tissue samples and to the LC/MS/MS equipment available.

**Tissue Processing:** Partially thawed blood (50μL) or tumor tissue was cut (~20 mg) and homogenized immediately in 500-μL conical polypropylene vial with: 4 parts (g/vol) of GSH-trapping solution (20 mM ethylmaleimide (NEM; Sigma), 2% SSA and 2 mM EDTA in 15% methanol), 10 parts (g/vol) of 1% formic acid (Fluka) and two 2.5mm Zr-silica beads (Biospec Products Inc.) in FastPrep (Thermo-Savant) homogenizer at speed 5 for 40 s. After 30 min incubation at room temperature, 150 μL of the homogenate was transferred into a 2-mL conical polypropylene vial, 500 μL of chloroform (B&D) was added, agitated in FastPrep at speed 5 for 40 s, followed by centrifugation at 16,000 g for 5 min. GSH analysis: 5 μL of supernatant, 1 mL of deionized water, and 100 μL of 1 μM GSH-NEM-d3 (internal standard) was combined and placed into autosampler at 4 °C. GSSG analysis: 20μL of supernatant and 20 μL of 1 μM GSSG-d6 (internal standard) in mobile phase A (see below) was combined and placed into autosampler at 4 °C.
LC-MS/MS Analysis

The measurement of GSH (as GSH-NEM derivative) and GSSG was performed on Shimadzu 20A series liquid chromatography (LC) and Applied Biosystems/SCIEX API5500 QTrap tandem-mass spectrometer (MS/MS). LC conditions: Column Agilent ZORBAX Eclipse Plus, C18 4.6x50 mm 1.8 μm particle size (P/N 959941-902) analytical column and Phenomenex, C18 4x3 mm guard cartridge (P/N AJ0-4287) at 45 °C. Mobile phase solvents (all MS-grade): A - 0.1% formic acid in water, 2% acetonitrile; B – acetonitrile. Elution gradient at 1 mL/min: 0-3 min 0-50% B, 3-3.1 min 50-95% B, 3.1-3.5 min 95%B, 3.5-3.6 min 95-0%B. Run time: 7 min. MS/MS conditions: MRM transitions (m/z) followed for GSH-NEM, GSH-NEM-d3, GSSG, and GSSG-d6 were 433-304, 436.2-307.1, 613.1-355, and 619.1-361, respectively. Calibration samples (n=6) in mobile phase A were prepared in the following appropriate ranges: 0.375-6.00 mM for GSH and 1.88-30 μM for GSSG analysis and analyzed alongside the study samples. Quantification was performed by Analyst 1.6.2 software.

Histologic Quantification of Metastasis

Lungs were paraffin-embedded and sectioned with 5uM thickness in six 200uM steps through the coronal plane of the lungs. The tissue was stained with hematoxylin and eosin. All sections were scanned with a 5X objective using a Zeiss AxioImager 2 microscopy system, and images of all identified metastatic lesions were obtained. Using ImageJ, the area of individual metastatic lesions of the lungs was measured.
**Modeling of Metastatic Colonization and Growth within Lungs**

A mathematical model was derived to be used for assessment of tumor metastases (See Figure 2.9). The model includes cells needed to establish metastatic colonization and secondary tumor growth rate within the lung. Histologic data of the number and size distribution of tumor nodules were used as input variables for this model. Using the growth rate of the primary tumor and the total number and area of metastatic pulmonary lesions from a pilot experiment it is possible to estimate the time that metastases form, as well as the growth rate in the lung.:

$$t_{ij} = (T - b^{-1}(\log V_{ij}(t) - \log V_{ij}(t_{ij})))$$  \hspace{1cm} (3.1)

where $T$ = the time of euthanasia, $V_{ij}$ = initial tumor volume within the lung, $t_{ij}$ = time a metastatic fragment of size $V_{ij}$ colonized the lungs, and $b$ = growth rate. This formula was validated using results from a pilot experiment that had growth rate data from the primary tumor and the physical measurements of tumor within lungs.

**Statistics**

**Bioluminescence Quantification of Metastasis:** Single comparisons were conducted using Student's T-test.
Counts of Gross Pulmonary Metastasis:

Because these counts were measured on mice observed over different lengths of time, we modeled the incidence rate of metastasis, assuming the incidence rate to be constant over the observed life of the mouse.

The observed number of metastases was modeled as a Poisson random variable, with a mean $\lambda(d,p)T$, where $\lambda(d,p)$ is the incidence rate, that depended on radiation dose $d$ and whether Saline ($p = 0$) or porphyrin ($p = 1$) was given. The dependence of the incidence on treatments was modeled as follows:

$$\log(\lambda(d,p)) = \mu + \alpha_d + \beta_p + \alpha\beta_{dp}$$

Where $\mu$ is the baseline mean, representing 90% RT dose and Saline, $\alpha_d$ is the effect of other dose, $\beta_p$ is the effect of Porphyrin and $\alpha\beta_{dp}$ is the interaction between radiation and porphyrin. Model was fit by maximum likelihood in the R computing package (www.r-project.org).

GSSG/GSH:

We analyzed the ratio using a model of the form:

$$r_{dptk} = \mu + \alpha_d + \beta_p + \gamma_t + \alpha\beta_{dp} + \alpha\gamma_{dt} + \beta\gamma_{pt} + \epsilon_{dptk}$$

Where $r_{dptk}$ is the GSS/GSSH ratio for the $d$-th RT dose, $p$-th dose of porphyrin (Saline or Porphyrin), $t$-th time of observation (abbreviated or extended) and $k$-th replicate. Model (3.3) explains variation in this ratio in terms of $\mu$, the baseline mean, representing 90% RT dose
and Saline, $\alpha_d$ is the main effect of other dose, $\beta_p$ is the effect of Porphyrin, $\gamma_t$ is the effect of extended observation time and the other terms are interactions between each of the factors. The model was fit using ordinary least squares.

RESULTS:

_Influence of MnP on Primary Tumor Growth and the Development of Metastasis_

Mice (n=6/group) were treated with either MnP or saline to understand the influence of MnP alone on primary tumor growth and the development of pulmonary metastasis (Figure 3.3A). MnP did not alter primary tumor growth rate compared to the saline control group (Figure 3.3B); however, metastatic burden quantified via bioluminescence imaging was significantly increased ($p=0.019$) in the mice treated with MnP (Figure 3.3C).

_Influence of MnP on Tumor Surgical Recurrence and the Development of Metastasis_

Mice (n=5/group) were treated with either MnP or saline in conjunction with surgical excision of the primary dorsal mammary tumor to understand the influence of MnP on tumor surgical recurrence and the development of pulmonary metastasis (Figure 3.4A). MnP did not alter tumor surgical recurrence nor the rate of secondary tumor growth compared to the saline control group (Figure 3.4B); however, there was a trend in the luciferase imaging data that is consistent with metastatic burden being decreased in mice treated with MnP ($p=0.081$) (Figure 3.4C).
Influence of Marginal Miss and MnP on Radiation Tumor Growth Time and the Development of Metastasis

Three variations of this experiment were performed, as described in Chapter 2, but with the addition of adjuvant MnP (Figure 3.2). Mice (n=5-6/group) were treated with either MnP or saline in conjunction with radiation (0Gy, 15Gy) administered to different volumes (0%, 50%, 90%, 100%) of primary dorsal mammary tumors to understand the influence of marginal miss and MnP on tumor growth delay and the development of pulmonary metastasis. For detailed information on the evolution of experimental design between Experiments A, B, and C, please see Chapter 2.

Experiment A: Metastatic burden quantified via bioluminescence imaging was again increased in mice treated with MnP alone compared to saline alone. Metastases trended towards a decrease in mice treated with MnP and either 50% or 100% irradiated tumor volume compared to mice treated with saline and the corresponding volume of tumor radiation, however, the difference was not statistically significant (Figure 3.5A). MnP as an adjuvant to 100% RT extended tumor growth time compared with mice that received saline + 100%RT compared to the saline-treated mice + 100% irradiated tumors (Figure 3.5B). By allowing mice to survive until their endpoint, these animals were at risk for developing metastases for a longer period than the unirradiated control mice. As validation of this point, we examined the relationship between total luminescence signal from lungs and cumulative tumor volume as well as time of euthanasia (Figure 3.5C). There was a linear increase in
metastatic burden in lungs vs. either parameter. Individual tumor growth curves per treatment group for Experiment A are presented (Figure 3.6)

Experiment B: The previously observed pattern of MnP alone increasing metastasis was not observed (Figure 3.7A). The combination of MnP and RT appeared to decrease metastasis; however, the marginal miss groups (90%) had less metastatic burden than the non-irradiated (0%) and completely irradiated (100%) groups. This pattern of metastasis results was unexpected and difficult to interpret biologically. (Figure 3.7A). The variability in the distribution of pulmonary metastasis results across treatment groups was improved compared to Experiment A (Figure 3.7B). Individual tumor growth curves per treatment group for Experiment B are presented (Figure 3.8)

Experiment C: As discussed in Chapter 2, the metastasis data acquired from the bioluminescence signal in Experiment C was invalid due to faulty constitutive luciferase reporter gene signal (Figure 3.9A, 3.9C-E). Mice with 90% and 100% RT tumors extended tumor growth time compared to mice with non-irradiated tumors (0%), and there appears to be a treatment effect of extended tumor growth time with adjuvant MnP compared to saline for the mice with non-irradiated (0%) and marginal miss (90%) groups, but not the 100% irradiated tumor groups (Figure 3.9B). Individual tumor growth curves per treatment group for Experiment C are presented (Figure 3.10)
Manual counts of grossly visible metastatic lesions on the surface of the lungs were used for analysis. MnP significantly increased metastasis compared with saline for all treatment groups. \( p < 0.0001 \) (Figure 3.11).

The metastasis results of Experiments A, B, and C are summarized in Table 3.1.

Oxidative stress was measured within the primary tumors at two time points: 1) 24 hours postRT and 2) time of euthanasia. MnP reduced oxidative stress within tumors when combined with radiation at both the 24hr time point and at euthanasia \( p = 0.046 \) (Figure 3.12A). MnP did not alter the redox status of whole blood at either time point (Figure 3.12B). Oxidative stress in circulating tumor cells was not measured.

**DISCUSSION**

This study investigated how adjuvant MnP±RT influenced primary and lung metastatic tumor growth in response to radiation marginal miss, using an orthotopic tumor model. We hypothesized that adjuvant MnP would reduce radiation-induced oxidative stress, downregulate HIF-1 in tumor bystander cells, and abrogate or inhibit metastases associated with radiation marginal miss. Instead, we found that MnP significantly increased pulmonary metastasis compared to saline treatment groups when used alone as well as in combination with either full irradiation or a scenario where a marginal miss was simulated.
While these results were initially surprising to us, a literature review uncovered a number of studies which have shown that drugs with antioxidant effects can promote breast cancer cell survival in circulation\textsuperscript{207,208}. Detachment of breast cancer cells from the extracellular matrix results in an increase in cellular oxidative stress and this can lead to apoptosis due to ATP deficiency\textsuperscript{207}. In these studies, it was found that antioxidants countered this oxidative stress and increased the survival of detached breast cancer cells \textit{in vitro}\textsuperscript{207}. This survival was possible due to restoration of the ATP deficiency via a metabolic switch to fatty acid oxidation\textsuperscript{207}. Downregulation of endogenous antioxidants, such as catalase and SOD, made it impossible for detached breast cancer cells to undergo this metabolic switch and survive\textsuperscript{208}.

Further, it was demonstrated \textit{in vivo} that antioxidants increased lung colonization of these breast cancer cells in mice\textsuperscript{208}. A recent study using patient-derived melanoma xenografts similarly shows that tumor cells which underwent reversible metabolic changes during metastasis increased their capacity to withstand oxidative stress and successfully metastasize\textsuperscript{209}. Further, treating these mice with exogenous antioxidants increased distant melanoma metastasis\textsuperscript{209}.

From our previous studies, the combination of MnPs and radiation sensitized 4T1 tumors through the following mechanism: MnPs reduced radiation-induced oxidative stress leading to the downregulation of HIF-1 expression. These prior studies, however, were never designed to evaluate the effects of MnP on metastasis. This is the first study to investigate how adjuvant MnP alters metastasis using an orthotopic tumor model.
Oxidative stress was reduced by MnP compared to saline for all treatment groups at both the 24 hr post-radiation time point and at endpoint. Reduced oxidative stress with single agent MnP, as well as the combination of adjuvant MnP and marginal miss extended tumor growth time compared to the corresponding saline treatment groups; however, there was no tumor growth time extension seen with adjuvant MnP and complete tumor radiation compared to the corresponding saline control. The results which will show the expression of HIF-1 across the treatment groups are pending, so we are unable to know if adjuvant MnP led to a reduction in HIF-1 expression in the irradiated primary tumors, as would be expected according to the reported mechanism of MnP tumor radiosensitization. HIF-1 protein levels from the tumors will be analyzed globally via western blot and regionally via immunohistochemistry.

The MnP-induced redox shift within the irradiated primary tumors was found to be consistent with an antioxidant effect of MnP compared to the saline-treated controls. This MnP redox shift did not lead to a significant primary tumor growth delay across the treatment groups, but it did influence metastatic efficiency. We strongly suspect that the antioxidant shift underlies the increased metastatic burden in the mice treated with the MnP alone, as well as the combination of MnP and radiation. Clinically, it has recently been demonstrated for the first time that various radiation protocols (palliative and curative-intent) result in mobilization of viable circulating tumor cells (CTCs) into the circulation of patients with non-small cell lung cancer 24 hours after a first radiation fraction, as well as following additional fractions given
during definitive radiation treatment protocols\textsuperscript{210}. This phenomenon of increased CTCs measured after radiation was documented following blood samples collected out past the tenth fraction of radiation\textsuperscript{210}. A similar study has not been performed for breast cancer patients, as most undergo postoperative radiation, but mobilization of CTCs following radiation may explain our results of increased metastasis with the combination of MnP and radiation. Knowing that antioxidants can influence the survival of detached, circulating breast cancer cells \textit{in vitro} and \textit{in vivo}, it is likely that, in our study, radiation increased CTCs while the antioxidant state provided by MnP supported tumor cell survival in circulation and colonization in the lungs. We note that when considering the environment of the circulating tumor cells, the antioxidant shift was confined to the tumor cells; the redox status of the whole blood was not altered, so this can be considered truly an effect on circulating tumor cells. A visual representation of this proposed model is presented (\textbf{Figure 3.13}).

In the previous chapter, we showed that tumors subjected to radiation marginal miss had increased oxidative stress compared to controls, and this was associated with increased distant metastasis in these mice. Final results are pending, but we speculate that in that situation, the increased oxidative stress in the sublethally-irradiated tumor cells led to increased HIF-1 expression, increased cell motility, and metastasis. We believe that the mice treated with the combination of MnP and radiation in this study, however, developed increased metastasis through a different mechanism; specifically, that the antioxidant effects of MnP on the tumor cells supported CTC survival in circulation and promoted the formation
of distant metastasis. It will be very interesting to see how HIF-1 expression in the primary tumors is impacted by radiation and MnP. Based on previous studies, we would anticipate that the combination of radiation and MnP would result in downregulation of HIF-1, leading to a reduction in metastasis; however, metastasis was significantly increased in the mice in this study. The mechanism of increased metastasis could be due to shifts in CTC metabolism, in line with what has been reported by other groups to promote survival among detached and circulating breast cancer and melanoma cells\textsuperscript{207,209}. An important future direction would be to characterize metabolism of the circulating 4T1-Luc tumor cells to see whether those treated with MnP and radiation had switched to fatty acid oxidation, as reported by others.

The results of this study show that the combination of MnP and radiation increases metastasis in a preclinical orthotopic breast cancer model. This is simultaneously discouraging and frightening when considering adjuvant MnP for clinical use. Through modification of different aspects of the treatment protocol used in this study, both tumor sensitization and reduction in metastasis with adjuvant MnP might still be possible. The combination of MnP and radiation extended the tumor growth time of the orthotopic 4T1-Luc tumors, as had been reported previously. The dose of 15Gy was selected for this project because it is a clinically relevant dose used for single-fraction, conformal presurgical radiation therapy for patients with breast cancer\textsuperscript{211}. It is possible that increasing the radiation dose may enhance primary tumor sensitization and reduce pulmonary metastasis due to increased initial tumor cell
death. Previous studies have coupled MnP with fractionated radiation therapy and seen significant tumor sensitization. It is possible that fractionated radiation may be more effective at improved local tumor sensitization and reducing metastasis when coupled with MnP than single dose radiation protocols due to repeated episodes of radiation and MnP-induced redox modulation within the tumor microenvironment; alternatively, enhancement of metastasis via the antioxidant effects of MnP on shed irradiated CTCs may be intensified with repeated radiation fractions. Further, the dose of MnP used in this study might have been too low to maximize tumor radiosensitizing effects. Finally, the schedule of MnP administration might have influenced the results. When used in the time around radiation administration, and under increased radiation and MnP dosing conditions, a single dose or a few doses of the compound following radiation may be sufficient to shift the tumor redox microenvironment to one which supports tumor sensitization; however, if MnP is given in a prolonged manner following therapy, as was done in these experiments, antioxidant effects may support the survival of CTCs and promote metastasis.

It is important to compare the results from the radiation study with the data from the surgery and MnP experiment. In this study, the combination of MnP and surgery trended toward a reduction in metastasis compared to saline-treated controls. At this point in the project, quantification of metastasis was performed via bioluminescence imaging, and as noted, we had concerns overtime with the validity of this reporter cell line. It is possible that the reporter cell line provided faulty results and there was no reduction of metastasis when
adjuvant MnP was added to surgery. Alternatively, we consider that the redox status of a postoperative, inflammatory microenvironment for microscopic tumor cells may be vastly different than that of a post-radiation, bulky tumor. In the surgical scenario, MnP might have shifted the redox status to a more pro-oxidant state, increased tumor cell death locally and reducing the number of tumor cells healthy enough to detach and successfully metastasize. Additional experiments investigating the use of adjuvant MnP for surgical oncology are warranted.

The fact that MnP may have different effects on tumor metastasis in a surgical setting compared to radiation is intriguing when considering clinical management of cancer patients. Depending on the tumor type, many cancers are treated with a combination of surgery, radiation, and chemotherapy. MnPs have been shown to have beneficial adjuvant effects when combined with chemotherapy. Additional experiments which investigate how shifts in the tumor redox status via MnP in conjunction with various combinations, timelines, and protocols of surgery, radiation, and chemotherapy may all result in very different biological and clinically relevant outcomes.

It is clear that it is incredibly important that redox modulators, like MnPs, are used with great caution for tumors with high metastatic propensity. While it may be possible to harness the benefits of modulating the tumor redox environment for improved local therapeutic outcomes, this can only be done if adjuvant MnP will not enhance the development and
progression of distant metastasis. However, antioxidants have been implicated recently in other aspects of cancer biology, as well. For example, glutathione, an antioxidant, has been found to be necessary for the initiation of some cancers, and there is provocative new evidence that antioxidants can promote cancer initiation and progression\textsuperscript{213-216}. In fact, not only has increasing dietary antioxidants generally not reduced cancer incidence in clinical trials\textsuperscript{217}, dietary antioxidants have been shown to increase cancer incidence and death from lung and prostate cancer\textsuperscript{218-220}, as well as development and progression of breast cancer\textsuperscript{221 222}. There are reports which counter these findings, demonstrating that antioxidants can inhibit the metastasis of some cancer cell lines; however, the mounting evidence of the clinical risks of antioxidants in cancer progression suggests that there is a critical need for a better understanding of the biology and risks behind redox modulating compounds.
**Figure 3.1 Graphical depiction of the central hypothesis.** Partial tumor radiation will generate reactive oxygen species in the irradiated tumor which will increase HIF-1 expression in the sublethally irradiated tumor region via bystander effects. This overexpression of HIF-1 will lead to aggressive tumor changes, such as angiogenesis, epithelial-mesenchymal transition, tumor cell migration and invasion, and metastasis. Modulation of oxidative stress by manganese porphyrin (MnP) will inhibit overexpression of HIF-1 in the bystander cells and abrogate these effects.
Figure 3.2 Experimental design for MnP and radiation marginal miss experiments.

**Experiment A** investigated the effects of MnP on radiation marginal miss compared to controls with 0%, 50%, and 100% tumor volume radiation administered as tumors reached treatment size (Longest diameter >7mm). Mice were treated with a loading dose of MnP (0.2 mg/kg) or saline, followed by primary tumor radiation 24 hours later. MnP or saline was administered at a maintenance dose (0.1mg/kg) every Monday, Wednesday, and Friday until completion of the study. This dosing protocol was used throughout Experiments A, B, and C, as indicated. Mice reached their endpoint when their tumors were >1500mm³, they developed neurologic deficits, or demonstrated >20% body weight loss. **Experiment B** was modified so radiation marginal miss was simulated with 90% instead of 50% tumor volume radiation. Tumors were irradiated as they reached treatment size (Longest diameter >7mm). Also, a five day window was set as the defined endpoint according to when the first mouse with a tumor that reached 1500mm³. During this five day window, mice were euthanized when: 1) their tumor reached 1500mm³, 2) they developed neurologic deficits, 3) >20% body weight loss or 4) they were euthanized at the end of the five day window, regardless of the size of their primary tumor. **Experiment C** was designed to keep the time between tumor implantation and irradiation consistent. Radiation treatment was performed nine days
following tumor implantation. Animals were eligible to be irradiated if their tumors were 7-12mm diameter. Mice with tumors that were not the appropriate target volume on the defined radiation treatment day were excluded from the study. All animals were followed until tumors reached 1500mm³, until they experienced paralysis from local tumor invasion, or developed >20% body weight loss.
Figure 3.3 Single agent MnP increases distant metastasis. (A) Experimental design to determine how MnP treatment influences the development of pulmonary metastasis in an orthotopic dorsal mammary tumor model compared to saline-treated controls. Three days following surgical implantation of tumor cells, mice were treated with a loading dose of MnP (0.2 mg/kg) or saline. MnP was administered at a maintenance dose (0.1mg/kg) every Monday, Wednesday, and Friday until completion of the study. (B) MnP did not alter local tumor growth compared to controls. (C) Treatment with MnP significantly increased pulmonary metastasis quantified via bioluminescence imaging compared to saline treated controls (p=0.019).
Figure 3.4 Surgery and adjuvant MnP reduces distant metastasis. (A) Experimental design to determine how MnP treatment influences the development of pulmonary metastasis when combined with surgical excision in an orthotopic dorsal mammary tumor model compared to saline-treated controls. Mice were treated with a loading dose of MnP (0.2 mg/kg) or saline, followed by surgical excision of the primary tumor 24 hours later. MnP was administered at a maintenance dose (0.1 mg/kg) every Monday, Wednesday, and Friday until completion of the study. (B) MnP did not alter local tumor recurrence rates compared to controls. (C) Treatment with MnP and surgery reduced pulmonary metastasis quantified via bioluminescence imaging compared to saline treated controls.
Figure 3.5 MnP and radiation marginal miss Experiment A. (A) Metastatic burden was quantified via total bioluminescence signal recorded from dissected lungs. Pulmonary metastasis was increased in the mice treated with marginal miss (50%) and complete (100%) tumor radiation compared to non-irradiated controls (0%). MnP alone increased pulmonary metastasis compared to the saline alone group, and, when combined with both 50% and 100% tumor radiation, MnP decreased metastasis compared to saline and radiation. There was radiation treatment volume effect whereby the greater the tumor volume irradiated, the higher the measured bioluminescence signal. (B) This radiation treatment volume effect translated to survival time. Mice with tumors treated with 100% RT lived longer than mice with 50% irradiated tumors or non-irradiated (0%) controls, respectively. (C) These metastasis results were associated with the influence of survival time. A relationship between total luminescence signal from lungs and both maximum time and cumulative tumor volume was reported. There was a linear increase in metastatic burden in lungs vs. either parameter.
Figure 3.6 Variation in tumor growth curves for Experiment A. Mice with tumors treated with 100% RT lived longer than mice with 50% irradiated tumors or non-irradiated (0%) controls. MnP extended survival for 100% irradiated tumors compared to saline + 100% RT.
Figure 3.7 MnP and radiation marginal miss Experiment B (A) Metastatic burden was quantified via bioluminescence imaging of dissected lungs. Pulmonary metastasis was decreased in the mice treated with marginal miss (90%) compared to complete (100%) tumor radiation and non-irradiated controls (0%). There was a pattern of decreased metastasis with MnP across the treatment groups compared to saline-treated controls. (B) The variability in the distribution of pulmonary metastasis results across treatment groups was improved compared to Experiment A.
Figure 3.8 Variation in tumor growth curves for Experiment B. Mice reached their endpoint within a window between Day 3 and Day 8 after radiation treatment. Individual tumor growth curves per treatment group are presented.
Figure 3.9 MnP and radiation marginal miss Experiment C

(A) Metastatic burden was quantified via bioluminescence imaging of dissected lungs. Pulmonary metastasis was increased in the mice treated with marginal miss (90%) compared to complete (100%) tumor radiation and non-irradiated controls (0%). (B) Mice with marginally-missed tumors (90%) lived longer than those with completely irradiated (100%) or non-irradiated (0%) controls. Treatment with MnP extended survival for mice with non-irradiated tumors (0%) or completely irradiated tumors (100%) compared to the corresponding saline treated controls. (C) No bioluminescence signal was detected from this representative primary tumor, therefore the lack of signal from the corresponding lung tissue could not reliably be used to indicate the presence or absence of pulmonary metastasis. (D) Mice with dorsal mammary tumors at the beginning of the project, prior to Experiment A, have strong bioluminescent signal. (D) No bioluminescence signal was detected from this representative primary tumor. Quantification of the signal registered from the corresponding lung tissue could not reliably be used to indicate the degree of pulmonary metastasis in this experiment due to this evidence of a faulty reporter gene.
Figure 3.10 Variation in tumor growth curves for Experiment C. Mice with marginally-missed tumors (90% RT) lived longer than mice with 100% irradiated tumors or non-irradiated (0%) controls. Treatment with MnP extended survival for mice with non-irradiated tumors (0%) or completely irradiated tumors (100%) compared to the corresponding saline treated controls.
Figure 3.11 Quantification of pulmonary metastasis in Experiment C. Gross metastatic nodules on the surface of the lungs were manually counted. MnP significantly increased pulmonary metastasis across all treatment groups (p= <0.0001)
Figure 3.12 Oxidative stress in primary 4T1-Luc orthotopic dorsal mammary tumors. (A) MnP significantly reduced oxidative stress in the primary tumors across all treatment groups compared to corresponding saline-treated controls (p=0.046). There was a significant difference in oxidative stress measured in tumors analyzed at 24 hours following radiation compared to endpoint across all treatment groups (p=0.0079). (B) MnP did not alter the redox status of whole blood at either time point.
Figure 3.13 A proposed model of the influence of MnP and radiation to the primary tumor on the development of distant metastasis. MnP serves as an antioxidant for 4T1 tumor cells. Radiation increases the number of circulating tumor cells released into the vasculature. Antioxidant MnP supports the survival of detached, circulating 4T1 cells and leads to increased metastatic efficiency and burden in the lungs. The role of HIF-1 in this model is to be determined.
**Table 3.1.** Summary of results from Experiments A, B, and C with respect to how MnP influenced pulmonary metastasis compared to corresponding

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CHAPTER 4

Potential for a novel manganese porphyrin compound as adjuvant canine lymphoma therapy

INTRODUCTION:

Cancer therapy should operate with a wide therapeutic index whereby tumor cells are preferentially killed and normal tissues are protected. Unfortunately, the most common cancer treatments are toxic to both tumor and normal tissues and the therapeutic index is narrow. Many cancer therapeutics increase cellular oxidative stress and this stress can induce cell death. Increased oxidative stress has been observed after radiation therapy, several common chemotherapy agents, and even targeted agents. While the increase in oxidative stress contributes to tumor cell cytotoxicity, it can also be damaging to normal tissues. We have designed a novel manganese porphyrin, MnTBuOE-2-PyP^5+ (MnBuOE) (Figure 4.1A), which has been shown to sensitize tumor cells to chemotherapy and radiotherapy while protecting normal tissue by modulating tissue reduction-oxidation (redox) status.

MnBuOE, has divergent effects in tumor and normal tissue due to the inherent differences in basal cellular oxidative stress levels. Normal tissues have low levels of reactive species, and, in this context, MnBuOE serves as an antioxidant, marginally suppressing NF-kB activity, triggering anti-inflammatory responses, preventing cycling oxidative stress, and protecting
normal cells from damage. In tumors, however, an excessive baseline level of oxidative stress exists. The oxidative stress is driven by accumulation of the long-lasting reactive oxygen species, hydrogen peroxide. This excessive baseline level of oxidative stress is further enhanced by cancer therapies. As a cancer therapy adjuvant, MnBuOE serves as a pro-oxidant, stimulating the peroxide-induced oxidation of NF-kB, suppressing mitochondrial respiration, inhibiting glycolysis, and driving tumor cytotoxicity.

MnBuOE is primed for clinical use as an adjuvant cancer therapy and normal tissue protector. The compound has been optimized for pharmacodynamics with minimal toxicity and has entered Phase I/II Clinical Trials at Duke University as a radioprotector of normal tissue for patients with glioma who are treated with radiotherapy and temozolomide (Clinical Trial NCT02655601, clinicaltrials.gov).

A manganese porphyrin derivative from MnBuOE, MnTE-2-PyP5+, has shown benefits as a chemosensitizer for lymphoma. Lymphoma is a hematologic malignancy which develops within the lymph nodes. Jaramillo et al. demonstrated that MnTE-2-PyP5+ enhanced the chemotherapeutic response of murine lymphoma cell lines and primary human lymphoma samples in vitro and ex vivo, respectively. Chemosensitization of lymphoma cells by adjuvant manganese porphyrin would have significant implications for patients undergoing chemotherapy. Unfortunately, preclinical rodent models of lymphoma may not accurately portray the natural biology of the disease. Instead, important information regarding the
clinical use of adjuvant MnBuOE in treating lymphoma may be gleaned from a comparative canine oncology trial.  

Dogs develop a broad spectrum of naturally occurring cancers that share strong similarities with human cancers, they are an out-bred population, and, like human patients, pets receive state-of-the-art medical care; studying dogs with cancer offers a remarkable opportunity for preclinical modeling. For the interest of this study, we are considering canine lymphoma and the potential to perform a comparative oncology clinical trial to investigate the utility of MnBuOE as an adjuvant to canine lymphoma therapy. Positive results from a canine lymphoma trial would provide support for moving MnBuOE into clinical trials as an adjuvant to human lymphoma therapy. Many similarities exist between human and canine lymphoma: both are spontaneously occurring and genetically diverse, most patients are diagnosed with the Non-Hodgkin's type of lymphoma, and they are both treated with CHOP-based chemotherapy treatment protocols. The clinical value in studying pet dogs with lymphoma as a model of the human disease is actively being researched and reported.

Before introducing MnBuOE into the veterinary clinic to perform a companion canine lymphoma clinical trial, the goal of this study was to determine the safety and optimal dosing regimen of MnBuOE in normal dogs.
METHODS:

*Dogs:*

All animal care and experimental procedures were approved by and complied with the regulations of the North Carolina State University Institutional Animal Care and Use Committee, as described in the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985). Three adult male Beagle dogs (4 years old; one reproductively intact, two neutered; weight: 11.6-13.1 kg) were used for all phases of this study. Baseline physical examination and systemic health analysis with standard laboratory assessments (complete blood cell count, biochemistry panel, urinalysis) confirmed that the dogs were in good health prior to initiation of the study.

*MnBuOE structure and preparation:*

The chemical structure of MnBuOE is shown in **Figure 4.1A**. MnBuOE was synthesized and purified according to the procedures described elsewhere\(^{205,206}\)

*Monitoring:*

Safety was assessed by evaluation of standard physical and laboratory parameters of systemic health. A full physical examination was performed prior to initiation of the study and on each treatment and sampling day. Indirect blood pressure monitoring was performed using Cardell Vet Monitor 9401 Blood Pressure Monitor. Pulse was quantified as heart beats per minute according to manual counts, as well as quantified with a blood pressure monitor.
There was good correlation between pulse rates counted with manual methods compared to the blood pressure monitor (Figure 4.2). Standard laboratory assessments (complete blood cell count, biochemistry panel, urinalysis) were repeated throughout the phases of the study to assess systemic health.

**Single-Dose Pharmacokinetic Study**

A single-dose pharmacokinetic study was first performed over 14 days to ascertain the full pharmacokinetic profile. Prior studies in mice demonstrated that a dose of 1 mg/kg is adequate to achieve pharmacologically active levels in normal and tumor tissues. Based on pharmacodynamic studies conducted on mice and monkeys, this dose was projected to be well below the maximally tolerated dose in dogs.

Intravenous catheters were placed in the jugular vein of each dog under heavy sedation (dexmedetomidine, 500mcg/m²) 24-hours prior to initiation of the pharmacokinetic study to facilitate blood sampling. Baseline pulse and blood pressure (Cardell Vet Monitor 9401 Blood Pressure Monitor) were recorded prior to administration of MnBuOE. MnBuOE (5.64 mg/mL) was injected subcutaneously in the dorsal intrascapular region. Following injection, 3-6 mL of blood was collected after 10 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 8 hours, 24 hours, 48 hours, 72 hours, 7 days, and 14 days into lithium heparin tubes (Vacutainer, 1.3mL). Whole blood was centrifuged at 1500 x g for 20 minutes, and plasma was collected and stored in 1% citric acid at -80°C.
Defining Maximally Tolerated Dose

The maximally tolerated dose (MTD) was defined as the dose of MnBuOE which did not induce clinically intolerable side effects in the dogs. This MTD dose would be used for the multiple dose pharmacokinetic study.

Multiple Dose Pharmacokinetic Study

The dosing regimen selected for the multiple dose pharmacokinetic study was based on the $t_{1/2}$ defined in the single dose pharmacokinetic study. Blood samples were collected prior to and one hour following administration of MnBuOE. Blood samples were processed and stored as described for the single-dose pharmacokinetic study. Pulse was counted manually prior to and one hour following administration of MnBuOE. This multiple dose pharmacokinetic study was performed twice to ensure plasma drug levels were maintained as predicted and that these results were reproducible. The first study involved a treatment period of 3 weeks, and the second study spanned a treatment period of 2 weeks leading up to euthanasia with necropsy and tissue harvest.

Samples from organs and tissues of interest were harvested during necropsy and placed over dry ice until stored at -80°C. Drug concentrations were analyzed via LC/MS-MS.
Histopathologic Evaluation

Tissue samples collected at necropsy were stored in 10% neutral buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin. Histopathologic evaluations of the tissues were performed by a veterinary pathologist (KB).

Measurement of MnBuOE in Plasma and Organs

Plasma and tissue processing

Organs were cryo-pulverized in a Bessman tissue pulverizer (BioSpec Products, Bartlesville, OK) under liquid nitrogen and then homogenized in a rotary homogenizer (PTFE pestle and glass tube) with 2 volumes of deionized water. An aliquot of either plasma or tissue homogenate was transferred into a 2 mL polypropylene screw-cap vial and a double volume of 1% HCl in methanol was added, agitated in FastPrep apparatus (Q-biogene, Carlsbad, CA) at speed 6.5 for 20 s, and centrifuged 10 min at 16,000 g. For plasma an aliquot of the supernatant was pipetted into a HPLC autosampler polypropylene vial and 80 µL of 0.1%HFBA in H2O was added. It was followed by another cycle of centrifugation for 5 min at 4500 g (4°C), after which the sample was immediately analyzed by LC-MS/MS. For other organs, an aliquot of the supernatant was pipetted into a 5 mL polypropylene tube (10 × 50 mm) and the solvent was completely removed in a Savant Speed-Vac evaporator at 40°C within 1 h. The dry residue was dissolved in a 20 µL of mobile phase B (see below) and sonicated for 5 min, then 80 µL of mobile phase B was added, the mixture was sonicated again for 5 min and centrifuged for 5 min at 4500 g at 4°C. Finally, the tube content was
transferred to the HPLC autosampler polypropylene vial equipped with silicone/PTFE septum, followed by another cycle of centrifugation for 5 min at 4500 g (4°C), placed in autosampler 4°C, and analyzed by LC-MS/MS.

**Liquid Chromatography-Tandem Mass Spectroscopy (LC-MS/MS)**

Quantitative analysis of MnBuOE was performed on a Shimadzu 20A series HPLC (LC) - Applied Biosystems MDS Sciex 5500 QTrap tandem mass spectrometer (MS/MS) at PK/PD Core Laboratory of Duke Cancer Institute. The use of HFBA as an ion-pairing agent increases overall lipophilicity/volatility and greatly improves retention and ionization efficiency of the analytes, affording an abundance of \([\text{MnP}^{5+} + 2\text{HFBA}]^{3+}\) and \([\text{MnP}^{5+} + 3\text{HFBA}]^{2+}\) ions. Solvents employed were: A = 9:1 water:acetonitrile (0.05% HFBA); B = acetonitrile (0.05% HFBA). Analytical column: 2 x Phenomenex AJ0-4287, C18, 4 x 3mm at room temperature. Elution gradient: 0-1 min 0-70%B, 1-2 min 70%B, 2-2.1 min 70-100%B, 2.1-2.6 min 100%B, 2.6-2.7 min 100-0%B. Run time: 4 min. Mass transitions used for quantification: \(\text{MnTnBuOE-2-PyP}^{5+}\) at m/z = 857.3/599 and \(\text{MnTnBuOE-2-PyP}^{5+}-d_8\) (internal standard) at m/z = 862.2/603.9. Calibration samples in 1–1000 nM or 0.1–10 μM range (depending on the expected levels) were prepared by adding known amounts of serially diluted pure standards into plasma or corresponding tissue homogenates and were analyzed along with study samples. Response was calculated as the ratio between the standard peak area and internal standard peak area.
RESULTS:

*Single-Dose Pharmacokinetic Study*

The complex PK profile obtained is presented in Figure 4.1B&C. Relevant PK parameters (e.g. $C_{\text{max}}$, $T_{\text{max}}$, AUC, clearance, $t_{1/2}$) were obtained by compartmental and non-compartmental modules within WinNonlin v. 2.1 software (Pharsight Corp. Cary NC) and are presented in Table 4.1. Plasma MnBuOE levels peaked at 30 minutes post-injection with a mean concentration of 3.993 $\mu$M (Figure 4.1B), followed by a complex tissue distribution profile (non-linear trace in log-linear plot) within first 6 hrs post injection (Figure 4.1B). From 6 to 48 h, however, a single exponential (linear in log-linear plot, Figure 4.1C) elimination process from the central compartment (e.g. plasma and weakly bound drug to plasma proteins, membranes, etc) was observed with the half-life $t_{1/2} = 7$ h, leaving only 1% of MnBuOE remaining in plasma. From 48h to 14 days (Figure 4.1C), a complex process (presumably a slow elimination from “deep” compartments) was observed with the estimated “terminal” half-life of 20 days. Selected PK parameters from compartmental and non-compartmental PK calculations are presented in Table 4.1. Compartmental (first order absorption – 2 compartment model) calculations including 0-48 h data was performed on the averaged PK profile for the purpose of obtaining PK parameters needed for the simulation of the multi-dose regimen.
Defining Maximally Tolerated Dose

Results at 1mg/kg: Administration of the 1mg/kg dose resulted in unexpected and unacceptable toxicities in all of the dogs. The first toxicity was an acute anaphylactic drug reaction which developed 15-20 minutes following injection of MnBuOE. This reaction was characterized by hyperemic mucus membranes, head shaking, urticaria, restlessness, and protrusion of the third eyelids. These clinical signs were resolved with treatment with an antihistamine medication (diphenhydramine, 2mg/kg). The second toxicity was a severe sinus tachycardia, which peaked 1 hour after MnBuOE injection. Average heart rates at this time point were 210 beats per minute (Figure 4.3A). By comparison, resting heart rate for these dogs averaged 88 beats per minute. This tachycardia persisted for at least 6 hours (Figure 4.3A), and pulses remained increased compared to resting heart rates 24 hours later (Figure 4.3B). The tachycardia was not associated with changes in blood pressure (Figure 4.4), nor was the tachycardia alleviated by the antihistamine medication (diphenhydramine) or intravenous fluid therapy (Figure 4.5).

Local drug reactions in the subcutaneous site of injection occurred with two of the dogs. One dog developed non-painful, mild hyperemia the time of injection, and this resolved without treatment 48 hours later. The other dog developed a non-painful, subcutaneous thickening 3 days after MnBuOE injection; this reaction resolved without treatment 4 days later. Alkaline urine (pH=9) was documented in two of the three dogs at 48 hours after MnBuOE administration (Table 4.2).
Results at 0.5mg/kg: The dose of MnBuOE was reduced to 0.5mg/kg and tested in one dog. MnBuOE was injected one hour after pre-administration of diphenhydramine (2 mg/kg), famotidine (0.5mg/kg), and prednisone (1 mg/kg). The anaphylactic reaction seen with MnBuOE alone did not occur with pretreatment with these drugs. Based on this observation, the remainder of the studies reported in this paper included pre-medication with anti-histamines and steroids. No local drug reaction at the injection site was noted. However, the dog still developed a moderate tachycardia following administration of the 0.5mg/kg dose, peaking at one hour after injection with an average of 144 beats per minute; the heart rate 24 hours later remained slightly increased (Figure 4.3A-B). This degree of tachycardia was deemed too high to justify using this drug at this dose in a companion canine lymphoma clinical trial.

Results at 0.25 mg/kg: No anaphylactic or local drug reactions were observed in the first dog treated with 0.25mg/kg, so this dose was repeated in the other two dogs. A mild increase in heart rate developed (range, 104-116 beats per minute) following injection, but had normalized by 24 hours (Figure 4.3A-B). This level of increase in heart rate was considered clinically acceptable for a canine lymphoma trial. Laboratory health assessments were repeated 48 hours following administration of 0.25 mg/kg MnBuOE. No remarkable changes in serum biochemistries were seen. Alkaline urine (pH=9) was seen in the one dog from
which urine was analyzed (Table 4.2). Based on these findings, 0.25mg/kg of MnBuOE was defined as the MTD.

Multiple Dose Pharmacokinetic Studies

MnBuOE was administered at 0.25 mg/kg Monday, Wednesday, and Friday (MWF). Dogs were treated concurrently with diphenhydramine (2 mg/kg every 12 hours), famotidine (0.5 mg/kg every 12 hours), and prednisone (1 mg/kg every 12 hours) throughout these studies. Blood/plasma was collected at two time points: 1 h post dose (expected high levels but “safely” away the peak at $C_{\text{max}} = 0.5$ h) and just prior to injection (trough level). The results are presented on Figure 4.6A. As expected, the trough levels measured were very low. An important finding is that no accumulation of the drug in plasma was observed during the entire long-term treatment. i.e. high levels never exceeded the value observed after the first injection (based on which the MTD-0.25 mg/kg was established). This would imply that no change in the expected mild acute side effects should be observed. Mildly to moderately increased heart rate one hour after each injection was observed (Figure 4.6B). The change in pulse increased over time for both treatment week and day of the week. Baseline pulse prior to injection was not altered with multiple doses of MnBuOE.

First study: Laboratory health assessments were repeated on the final day of the initial 3 week study. Mildly increased liver enzyme activity (mean ALP 203 IU/L, reference range 16-140 IU/L; mean ALT 133 IU/L, reference range 12-54 IU/L) and mild
hypocholesterolemia (mean 96 mg/dL, reference range 124-344 mg/dL) was documented in all three dogs, as well as a slight to mild anion gap metabolic acidosis (mean 21.5, reference range 11.2-19.9) (Table 4.2). Alkaline urine (pH=9) was reported for two of the three dogs. Prolonged treatment with prednisone induced shifts in white blood cell counts in a pattern consistent with administration of exogenous glucocorticoids (n=3/3). One of the dogs developed a urinary tract infection and was successfully treated with enrofloxacin (10 mg/kg every 24 hours for 10 days).

Second study: Following a 44 day wash-out period, the dogs were treated with the MWF protocol, beginning on a Monday, for a total of 5 doses of MnBuOE. Forty-eight hours after the last dose of MnBuOE, the dogs were euthanized (intravenous injections of acepromazine (0.2 mg/kg), butorphanol (2.0 mg/kg), and pentobarbital sodium (390mg/mL; 1mL/lb) and tissues were harvested.

**Histopathologic Evaluation**

Varying degrees of bronchointerstitial/interstitial pneumonia (n=3/3), hydropic degeneration of the liver (n=3/3), tubular degeneration and necrosis of the kidney (n=3/3), and necro suppurrative/granulomatous steatitis in the subcutaneous adipose tissue of the injection site (n=3/3) were identified (Table 4.2). Lymph nodes appeared mildly reactive, but this was not considered clinically significant.
**Tissue Drug Levels**

The highest recorded tissue drug levels were in the lymph nodes (3.98 - 5.99 μM), followed by the kidney and liver (2.58, 1.97 μM, respectively) *(Figure 4.6C)*.

**DISCUSSION:**

In this study, we obtained critical information regarding the PK and toxicity of MnBuOE in normal dogs as a prelude to a planned clinical trial in canine patients with lymphoma. Following a single dose of 1 mg/kg MnBuOE administered subcutaneously, the peak plasma drug concentration occurred at 30 minutes. The $t_{1/2}$ was defined as 7 hours via primary elimination and 20 days via other processes. A dosing regimen was determined according to the $t_{1/2}$, and the dogs were injected with MnBuOE at the MTD on a Monday, Wednesday, Friday schedule for 2-3 weeks during two multi-dose PK studies. At completion of the final multi-dose PK study, tissue drug levels were analyzed. The highest recorded tissue drug levels were in the peripheral lymph nodes (3.98 - 5.99μM), followed by the kidney and liver (2.58, 1.97 μM, respectively).

The most important result from single-dose 14-day PK study was that the 99% of drug was eliminated from plasma after 48 h. This implies that in a multi-dose treatment the same dose as initial may be given every 2 days without danger of drug accumulation above the established single-dose MTD. The data obtained from 48 h to 14 days suggest a slow elimination from multiple “deep” compartments (cell cytosol and organelles). Another
important result is that $C_{\text{max}} = 4 \, \mu\text{M}$ obtained in this study with dogs is much higher than the value observed in mice ($C_{\text{max}} \sim 0.3 \, \mu\text{M}$ [dose adjusted from 6 mg/kg in $^{235}$] and $C_{\text{max}} = 1 \, \mu\text{M}$ [1 mg/kg]), rats ($C_{\text{max}} = 1 \, \mu\text{M}$ [1 mg/kg], and non-human primates ($C_{\text{max}} = 1.6 \, \mu\text{M}$ [1 mg/kg, $^{235}$]). This can explain the unexpected serious side effects and the lower than expected MTD established in this study with dogs.

Based on the results from the multiple-dose PK study, it was confirmed that the plasma peak concentration is under control (no accumulation observed over time, Figure 4.6A) which suggests that no acute toxicity signs should worsen in the course of the long-term therapy. Tissue levels revealed accumulation of the organs with the drug. Particularly encouraging for this study are highest levels observed in lymph nodes. If plasma peak concentration were the only controlling factor for the acute toxicity, the observed accumulation would be only beneficial for the treatment. However, pulse data as well as laboratory assessments suggest that controlling plasma level is not sufficient and that long-term multiple-dose MTD should be lower than 0.25 mg/kg and/or frequency of dosing extended to once-weekly, depending on the application.

As we prepare to move toward testing the utility of adjuvant MnBuOE in the treatment of naturally occurring canine lymphoma, the finding that the highest drug levels were measured in the lymph nodes is particularly encouraging. The high tissue drug levels in lymph nodes has not been documented previously; this was the first animal study to perform such
measurements. Although it is a different cell type, we have reported a 3-fold higher accumulation of manganese porphyrins in the nucleus of macrophages compared to the cytosol\textsuperscript{236}. It is likely that MnBuOE is also accumulating in the nucleus of lymphocytes. Lymphocytes are a cell type with a very high nuclear to cytosolic ratio and they are tightly packed with a high cell density within the parenchyma of lymph nodes\textsuperscript{237}. The preferential accumulation of MnBuOE in lymph nodes is likely due to both the accumulation of the drug within the relatively large nucleus of lymphocytes compared to other cells and the high lymphocyte cell density in the lymph node. Although MnBuOE lymph node accumulation was recorded in normal, healthy dogs in this study, it is important to consider that manganese porphyrin compounds also accumulate in tumor tissue. Recently, we demonstrated that MnTE-2-PyP5+, a manganese porphyrin compound similar to MnBuOE, accumulates preferentially in tumor tissue compared to normal tissue\textsuperscript{204}. Evidence that manganese porphyrin compounds accumulate in both primary tumor tissue and lymph nodes strengthens the justification to use MnBuOE in a canine lymphoma trial. The lymph nodes of canine lymphoma patients will likely accumulate MnBuOE, as was seen with the normal dogs in this study, but also because they will be filled with tumor cells. For these reasons, patients with lymphoma may benefit substantially from adding adjuvant manganese porphyrin to chemotherapy protocols given its chemosensitization properties against lymphoma cells\textsuperscript{212,227}. The high lymph node drug level is also an important finding when considering other types of cancers which metastasize to lymph nodes; for these cancers, improved treatment outcomes may also arise from adjuvant MnBuOE.
There are many benefits to studying canine cancer patients in order to determine the clinical utility of adjuvant MnBuOE in treating lymphoma. Compared to rodent models, dogs develop a natural form of lymphoma which is similar to the human disease\textsuperscript{232,233}. Also, the progression of canine lymphoma from diagnosis until death is much longer than rodent models, which allows for more time for various levels of analyses. Without treatment, most dogs with lymphoma die of the disease within four to six weeks; however, with conventional CHOP-based chemotherapy protocols, complete remission is induced in approximately 60-90\% of dogs. Despite this first remission rate, the median survival times for dogs treated for lymphoma range from 6 to 12 months. This survival time is shorter than the human course of disease, and as such, clinical trials done on this time scale will lead to a quicker turn-around for results regarding patient treatment outcomes. Additionally, most dogs which develop lymphoma are older and have developed similar co-morbidities as the comparable human patient cohort. This presents an opportunity to screen for side effects in cancer patients that might not have been discovered during preliminary testing with younger, healthy research dogs. Finally, there are fewer restrictions in obtaining multiple clinical samples from canine patients compared to human patients, so a greater depth of analysis of underlying biology during a clinical trial may be possible.

The greatest obstacle in treating both human and canine lymphoma is the development of drug resistance. Treatment with adjuvant MnBuOE may be a way around this drug
resistance. The combination of manganese porphyrins and certain chemotherapy agents creates an environment of high oxidative stress within tumor cells. This pro-oxidant mechanism of chemosensitization by manganese porphyrins creates a scenario whereby lymphoma cells will be less likely to survive and develop drug resistance. This could lead to lengthened remission duration. Yet another advantage to performing comparative oncology studies in dogs is the ability to initiate a Phase I clinical trial prior to patients receiving and failing standard-of-care treatment protocols due to drug resistance. Given that phase I human lymphoma clinical trials typically are only able to evaluate treatment outcomes for patients whom have already failed frontline therapy, reports of improved clinical outcomes for dogs treated upfront with experimental therapies, such as adjuvant MnBuOE, could provide support for moving forward with subsequent human clinical trials. Such positive canine trials could also provide motivation toward introducing novel therapies earlier into the course of treatment for human patients.

MnBuOE has been shown to protect normal tissues from radiation and chemotherapy injury in laboratory studies, and human clinical trials have begun testing MnBuOE as a radioprotector. Supporting and complementary information regarding the protective use of MnBuOE against normal tissue damage may arise from comparative canine oncology trials. For example, a canine cancer clinical trial could present the opportunity to investigate the utility of MnBuOE as a cardioprotectant against doxorubicin. Doxorubicin is an anthracycline drug which is administered in standard chemotherapy protocols to treat
multiple human and canine cancers. It is known to have cumulative, dose-limiting toxic effects to the cardiac tissue of both species\textsuperscript{238,239}. While manganese porphyrin enhanced the cytotoxicity of lymphoma cells treated with chemotherapy drugs, Jaramillo et. al also found that MnTE-2-PyP5+ protected cardiomyocytes from doxorubicin toxicity \textit{in vitro}\textsuperscript{212}. Therefore, inclusion of MnBuOE into lymphoma treatment protocols may improve treatment outcomes for patients via sensitization of lymphoma cells to chemotherapy, particularly those harbored within the lymph nodes, as well as protection of the sensitive cardiac tissue from doxorubicin-induced toxicity - thus improving the therapeutic index.

Although there is more flexibility in performing a comparative oncology trial, it is imperative that the canine patients are treated safely and responsibly; after all, these dogs are companion animals. Therefore, before testing MnBuOE in canine cancer patients, it was necessary to understand the safety and optimal dosing regimen in normal dogs. The 1 mg/kg dose of MnBuOE induced an anaphylactic drug reaction and a severe, prolonged tachycardia. The acute drug reaction was prevented with premedications (steroids, anti-histamines) and the tachycardia was alleviated by reducing the MnBuOE dose. Neither intravenous fluid therapy nor anti-histamine medication affected the tachycardia (Figure 4.5), indicating that this toxicity is most likely a primary tachycardia and not secondary to hypotension. The MTD was defined as 0.25 mg/kg. Aside from a mild to moderately increased heart rate one hour post-injection that increased in severity over time, the dogs had no clinical evidence of toxicity throughout the multi-dose PK study. This change in heart rate throughout the multi-
dose PK study is most likely due to accumulation of MnBuOE in the cardiac tissue. The acute anaphylactic drug reaction and tachycardia post-injection have not been described in other species and may be specific to canines.

Laboratory tests performed throughout the studies identified changes to the organ systems functions of the dogs when treated with MnBuOE. Following treatment of MnBuOE as single doses of 1 mg/kg and 0.25 mg/kg, as well as prolonged treatment in the first multi-dose study, alkaline urine was documented (n=1-2/3). A slight to mild high anion gap metabolic acidosis developed in all three dogs following the three-week multi-dose PK study. These results combined are indicative of renal damage. One dog developed a urinary tract infection during the study; however, this was most likely secondary to introduction of bacteria into the bladder during urine collection via cystocentesis. To prevent the anaphylactic drug reaction associated with MnBuOE administration, dogs were treated with prednisone and antihistamine medications throughout the multi-dose PK study. Prednisone can induce changes in liver enzyme activity, and, in fact, mildly increased liver enzyme levels were recorded in the three dogs; however, mild hypocholesterolemia was also found in the three dogs, which may indicate that the changes in the liver are due to damage from the MnBuOE.

Histopathologic evaluation of tissues revealed mild to moderate inflammatory and degenerative changes in the kidney, liver, and lungs. Consistent with the laboratory results,
acute, mild to moderate tubular degeneration and necrosis of the kidney was reported (n=3/3), as well as marked hydropic degeneration of the liver (n=3/3). Interestingly, mild to moderate sterile bronchointerstitial/interstitial pneumonia was discovered (n=3/3). While MnBuOE causing these changes in the lungs cannot be ruled out, the characteristics of this pneumonia could also point to an acute injury from inhalation of a noxious cleaning agent from the kennel in which they were housed. Finally, mild necrosupporative/granulomatous inflammation was found in the subcutaneous adipose tissue of the injection site (n=3/3). These findings have not been reported in other species and may be specific to canines.

Again, because manganese porphyrin compounds accumulate in primary tumors and lymph nodes, it may be possible to reduce the treatment dose and/or frequency of administration of MnBuOE for a canine lymphoma trial while maintaining clinical efficacy in order to reduce or prevent the identified toxicities.

As a final point, the potential clinical benefit of manganese porphyrin compounds, such as MnBuOE, in human and veterinary medicine extends far beyond their use as cancer adjuvant drugs. The various derivatives of manganese porphyrin compounds, such as MnBuOE, have proven efficacious other diseases or conditions that have oxidative stress contributing to the pathology. Examples tested thus far include radiation induced pneumonitis and erectile dysfunction, diabetes, islet cell transplants, and spinal cord damage \(^{226}\). Now that the primary PK data and toxicities associated with MnBuOE in dogs have been determined, future studies
will involve optimizing safe dosing regimens so comparative canine clinical trials can be performed.
Figure 4.1: Pharmacokinetic profile of MnBuOE in dog after single subcutaneous injection. (A) Chemical structure of MnBuOE. (B) Concentration of MnBuOE in plasma during initial 12 h after single subcutaneous injection of 1mg/kg (in 100 mL saline) to 3 dogs (average line presented for visualization only). Absorption from subcutaneous space (T<sub>max</sub> = 30 min) is followed by a complex distribution into various organs/compartments. (C) A single (linear in log-lin plot) plasma elimination process, leaving only 1% drug remaining, starts after first 8 h and ends in 2 days, followed by a complex elimination profile, presumably from cellular compartments of various affinity for the drug, extending beyond 2 weeks.
Figure 4.2. Correlation of pulse measured by machine or manual counts. Pulse was quantified via machine (indirect blood pressure monitor) and manual counting (palpation or auscultation). There was a strong correlation (0.8973) between pulse quantified with the two methods. Pulse was quantified via manual counting for the multiple dosing experiment.
Figure 4.3: Pulse measured after single MnBuOE injection. (A) Subcutaneous administration of MnBuOE induced a dose-responsive acute and persistent increase pulse rate (n= 3/3). A severe tachycardia was documented following administration with 1 mg/kg MnBuOE. Reducing the MnBuOE dose to 0.5 mg/kg resulted in a mild to moderate tachycardia. Reducing the dose to 0.25 mg/kg MnBuOE increased the pulse rate but did not induce a clinical tachycardia. Dotted line indicates the defined acceptable resting pulse rate for the dogs. (B) Subcutaneous administration of MnBuOE induced a dose-responsive increase in pulse rate that persisted 24 hours post-injection. Treatment with 0.25mg/kg MnBuOE did not alter pulse rate 24 hours post-injection.
Figure 4.4 Variations in indirect blood pressure following administration of single dose of MnBuOE. Subcutaneous administration of MnBuOE caused subclinical fluctuations in recorded indirect blood pressures. Administration of MnBuOE at 0.25mg/kg resulted in the most stable maintenance of initial blood pressures.
Figure 4.5 Variations in heart rate and blood pressure following administration of single dose of MnBuOE. Dog 2 and Dog 3 were treated with diphenhydramine (2 mg/kg intramuscularly) upon the first evidence of anaphylactic drug reaction (20 minutes post-injection MnBuOE). Dog 3 was treated with 3 intravenous boluses of lactated ringer’s solution (10-12 mL/kg over 15 minutes). Tachycardia persisted following treatment with diphenhydramine (n = 2/2) and intravenous fluid therapy (n= 1/1). The effects of treatment with diphenhydramine and/or intravenous fluid therapy on indirect blood pressure measurements are unclear.
Figure 4.6: Tissues after 2-week multi-dose subcutaneous treatment. (A) MnBuOE was administered subcutaneously at 0.25 mg/kg every Monday, Wednesday, and Friday for 3 weeks. Plasma MnBuOE was measured pre-injection and 1-hour post-injection. MnBuOE concentration was highest following the initial dose (1.4mM) then plateaued with subsequent doses with a range of approximately 0.85 to 1.1 mM. Owing to 7 h half-life of the elimination and even shorter initial distribution-phase half-life, MnBuOE was essentially cleared prior to each subsequent dose; consequently, no plasma accumulation was observed. (B) Subcutaneous administration of MnBuOE at 0.25 mg/kg increased pulse rate one hour post-injection. When MnBuOE was administered Mon/Wed/Fri for 3 weeks, the change in pulse increased over time for both treatment week and day of the week. Pre-injection pulse rate was not altered with multiple dosing of MnBuOE. (C) Dogs received 0.25mg/kg MnBuOE Mon/Wed/Fri for a total of 5 doses prior to euthanasia. Tissues were harvested 48 hours post-last injection. Even after 48 hours, most tissues retained higher concentration of the drug than the C\text{max} in plasma, suggesting that the drug accumulates during the given dosing regimen. Drug levels were highest in peripheral lymph nodes (prescapular, submandibular, popliteal) and lowest in brain tissue.
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Table 4.1. PK Parameters. Non-compartmental parameter estimation module within WinNonlin software was performed on concentration/time sets from individual dogs after single 1 mg/kg dose to obtain critical PK parameters. Compartmental modeling (absorption + 2 compartments) using average single-dose profile was performed to estimate the PK parameters in order to simulate the multi-dose regimen. Since the 4-48 h process dominates, selected PK parameters are in very good agreement; complexity of the overall profile and scarcity of the early data precludes rigorous compartmental modeling of all the processes observed.
Alkaline urine (pH = 9) was documented following single doses of MnBuOE at 1mg/kg and 0.25mg/kg as well as following multiple doses of MnBuOE at 0.25 mg/kg.  Mildly increased liver enzyme activity (n= 3/3) and mild hypocholesterolemia (n= 3/3) was seen following 3 weeks of multiple doses of MnBuOE at 0.25 mg/kg and concurrent oral medications.  Slight to mild metabolic acidosis (n= 3/3) was also seen following this multiple dosing protocol. Single doses of MnBuOE (1 mg/kg, 0.25 mg/kg) and multiple doses of MnBuOE at 0.25 mg/kg did not alter blood cell volumes.  Three weeks of treatment with prednisone induced shifts in white blood cell counts in a pattern consistent with administration of exogenous glucocorticoids. Tissue samples were stored in 10% neutral buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin. Histopathologic evaluations of the tissues were performed by a veterinary pathologist (KB). Varying degrees of bronchointerstitial/interstitial pneumonia (n=3/3), hydropic degeneration of the liver (n=3/3), tubular degeneration and necrosis of the kidney (n=3/3), and necrosuppurative/granulomatous steatitis in the subcutaneous adipose tissue of the injection site (n=3/3) were identified.
CHAPTER 5

**Future Directions**

As with all projects, additional experiments could be performed to further investigate the findings of my thesis project. We have discussed pending results which will be completed to conclude publications; the ideas presented here are separate from those, and represent ideas for successive studies. Briefly, I will describe future directions which could be explored to continue these research efforts.

**Partial Tumor Radiation and Radiation Marginal Miss**

*Window Chamber Studies* - Our results characterized the local microenvironmental changes that occur following partial tumor radiation with a single microbeam (MRT) compared to non-irradiated and completely irradiated controls. We demonstrated that MRT induced unique vascular responses, increased HIF-1 expression, and the development of regional metastasis along the tumor-associated vessels. We hypothesized that these radiation bystander effects were occurring in the tumor rim via the generation of oxidative stress from the irradiated tumor region leading to increased HIF-1 in the sublethally irradiated tumor cells; however, we have not investigated differences in oxidative stress in the tumor microenvironment of the MRT, non-irradiated, and completely irradiated tumor samples. These formalin-fixed, paraffin embedded samples could be processed for 8-OHdG immunohistochemistry (IHC) as a marker of oxidative stress. If the IHC results are consistent with our hypothesis, we would expect to see increased 8-OHdG staining
throughout a completely irradiated tumor, within the irradiated portion of an MRT-treated tumor as well as radiating out through to the tumor rim, and reduced staining compared to the irradiated controls for the non-irradiated tumors.

In this study, a single fraction of 50Gy was administered to the window chamber tumor in the form of a microbeam or widefield radiation. This a very large dose of radiation to be administered as a single fraction and it is inconsistent with what would be prescribed to a patient's tumor in a clinical situation. Additional window chamber studies investigating the local effects of partial tumor radiation with a lower radiation dose are warranted.

Orthotopic tumor studies - It has been shown that the bystander effect can result in genetic changes and chromosomal instability in affected cells\(^92\). Additional experiments could be performed to determine alterations in the genome of cells in the sublethally irradiated tumor margin, the tumor cells which have metastasized to the lungs, as well as isolated circulating tumor cells. Information about the phenotype and genotype of these cells at each location (primary tumor, circulation, metastatic site) could provide important biological data for the changes occurring due to the radiation bystander effect at various the stages of tumor progression; specifically, we would determine whether the phenotypic and/or genotypic bystander effects are transient in these cells to facilitate and support cellular migration/invasion and metastasis or permanent changes. Another supporting animal experiment could be performed whereby tumor cells which have metastasized to the lungs
following radiation marginal miss are isolated and implanted into the dorsal mammary tissue of recipient mice. Through repeating the marginal miss experiment as described in Experiment C, it would be possible to see whether the tumor cells have maintained an increased aggressive phenotype/genotype and if distant bystander effects are enhanced compared to the cohort of mice with the naive 4T1-Luc cells.

**Tumor Microenvironmental Redox State Drives Distant Response to Radiation**

In Chapter 3, we discussed possible alterations that could be made to the radiation and MnP administration protocol that might change the outcomes of this study - specifically, that treatment of mice with orthotopic mammary tumors with MnP alone or in combination with radiation increased metastasis compared to saline-treated controls. Proposed changes included repeating the study but with an increased single fraction radiation dose, fractionated radiation protocol, increasing the MnP dose, and/or restricting administration of MnP to times before and shortly after tumor radiation.

While the results comparing HIF-1 expression in the primary tumors across the treatment groups are pending, a clear mechanism of these effects remain undefined. Based on previously published studies, antioxidant effects of MnP were associated with decreased HIF-1 expression in irradiated tumors. Therefore, MnPs affect both oxidative stress and HIF-1 in these experiments. In order to investigate the influence of HIF-1 on marginal miss effects separately, alternative agents which downregulate HIF-1 could be studied, such
as digoxin or CRLX101. Additionally, we hypothesize that the antioxidant properties of MnP are promoting survival and colonization of CTCs, leading to increased metastasis in our study. Recent reports have found that, in the presence of antioxidants, breast cancer and melanoma CTCs undergo a metabolic shift to support their survival as detached, circulating tumor cells \(^{207-209}\). To determine whether mice treated with MnP, with or without radiation, have CTCs with metabolic shifts which favor their survival compared with saline-treated controls, future experiments could be performed with the aim of isolating and characterizing the metabolism of circulating 4T1 cells. This could be done via FACS for 4T1 cells using endogenous markers such as EpCAM or a fluorescent reporter gene. Duke has a tumor metabolomics shared resource, which could be used to investigate any metabolic changes that might have been induced.

MnP enhanced metastasis compared to saline-treated controls when given as a single agent and when combined with a single fraction of radiation to bulky tumors; however, results from another experiment suggested that MnP may reduce metastasis when combined with surgical excision. MnPs may shift the redox status of tumor cells differently according to the microenvironment of a tumor which has been treated with surgery, radiation, and/or chemotherapy due to variability in oxidative stress the cells encounter. Variations in MnP treatment effects could be tested according to experiments simulating clinically-relevant cancer therapy protocols; for example, radiation to macroscopic vs. microscopic disease, chemoradiotherapy, or surgery followed by chemotherapy or chemoradiotherapy.
Potential for a Novel Manganese Porphyrin Compound as Adjuvant Canine Lymphoma Therapy

The pharmacokinetics and toxicity of MnBuOE has been defined in normal dogs. MnBuOE was associated with a number of toxicities; however, it was determined that the compound reaches very high drug levels in the plasma of dogs, leading to these toxicities and the relatively low maximally tolerated dose, compared with what has been seen in other species (mouse, rat, primate). Importantly, it was found that MnBuOE accumulates to a high degree in canine lymph nodes, an important result for using this drug as an adjuvant cancer therapy for a number of tumor types, particularly lymphoma. This preferential accumulation in lymph nodes has not been evaluated in other species, and thus represents an entirely new finding. This translates to the potential to reduce the dose and the dosing frequency of MnBuOE for canine patients with lymphoma.

However, based on the results of the 4T1 metastasis study, where MnP increased metastasis compared to saline-treated controls, great consideration must be taken before introducing MnBuOE or a derivative compound into a comparative oncology clinical trial with canine patients. Until additional preclinical studies can demonstrate consistent benefits of adjuvant MnP when combined with surgery, chemotherapy, and/or radiation without increasing distant metastasis, MnBuOE should not be administered to patients with cancers with a potential to metastasize. Multicentric lymphoma in dogs is not considered a "metastatic cancer", as it is a
hematologic malignancy, so the risks of adjuvant MnPs enhancing CTCs in this tumor type may not be clinically relevant.
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