An Investigation of Vascular Strategies to Augment Radiation Therapy

by

Ahmed Nagy El Kaffas

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Medical Biophysics
University of Toronto

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2014

Abstract

Radiation therapy is administered to more than 50% of patients diagnosed with cancer. Mechanisms of interaction between radiation and tumour cells are relatively well understood on a molecular level, but much remains uncertain regarding how radiation interacts with the tumour as a whole. Recent studies have suggested that tumour response to radiation may in fact be regulated by endothelial cell response, consequently stressing the role of tumour blood vessels in radiation treatment response. As a result, various treatment regimens have been proposed to strategically combine radiation with vascular targeting agents.

A great deal of effort has been aimed towards developing efficient vascular targeting agents. Nonetheless, no optimal method has yet been devised to strategically deliver such agents. Recent evidence suggesting that these drugs may “normalize” tumour blood vessels and enhance radiosensitivity, is supporting experiments where anti-angiogenic drugs are combined with cytotoxic therapies such as radiotherapy. In contrast, ultrasound-stimulated microbubbles have recently been demonstrated to enhance radiation therapy by biophysically interacting with endothelial cells. When combined with single radiation doses, these microbubbles are believed to cause localized vascular destruction followed by tumour cell death. Finally, a new form of ‘pro-angiogenics’ has also been demonstrated to induce a therapeutic tumour response.
The overall aim of this thesis is to study the role of tumour blood vessels in treatment responses to single-dose radiation therapy and to investigate radiation-based vascular targeting strategies. Using pharmacological and biophysical agents, blood vessels were altered to determine how they influence tumour cell death, clonogenicity, and tumour growth, and to study how these may be optimally combined with radiation. Three-dimensional high-frequency power Doppler ultrasound was used throughout these studies to investigate vascular response to therapy.

Keywords
Three-dimensional high-frequency power Doppler ultrasound, SU11248, bFGF, vascular normalization, Sutent, Sunitinib, Dll4 mAb, Notch, anti-angiogenic, vascular targeting, Delta Like Ligand, radiation, ionizing radiation, radiosensitization, ultrasound-stimulated microbubbles
Co-Authorships

The work presented in this thesis is based on the following manuscripts:

Chapter 1:

Chapter 2:

Chapter 3:
Submitted to Microvascular Research Journal: Ahmed El Kaffas, Anoja Giles, Gregory J. Czarnota, “Sunitinib Effects on the Radiation Response of Endothelial and Tumour Cells”, (Revisions; Microvascular Research MVR-13-37). AEK conducted all experiments under guidance from AG. AEK analyzed all data, except for in vivo clonogenic assays, which were counted by AG.

Chapter 4:
Submitted to PLoS ONE Journal: Ahmed El Kaffas, Joris Nofiel, Anoja Giles, Stanley K. Liu, Gregory J. Czarnota, "Delta-Like Ligand 4-Notch Blockade Synergistically Acts to Block Vascular Rebounds Following Ultrasound-Stimulated Microbubble and Radiation Therapy", (Revisions: PLoS ONE PONE-D-13-32157). SKL provided Dll4 mAb agent from lab and advice when planning/designing experiments along with AEK. Experiments and data collection were done by AEK, AG and JN. Analysis was done by AEK.
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I am grateful to Dr. Michael Kolios, who repeatedly committed to commuting for committee meetings. He has always inspired my scientific methodology. His deep insight and realistic optimism provided a source of balance throughout my graduate career. I wish to thank Dr. Bojana Stefanovic for her openness to trying new ideas, and her bold interest in understanding fields of studies outside her own. Dr. Stefanovic has always managed to bring interesting questions to committee meeting that have helped guide my work. I would also like to thank Dr. Martin Yaffe for his support and deep probing questions during discussions. Dr. Yaffe never failed to attend committee meetings in person, always bringing valuable insight.

Dr. Carl Kumaradas has been a key mentor that I must acknowledge. It was through him that I first discovered the biomedical sciences. In addition, I would like to thank Dr. Stanley Liu for the great scientific conversations, insight and career advice. I would also like to thank Mr. Firas Moosvi and Mrs. Melissa Yin for their relentless efforts to push for novel, high-risk, experiments on evenings and weekends, and for the good times.

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This thesis is dedicated to my wife and son, Salma Shehata and Rayyan El Kaffas, and my parents and brother, Nagy, Iman and Tarek El Kaffas. I would not be the intellectually curious and passionate individual that I am today without my parents and brother, and I simply would not have survived the past years without the constant love and support of my wife and son.
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<th>Definition</th>
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<td>SCID</td>
<td>severe combined immuno-deficiency</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>a human breast cancer xenograft cell line</td>
</tr>
<tr>
<td>LS174T</td>
<td>colon cancer xenograft cell line</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray (unit of radiation)</td>
</tr>
<tr>
<td>dB</td>
<td>decibels</td>
</tr>
<tr>
<td>kPa</td>
<td>kilopascals (unit of pressure)</td>
</tr>
<tr>
<td>AML</td>
<td>acute myeloid leukemia</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal uridine deoxynucleotidyl transferase dUTP nick end labelling</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>dB</td>
<td>decibels (logarithmic unit of measurement)</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous injection</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal injection</td>
</tr>
<tr>
<td>CD31</td>
<td>cluster of differentiation endothelial marker</td>
</tr>
<tr>
<td>ISEL</td>
<td><em>in situ</em> end labelling</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>MI</td>
<td>mechanical index</td>
</tr>
<tr>
<td>ASMase</td>
<td>acid sphingomyelinase gene</td>
</tr>
<tr>
<td>asmase</td>
<td>acid sphingomyelinase protein</td>
</tr>
<tr>
<td>CA9</td>
<td>carbonic anhydrase 9</td>
</tr>
<tr>
<td>Dll4</td>
<td>delta-like ligand 4</td>
</tr>
<tr>
<td>Dll4 mAb</td>
<td>delta-like ligand 4 monoclonal anti-body</td>
</tr>
<tr>
<td>USMB</td>
<td>ultrasound-stimulated microbubbles</td>
</tr>
<tr>
<td>XRT</td>
<td>radiation therapy</td>
</tr>
<tr>
<td>VTA</td>
<td>vascular targeting agents</td>
</tr>
<tr>
<td>VTS</td>
<td>vascular targeting strategy</td>
</tr>
<tr>
<td>AA</td>
<td>anti-angiogenic</td>
</tr>
<tr>
<td>VDA</td>
<td>vascular destructive agent</td>
</tr>
<tr>
<td>TCD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>tumour control dose 50%</td>
</tr>
<tr>
<td>CW</td>
<td>continuous wave</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
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Chapter 1
Introduction

“Science goes where you imagine it.”
-Judah Folkman

1.1 Thesis Overview

Folkman first hypothesized and subsequently demonstrated the importance of angiogenesis in tumour growth and metastasis. More recently, a series of studies have suggested that tumour vascular effects are necessary to maximize tumour response to radiation therapy. This has motivated researchers to ask fundamental questions in regards to the role of tumour vasculature in cancer therapy response. Knowledge derived from such questions could potentially lead to therapeutic strategies that target tumour vasculature above and beyond classic clonogenic tumour cell death. Through the use of drugs that target angiogenesis and biophysical agents that perturb existing vasculature, it may be possible to modulate tumour vasculature in order to optimize treatment response. In this thesis, I propose to study the regulating role of blood vessels in radiation-based tumour response to therapy, and to subsequently investigate novel combinatory vascular targeting strategies. Altogether, the aim of such combination therapies is to take advantage of the vascular response induced by these chemical and biophysical agents in order to strategically administer radiation therapy. Through studying how these treatments affect tumour microvasculature, it is intended to better understand the role of blood vessels in treatment response.

1.2 The Vascularised Tumour

The first recorded observation of tumour vasculature was made in 1863 by Rudolf Virchow. A series of similar observations were then described in following decades. It was in 1935 that the term ‘angiogenesis’ was first coined to describe blood neo-vessel formation in the placenta; likewise, ‘angiogenesis’ was used four years later to describe the same process in wound healing and in tumour growth. Although tumour blood vessels were beginning to be recognized as integral in tumour progression, it was not until later that Judah Folkman first articulated key hypotheses regarding the importance of vascular development in tumours. Along with others, he then subsequently demonstrated that tumour angiogenesis is necessary for tumour...
survival and growth past 2-3 mm³ in volume. Taken together, his contributions suggest that inhibiting angiogenesis and/or targeting tumour blood vessels in cancer therapy is an important avenue of cancer treatment research. Today, tumour angiogenesis is widely accepted as fundamental in tumour progression and metastasis, and as a hallmark of cancer.

1.2.1 Tumour Angiogenesis

Generally, angiogenesis refers to the process of new vessel formation within tissue (Figure 1.1). Vascular development in tumours occurs following the activation of the ‘angiogenic switch’: a biological signalling ‘switch’ leading to angiogenesis. However, unlike in normal tissue processes, the tumour ‘angiogenic switch’ remains active and minimally regulated throughout the lifespan of a tumour in order to support a rapid and continuously growing tumour mass. In fact, tumours have commonly been referred to as ‘a wound that won’t heal’.

Tumour angiogenesis occurs mainly via endothelial sprouting, and involves the up-regulation of vascular endothelial growth factor (VEGF), interleukin (IL-8), epithelial growth factor (EGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF). More specifically, the process starts with a degradation of the extracellular matrix surrounding endothelial cells to promote endothelial migration/sprouting. This process also includes the detachment of vascular pericytes, henceforth increasing vessel porosity, and affecting existing blood flow and vessel pressure. An endothelial ‘tip’ cell leads the sprouting of new vessels, while a ‘stalk’ cell follows and proliferates to form a lumen. Functionally, VEGF-based vessel formation is regulated by the NOTCH-1 pathway and expression of delta-like ligand 4 (Dll4) in tip cells. This process also serves to direct the vessel sprout towards the VEGF signal source. During the final stages of vessel formation, endothelial cells produce PDGF to recruit pericytes, leading to vessel stabilization. Other reported modes of tumour vascularization include: ‘cooption’ of neighbouring persisting vessels, intussusceptions (splitting of existing vessels into two or more vessels) and recruitment of circulating endothelial progenitor cells from the bone-marrow. Reports have also suggested that tumour cells themselves can act as blood vessels in a process called vascular mimicry; however, this remains a controversial topic.

Angiogenesis can be triggered by a combination of factors including: 1) hypoxia, 2) oncogene-mediated growth signals, 3) metabolic and/or mechanical stress, 4) genetic mutations,
Figure 1.1 - Angiogenesis via vessel sprouting. Angiogenic factors (VEGF) are first released, causing a tip cell to start sprouting a vessel towards the tumour. Tip cell regulates stalk cells via NOTCH-1 Dll4 signalling. Newly formed tumour vasculature will provide tumour cells with oxygen and nutrients to enable tumour progression.
and/or 5) hormones and cytokine. Tumour angiogenesis is dependent on tumour type, site, cell proliferation rate and the stage of disease. It is generally accepted that rapid formation of new blood vessels is necessary to meet oxygen and nutrient requirements of all malignant tumour types. The continuous proliferative nature of malignant tumour cells results in poorly regulated tumour angiogenesis; no balance is ever reached between pro- and anti-angiogenic signalling factors. Because of this, tumour blood vessels tend to differ significantly from normal tissue blood vessels (Figure 1.2 and Table 1.1). Structurally, tumour vessels are tortuous, dilated, and leaky, lack pericyte coverage and have abnormal basement membranes. Moreover, vascular density and vessel diameters are physiologically different in tumours compared to blood vessels in normal tissue. Such structural characteristics are associated with aberrations in local tumour blood flow and unusual fluid dynamics.

Common tumour functional abnormalities are also caused by the abnormal tumour blood vessels. These include increased interstitial fluid pressure, decreased tumour oxygenation and increased intratumoural metabolites contributing to the hypoxic and physiologically complex microenvironment characteristic of many tumour lines.

1.2.2 The Architecture of Tumour Vasculature

In normal tissue, blood vessels tend to be organized into arterioles, capillaries and venules. Blood flows from arterioles into the capillaries of the microcirculation. Capillaries have small diameters (5-10 µm) and thin vessel walls; these provide tissue with necessary oxygen and nutrients to survive. Circulating blood then returns to the heart via the venules. Arteriole-venule pressure differences and microcirculation flow resistance governs blood flow dynamics; flow resistance is a function of the vascular architecture and blood viscosity. In contrast, the vascular architecture of tumour vessels differs from healthy tissue vasculature in that it lacks the organization and dichotomous branching patterns of normal vessels. Tumour vasculature is composed of branches with uneven diameters, chaotic patterns of interconnection, and is often comprised of capillary dead ends and vascular shunts (Figure 1.3 and 1.4). As a result, a combination of biophysical factors (i.e. stress on vessels from proliferating cancer cells, vascular architecture, vessel maturation, dead-ends and shunts, etc.) cause an increase in resistance to blood flow in tumours. This of course decreases overall tumour perfusion rates (blood flow rate per unit volume) and can often lower blood velocity by an order of magnitude in comparison to
Figure 1.2 - Electron microscopy images of normal and tumour vasculature. Scale bar is 125 µm.
<table>
<thead>
<tr>
<th>Normal Vasculature</th>
<th>Tumour Vasculature</th>
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<tr>
<td>Well constructed walls with full pericyte and basement membrane coverage</td>
<td>Absent or detached pericyte covering - Absent or thickened Basement membrane</td>
</tr>
<tr>
<td>Responds to innervations - Can increase blood flow in response to demand</td>
<td>Minimal response to innervations – Endothelium relies on cytoskeleton to maintain integrity</td>
</tr>
<tr>
<td>Organize hierarchical vessel diameter and distribution</td>
<td>Random vessel diameter variability and heterogeneous vascular densities</td>
</tr>
<tr>
<td>Low interstitial flow pressure (normal)</td>
<td>Low pO2 tension and high interstitial flow pressure - Increased permeability/leakiness</td>
</tr>
<tr>
<td>Resistant to radiation therapy - Can withstand high depletion of endothelial cells (in arterioles)</td>
<td>Generally more sensitive to radiation. Vessels can easily become dysfunctional</td>
</tr>
<tr>
<td>Slow endothelial proliferation</td>
<td>Rapid endothelial cell proliferation</td>
</tr>
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**Table 1.1** - Table highlighting main features that differentiate normal and tumour vasculature.
normal vessels. As the 'angiogenic switch' remains on during tumourigenesis, the vascular network is constantly changing leading to large fluctuations in blood flow, and at times, a reversal in flow direction. Irregular distributions of vessel density throughout the tumour microenvironment contributes to acute, chronic and cyclic hypoxia, which in turn is a major cause of conventional cancer treatment failure.

### 1.3 Tumour Vasculature and Radiation Therapy

Radiation therapy is administered to more than 50% of patients diagnosed with cancer in North America. Treatments are usually administered using small, fractionated doses (1.8 - 2 Gy) to permit healthy tissue recovery. On a molecular level, radiation acts by directly damaging cellular DNA, and indirectly by releasing free radicals, which also cause DNA damage, thus hindering clonogenic cell proliferation and inducing cell death. Nonetheless, it has been demonstrated that tumour-derived cell lines respond to radiation differently in vitro than in vivo. This phenomenon is potentially attributed to the temporal and spatial complexity of the tumour microenvironment, which includes the host-derived stroma. Abnormal tumour vasculature leads to disruptions in oxygen and other nutrient diffusion into the tumour core, resulting in hypoxic radioresistant regions (Figure 1.5). Moreover, low-dose fractions of radiation may promote radioresistance by causing the secretion of cytokines such as VEGF and bFGF that act to protect endothelial cells from radiation effects and augment the complexity of the tumour microenvironment. Radioresistance has been associated with poor prognosis and treatment response. Taken together, an understanding of how radiation acts on the tumour as a whole (including host-derived stroma) is lacking; gaining a better understanding of this would result in new and more efficient cancer treatment strategies. Recent studies indicate that tumour blood vessels play a crucial role in tumour response to radiotherapy, prompting clinicians and researchers to further study how tumour vasculature regulates tumour response to therapies.

#### 1.3.1 Can Radiation Alone Target Tumour Blood Vessels?

The role of blood vessels in tumour responses to ionizing radiation has been a topic of debate in recent literature. Although it is recognized that radiation therapy affects endothelial cells differently than epithelial and tumour cells, it remains unknown how the irradiated tumour vasculature contributes to overall tumour response. Substantive evidence
Figure 1.3 – Artist illustration highlighting the main abnormalities in tumour vascular architecture. Pink represents in-flow, blue represents outflow vessels.
Figure 1.4 – Three-dimensional illustration of tumour vascular architecture. Tortuous vessels, and multiple shunts are shown. Taken together, this abnormal architecture causes poor tumour perfusion. Eliminating shunts causes a ‘normalization’ effect, subsequently increasing flow in the larger vessels.
**Figure 1.5** – A) Illustration of conventional and well understood radiation-based DNA damage. B) An illustration of the complicated tumour microenvironment, which leads to complications in tumour response to conventional radiation therapy. Radiation-based DNA damage is highly dependent on the presence of oxygen, while in the physiologically complicated tumour microenvironment, hypoxic regions are less sensitive to radiation effects.
suggests that tumour blood vessels may act to regulate tumour response to radiotherapy \(^{2,29-34}\), challenging the canonical notion that tumour response is primarily dependent on an inherent radiosensitivity of clonogenic tumour cancer cells. This paradigm shift originated in a controversial study by Paris \textit{et al.} \(^{35}\) which suggested that endothelial cells are the primary lesion during gastrointestinal (GI) irradiation, further leading to secondary viable cell damage and the development of the radiation-induced GI syndrome. Their data demonstrated that radiation doses between 8 and 16 Gy caused intestinal crypt stem cell lethality via reproductive cell death. This lethality was linked to early upregulation of acid-sphingomyelinase (ASMase) dependent endothelial apoptosis causing dysfunction of crypt blood vessel networks \(^{35-40}\). The authors further demonstrated that bFGF administration before whole body irradiation countered the ASMase-dependent apoptosis signalling pathway, protecting gut endothelial and epithelial cells \(^{35}\). It was later suggested that microvascular function regulates expression of radiation-induced crypt stem cell clonogen damage in the evolution of radiation injury to the GI mucosa \(^{37}\). However, other researchers have challenged this notion, demonstrating that other factors (i.e. p53) control the radiation-induced gastrointestinal syndrome, independent of endothelial apoptosis \(^{41}\).

These findings prompted researchers to question whether vascular dysfunction in tumours could also regulate tumour response to therapy. A short article by Folkman and Camphausen \(^{42}\) speculated on the question: ‘What does radiotherapy do to endothelial cells’? It was suggested that if the microvasculature is the primary target of radiotherapy in the intestine, as posited by Paris and colleagues, and if damage to epithelial stem cell is a secondary event, then such a relationship may hold even in tumours, where endothelial cells also support surrounding tumour cells. It was also suggested that the differing tumour sensitivity to radiotherapy \textit{in vivo} and \textit{in vitro} might be due to the presence of host derived supporting cells (including endothelial cells). In 2003, a second controversial study by Garcia-Barros \textit{et al.} \(^{29}\) investigated tumour response to single large doses of radiotherapy (>8-10 Gy). Their data suggested that damage to the microvasculature of the tumour takes place within 6 hours via an ASMase-dependent pathway, followed by tumour cell death secondary to vascular collapse, suggesting that tumour endothelial cells are strongly linked to tumour cell damage. The ASMase enzyme is present in endothelial cells up to 20-fold more than in epithelial and tumour cells, making it an important apoptotic pathway in endothelial cells (Figure 1.6). On the other hand, standard 2 Gy fractionated radiation
<table>
<thead>
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<tbody>
<tr>
<td>• Endothelial membrane (morphologic) alterations</td>
</tr>
<tr>
<td>• Increased endothelium membrane permeability</td>
</tr>
<tr>
<td>• Cytoskeleton reorganization</td>
</tr>
<tr>
<td>• Decreased cell capillary/tube-like structure formation</td>
</tr>
<tr>
<td>• Decreased cell motility</td>
</tr>
<tr>
<td>• Decreased cell adhesion</td>
</tr>
<tr>
<td>• Blood flow alterations</td>
</tr>
<tr>
<td>• Platelet aggregation</td>
</tr>
</tbody>
</table>

**Table 1.2** - List of reported radiation effects on endothelial cells \(^{43,44-47}\).
was suggested to be less effective due to hypoxia, reperfusion and reactive oxygen species formation \textsuperscript{2,48,49}. The exact mechanism of the endothelial-tumour cell linkage is still unknown. It was suggested that it may involve leakage of a circulating factor, a bystander effect secondary to endothelial damage, or transient local ischemia/reperfusion produced by the acute microvascular dysfunction and its rapid reversal \textsuperscript{36}.

A series of letters were published opposing this potential new paradigm \textsuperscript{50,51}. The primary criticism relies on data published in 1993 by Budach \textit{et al.} \textsuperscript{52}, which demonstrated that the TCD\textsubscript{50} of transplanted tumours in severe-combined immunodeficient (SCID) mice was not significantly different from that of tumours implanted in wild-type C3H/Sed or NCr/Sed mice. This finding is important because SCID mice are suggested to exhibit 2.5-3 fold enhanced stroma sensitivity to radiation therapy. These observations were interpreted to indicate that tumour cure by radiation is dominated by the inherent radiosensitivity of tumour cells and independent of tumour stroma. Gerweck and colleagues \textsuperscript{53,54} revisited this work, providing further evidence that host derived endothelial cells do not regulate tumour response to radiotherapy. As a reaction to this argument, a study recently published by Garcia-Barros \textit{et al.} \textsuperscript{31} investigated the above-mentioned concerns in \textit{asmase} knock-out SCID mice. Results indicated that the \textit{asmase}\textsuperscript{-/-} SCID mice were almost completely resistant to radiation therapy, supporting previous evidence that tumour response is regulated by tumour vasculature. In addition, their results suggested that the SCID mutation, which radiosensitizes host stroma, only does so for double strand break pathways. On the other hand, the ASMase-mediated apoptosis pathway (predominant in endothelial cells) was not enhanced in SCID mice when compared to wild-type mice, indicative of the potential importance of the ASMase pathway in endothelial apoptosis. A second important criticism suggested that the original Garcia-Barros \textit{et al.} (6) results might be caused by a host immune response. This speculation was derived from observations that the TCD\textsubscript{50} of MCA/129 fibrosarcomas (used in (6)) was 15 Gy, which is particularly low for tumour transplants. Furthermore, these implants grew 2-4 fold faster in \textit{asmase}\textsuperscript{-/-} than in \textit{asmase} \textsuperscript{+/-} littermates. This criticism was however refuted in 2004, when it was shown that MCA/129 and B16F1 melanomas do not elicit a host immune response in wild-type mice and that the \textit{asmase}\textsuperscript{-/-} phenotype is not deficient in antitumour immunity \textsuperscript{30}.
Membrane alterations caused by high doses of ionizing radiation (> 8 Gy) cause the enzyme ASMase to hydrolyze sphingomyelin into ceramide, which then acts as an apoptosis messenger. ASMase is present up to 20-fold more in endothelial cells than in epithelial and tumour cells; it can hence be an important apoptotic pathway in endothelial cells. Methods of suppressing this pathway include pre-treatment of vasculature with bFGF and/or VEGF as well as asmase knock-out.

**Figure 1.6** - Diagram of endothelial apoptosis pathway independent of direct DNA damage. Membrane alterations caused by high doses of ionizing radiation (> 8 Gy) cause the enzyme ASMase to hydrolyze sphingomyelin into ceramide, which then acts as an apoptosis messenger. ASMase is present up to 20-fold more in endothelial cells than in epithelial and tumour cells; it can hence be an important apoptotic pathway in endothelial cells. Methods of suppressing this pathway include pre-treatment of vasculature with bFGF and/or VEGF as well as asmase knock-out.
The role of blood vessels in radiation response is complicated. A paradigm shift from the conventional concepts of classic radiobiology is suggested and is likely to follow through considering the evidence described above. This new paradigm assigns a more prominent role to the tumour endothelium component in radiation response. It further suggests that there may be strategies, which combine radiotherapy with chemical and/or biophysical vascular targeting agents to enhance tumour response.

1.3.2 Combining Radiation with Vascular Targeting Agents

Vascular targeting agents (VTA) are currently under investigation as potent tumour radiosensitizers. These are categorized into two types of agents: vascular disrupting agents (VDA), and anti-angiogenic agents. VDAs disrupt tumour vasculature leading to vessel collapse or destruction, whereas anti-angiogenics inhibit the growth and development of new blood vessels. A vast amount of evidence suggests an additive or synergistic tumour response to radiation when combined with such agents. Furthermore, studies have shown that VTAs can cause endothelial radiosensitization in vivo and in vitro. However, the exact mechanism of action of these agents when combined with radiotherapy remains unknown. Altogether, such agents could be used in two ways: before or during radiotherapy administrated as tumour radiosensitizing agents, or immediately after irradiation as a ‘maintenance therapy’. When used as radiosensitizing agents, these could alter the complex tumour microenvironment, leading to a normalization of tumour blood vessels and enhanced oxygen perfusion. Alternatively, these could enhance endothelial radiosensitivity, leading to enhanced vascular response after radiation. When used after a radiation treatment as ‘maintenance therapy’, these would act to prevent reperfusion of tumours by blocking functional vessel development. Theoretically, these could also enhance the effects of a vascular destructive treatment by minimizing endothelial cell survival and/or proliferation. An overview of various proposed vascular targeting strategies is presented in Figure 1.7.
Figure 1.7 - Potential radiotherapeutic strategies for enhancing tumour response to treatments. Vascular destruction can be initiated if single radiation doses are greater than 8 Gy (left-hand panel). It may also be possible to radiosensitize the endothelium to induce vascular destruction at lower radiation doses by pre-treating with biophysical or chemical vascular targeting agents. On the other hand, vascular normalization through anti-angiogenic therapy may enhance direct DNA damage to tumour cells by increasing oxygen levels in the tumour (middle panel). Finally, vascular rebounds are often observed after therapy, which are associated with tumour reperfusion and tumour cell repair. Blocking these rebounds via a maintenance therapy may be an essential step to increase/sustain tumour damage (right-hand panel).
1.3.2.1 Anti-Angiogenic Agents

Following the discovery of VEGF \(^{62}\), a rapid wave of early anti-angiogenic agents were developed to specifically target elements of the VEGF pathway, in order to promote vascular regression and tumour starvation. A wide variety of anti-angiogenics are currently available. These include agents that are in use in the clinic, as well as agents that are at various stages of clinical trials or preclinical testing \(^{63}\). These target various aspects of molecular pathways involved in tumour angiogenesis. Most clinically approved anti-angiogenic agents target VEGF by binding and neutralizing it (i.e Bevacizumab), or act as small molecule tyrosine kinase inhibitors, which prevent the activation of a wide-spectrum of receptors including VEGF receptors (i.e Sunitinib). These act by inhibiting downstream signalling pathways rather than binding directly to VEGF \(^{18,64-67}\). Only a handful of these agents are currently FDA approved for treating specific cancer types. There are other less common approaches which include agents that target vascular basement membrane degradation, endothelial cell migration, endothelial cell proliferation and tube formation that have been actively considered \(^{18,27,65,68,69}\). However, the majority of ongoing research focuses on targeting vascular growth factors or their receptors. More recently, agents that target the regulatory process of angiogenesis have been shown to have potential anti-tumour effects. These agents act by targeting the NOTCH-1 signalling pathway, blocking Dll4 messaging, and have been termed pro-angiogenic agents \(^{70-73}\). These tend to promote endothelial sprouting, leading to non-functional vessels due to the lack of a controlled regulatory process. Agents targeting Dll4 (anti-Dll4) have recently been made commercially available, and are currently in clinical trials \(^{73}\). These have also been demonstrated to synergize radiotherapy when delivered weekly after single dose radiation therapy \(^{74}\). Evidence suggests that these agents may be an ideal maintenance therapy, and may be used to prevent functional vessel rebounds following vascular shutdown to minimize tumour reperfusion and repair.

In the case of anti-angiogenics delivered alone, disappointing clinical results have thus far been observed \(^{64,70}\). This has motivated researchers to begin considering how these agents could be combined with other conventional cancer treatments. Teicher et al. \(^{75}\) first advocated the use of anti-angiogenic drugs as a supplement to radiation therapy in an early Lewis lung carcinoma animal study, where they demonstrated that this combination achieved a significant tumour growth delay \(^{75}\). It was later demonstrated that there may be a more-than-additive effect when combining radiation with anti-angiogenic agents \(^{28,58}\). Following this, Jain suggested that
there may be a timing and dose window where anti-angiogenic agents act to ‘normalize’ the
tumour vasculature by balancing “pro” and “anti” angiogenic factors. The term vascular
‘normalization’ has been generally associated with the pruning (vascular remodelling process
involving regression of unnecessary/superfluous blood vessels) of immature and leaky vessels
involving a remodelling of the remaining blood vessels to more closely resemble normal
vasculature. This is characterized by less leaky, less dilated and less tortuous vessels and more
normal basement membrane and pericyte coverage, with functional increase in tumour perfusion
and decrease in flow resistance. Moreover, with exposure to the right doses of anti-angiogenics,
and for a specified time-window, vascular density and vessel diameter becomes more
physiologically similar to normal blood vessels. These changes are accompanied by functional
changes including decreased interstitial fluid pressure, increased blood flow and tissue perfusion,
increased tumour oxygenation and an improved penetration of drugs. It has been suggested that
the accompanying period of enhanced oxygenation would potentially correspond to enhanced
radiosensitivity of some tumours. A number of earlier studies have yielded data supporting the
‘normalization’ theory using DC101 and thalidomide, both anti-angiogenic agents. Another
study demonstrated that radiation therapy to glioma (U87) tumours orthotopically implanted in
rodents was enhanced when combined with anti-angiogenic therapy. A number of publications
have suggested a normalization window can last for up to 14 days following the start of anti-
angiogenic therapy delivery. Zhou et al. (2008, 2009) have demonstrated indirectly that tumour
perfusion is enhanced in SF188V+ human glioma xenografts 14 days after 20 or 40 mg/kg of the
anti-angiogenic Sunitinib. Hillman et al. (2009, 2010) have further shown with dynamic contrast-
enhanced MRI that administration of 20 mg/kg (up to 18 days) Sunitinib to KCI-18 model of
human RCC xenografts in nude mice yields tumour vasculature features similar to those
expected when blood vessels are ‘normalized’. These studies have linked the enhanced
radiosensitivity of tumours to enhanced tumour tissue oxygenation after anti-angiogenic
administration and a potential vascular normalization process.

1.3.2.2 Vascular Disruptive Agents

Vascular disruption encompasses a wide variety of treatments, including physical
treatments such as hyperthermia and photodynamic therapies, biological response modifiers (i.e
tumour necrosis factor and interleukins), certain chemotherapies (i.e arsenic trioxide) and various
ligand-based approaches, which bind to tumour blood vessels. However, in recent years, the term
VDA has been predominantly linked to small molecule VDAs\textsuperscript{21,56}. There exist two major classes of small molecule VDAs. Agents derived from flavon acedic acid (i.e. 5,6-dimethylxanthenone-4-acetic acid (DMXAA)), which act through a cascade of direct and indirect effects leading to tumour haemorrhages and heavy necrosis. More common however are a class of tubulin-depolymerizer, which have become increasingly popular over the past decade (i.e. CA4P, CA1P and OXi4503). Tozer \textit{et al.}\textsuperscript{56} recently reviewed the action of this second group of chemical VDAs. These agents predominantly target dividing endothelial cells leading to a rapid shut-down of the tumour vasculature by destabilizing blood vessel walls and causing endothelial apoptosis, or changes in morphology within hours after administration. Vascular damage leads to increased vessel permeability, haemorrhages into tumour tissue, blood coagulation and increased infiltration of immune effector cells into the tumour. On a cellular level, VDAs severely affect the cytoplasm of endothelial cells by binding to tubulin causing microtubule depolymerisation and activating actin stress fibers, and with extended exposure, can lead to anti-angiogenic effects such as decreased proliferation and migration. The extensive vascular closure caused by VDAs leads to secondary cancer cell death mostly in the core of tumours. However, the effects of VDAs are likely less severe in well-oxygenated tissues such as the rim of tumours or normal tissue, often leading to post therapy tumour cell repopulation. This has led researchers to attempt using these agents by strategically combining them with radiation therapy. The reasoning was as follows: whereas radiotherapy is successful at targeting cancer cells in well-oxygenated regions of the tumour (i.e. the rim), it is often much less effective in the hypoxic tumour core. Vascular disrupting agents have been demonstrated to predominantly target areas of the tumour, which are poorly perfused, likely because of the elevated number of proliferating endothelial cells, sparing most of the rim of the tumour. The use of VDAs may then complement radiation therapy by causing damage in the hypoxic regions of the tumour.

As an alternative to the above-mentioned chemical VTAs, biophysical agents are also being examined as potential vascular strategies for enhancing radiation treatment response in tumours – specifically for enhancing endothelial radiosensitivity. Ultrasound-stimulated microbubbles are such agents under investigation. Over the last decade, microbubbles have been recognized as potent contrast agents for medical ultrasound imaging. These consist of microsphere-shaped bubbles on the order of 3-4 \( \mu \)m, filled with air or gas, and stabilized by a thin shell of biocompatible material. A number of agents are now approved for clinical use. One
example is Definity® (Lantheus Medical Imaging; N. Billerica, MA), which is composed of perfluoropropane within a lipid shell $^{81-84}$. Acoustic microbubble disruption has been noted to also perturb the function of surrounding cells (primarily endothelial cells). Disruptions have been associated with bubble-induced cavitational microstreams and microjets, stress fields and shockwaves capable of destroying cells or permeabilizing their membranes $^{85-87}$. More recently, it has been demonstrated that causing microbubbles to oscillate near the surface of endothelial cells can activate angiogenic pathways $^{88}$ and alter physical properties of the cellular membrane $^{89}$. Moreover, it has been suggested that bubble microstreams could be controlled by altering the ultrasound field parameters $^{90}$, leading to localized stretching and opening or contraction of the cellular membrane. The shell materials of microbubbles have also been shown to affect cells differently, suggesting that these could be tailored for specific applications $^{85}$. Proposed applications for the microbubble-cellular interaction include permeabilizing the blood-brain barrier for drug delivery, permeabilizing cells to introduce therapeutic agents or genes, and treating intravascular thrombi. Recent investigations have demonstrated microbubbles as novel endothelial radioenhancers. The cellular-microbubble relationship can be exploited by treating tumours with ultrasound-stimulated microbubbles, followed by radiation therapy. Preliminary experiments indicate that tumours implanted in mice, pre-treated with microbubbles followed by single doses of radiation, yield a significant amount of cell death and a synergistic tumour growth suppression effect. It is hypothesized that these microbubbles cause endothelial cell apoptosis by releasing ceramide via an ASMase dependent pathway, and act synergistically with radiation to maximize response to radiation $^{91}$, leading to localized vascular destruction effects. This observed phenomenon is potentially more effective than the administration of chemical VDAs because ultrasound-microbubble treatments can be localized to specific anatomical sites (i.e. focusing ultrasound), while VDAs are administered as a drug and have been reported to induce side effects $^4$.

**1.3.3 Summary of Vasculature And Radiation**

Taken together, the above-mentioned studies support the notion that blood vessels are essential in how tumours respond to radiation therapy. Questions remain on how to optimally target tumour vasculature and whether it is more effective to ‘normalize’ tumour blood vessels to improve direct clonogenic cell kill, or whether it is better to aim for vascular destruction. Moreover, with the development of a vast number of chemical and biophysical VTAs, it will be
necessary to comprehend the advantages and disadvantages of each in order to optimally combine these with radiotherapy. In addition, it may be necessary to investigate differing strategies for different tumour types – depending on aggressiveness, stage, location and vascularity. Finally, designs of clinical trials involving radiotherapy and anti-angiogenic drugs have recently been published, which suggests that there is a growing interest in bringing this type of combination into clinical use\textsuperscript{85}. Various imaging modalities have been developed to visualize the response of blood vessels to vascular targeting strategies. These tools will play an important role in effectively characterizing the proposed radiation-VTA combination strategies. Imaging methods that can be used non-invasively offer new ways to assess response to vascular targeting strategies.

1.4 Pre-clinical Imaging of Tumour Response to Vascular Targeting Strategies

In order to assess the response of tumour blood vessels to radiation and anti-vascular treatment strategies, various imaging techniques have been developed or extended from existing modalities. Such imaging now permits the quantification and characterization of the shutdown of tumour blood vessels and their hemodynamic changes over a course of therapy. Each modality has its own advantages and disadvantages, which emphasizes the need to use multiple imaging techniques to understand the role of blood vessels in tumour response to radiation therapy vascular strategies. In clinical settings, dynamic contrast enhanced ultrasound, MRI and CT have been demonstrated to yield valuable information characterizing the tumour vasculature during VTA therapy\textsuperscript{92}. However, to uncover mechanisms of action and to comprehend the dependence tumour cells have on their vasculature, multi-imaging modality studies will likely be required in pre-clinical studies. For the studies conducted in this thesis, we have used three-dimensional high-frequency power Doppler ultrasound to assess vascular treatment response; this modality will be reviewed in section 1.4. In the following subsections, we briefly review the use of common imaging techniques to evaluate tumour vasculature response to anti-vascular strategies in pre-clinical models. A summary of the main attributes of each of these imaging modalities and others is presented in Table 1.3.
1.4.1 Optical Imaging

Light-based imaging techniques are recognized to have superior spatial resolution over most available imaging modalities. For instance, intravital microscopy (IVM) is capable of resolving down to sub-micrometers in living tissue \(^6\). However, these have a limited imaging depth penetration. These optical modalities are only capable of imaging the periphery of tissue at depths up to 1-2 mm. This limitation can be attributed to light scattering and absorption within tissue. Although improvements have been implemented, none has surpassed 2 mm in penetration. Techniques using IVM include confocal microscopy and multiphoton laser scanning microscopy. For vascular imaging, IVM typically employs a fluorescent molecular probe that can be detected optically. Depending on the nature of the investigation, one can choose the probe size and fluorescence wavelength for various structural, functional and biological imaging applications \(^9\). Another more recent, light-based imaging technique is optical coherence tomography (OCT). This modality is capable of imaging at slightly deeper depths in tissue than microscopy-based techniques. Although OCT is relatively less expensive and simpler to use than IVM, it is limited to imaging structural and some functional properties, but cannot image fluorescent probes. It relies on flowing light scatterers (intrinsic contrast) to reconstruct images of blood vessels using Doppler or speckle correlation methods \(^9\). However, the reliance of OCT on intrinsic contrast gives it an advantage over IVM methods, since it is less affected by the abnormal and leaky tumour blood vessels during which background saturation can occur rapidly due to extravasations of fluorescent markers \(^9\). Methods associated with IVM are ideal for assessing vascular normalization characteristics (i.e decreased permeability, pericyte and basement membrane re-arrangement, etc.). Nevertheless, OCT may prove useful in assessing vascular density and blood vessel sizes, length and branches in tumours throughout therapy delivery. However, because of their limited penetration depth, studies utilizing these modalities are mostly limited to window chambered tumour models.

1.4.2 MRI Based Techniques

Dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) is commonly used for assessing tumour vasculature in pre-clinical animal models. It is ideal for imaging active changes in hemodynamic parameters following irradiation schemes. Unique MRI contrast agents are chelated with gadolinium, generating powerful magnetic lattice fields. These then shorten the
T1 time (longitudinal-relaxation of water protons), yielding a unique contrast between tissue types in MRI images \(^81,95\). Common hemodynamic parameters that DCE-MRI can be used to measure are tumour perfusion and vascular leakiness. One can do so by collecting serial images of a region of interest while injecting a bolus of contrast agent. The changing intensities in the region of interest are then fitted to a mathematical model, which can yield information on perfusion, permeability and/or blood volume. DCE-MRI is ideal for characterizing both vascular destruction and vascular normalization strategies in conjunction with radiation therapy. It can yield both structural as well as functional information. Recent work used MRI to develop a vascular normalization index as a methodology for assessing tumour response to anti-angiogenic agents \(^96\). Such a vascularisation index would be very useful in assessing tumour response when radiation is combined with anti-angiogenic agents. Overall, DCE-MRI’s greatest limitation is accessibility. MRI scanners are not widely available, limited only to large medical and research institutes, and require extensive coordination for daily use in longitudinal animal studies.

1.4.3 Computed Tomography

Nuclear medicine modalities such as PET and SPECT have dynamic tracer imaging capabilities also very good for imaging various tumour vasculature hemodynamics \(^97\). Available parameters are obtained from dynamic changes in X-ray attenuation after intravenous injection of a contrast agent. Available parameters include, perfusion, permeability tumour perfusion, blood flow and/or mean transit time. The major limitations to CT-based methods are its continuous exposure to radiation, limiting its use in longitudinal studies, and potential spatio-temporal resolution. Micro-CT systems are also available for superior imaging specific for rodent studies. These are however not widely available. Nonetheless, the wide array of measurable vascular parameters make this modality good for assessing vascular response to radiation strategies, which target blood vessels.

1.4.4 Contrast-Enhanced Ultrasound Imaging

Ultrasound microbubble contrast agents are well suited for assessing tumour vascular perfusion and perfusion rates as they are confined within vessel walls, and can be detected in the smallest vessels of a tumour \(^98–100\). Contrast enhanced imaging can be performed in conventional Doppler mode, enhancing the Doppler signal from smaller blood vessels with slower flows, or in contrast-mode. Contrast mode imaging exploits the unique non-linear harmonics from resonating
<table>
<thead>
<tr>
<th>Modality</th>
<th>Resolution**</th>
<th>Temporal</th>
<th>Spatial</th>
<th>Sensitivity</th>
<th>Depth</th>
<th>Contrast</th>
<th>Assessment</th>
<th>Cost</th>
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<tr>
<td>PET</td>
<td>min-hours</td>
<td>1–2 mm</td>
<td>10^{-11}–10^{-12} mol/l</td>
<td>all</td>
<td>F-18, Cu-64, etc.</td>
<td>F, M</td>
<td>$$$</td>
<td></td>
</tr>
<tr>
<td>SPECT</td>
<td>min-hours</td>
<td>1-2 mm</td>
<td>10^{-10}–10^{-11} mol/l</td>
<td>all</td>
<td>Tc-99m, In-111, etc.</td>
<td>F, M</td>
<td>$$</td>
<td></td>
</tr>
<tr>
<td>micro-CT (DCE)</td>
<td>min-hours</td>
<td>0.05-0.8 mm</td>
<td>μm</td>
<td>all</td>
<td>Iodinated Tracer</td>
<td>A, F</td>
<td>$$</td>
<td></td>
</tr>
<tr>
<td>MR (DCE)</td>
<td>min-hours</td>
<td>10–100 μm</td>
<td>10–3–10–5 mol/l</td>
<td>all</td>
<td>Gd-DTPA</td>
<td>A, F</td>
<td>$$$$</td>
<td></td>
</tr>
<tr>
<td>HF-US (DCE) &gt; 20 MHz</td>
<td>sec</td>
<td>50-150 μm</td>
<td>Single bubble</td>
<td>mm (Freq Dep)</td>
<td>Microbubbles (Intravascular)</td>
<td>F, M</td>
<td>$$</td>
<td></td>
</tr>
<tr>
<td>HF-US (Doppler) &gt; 20 MHz</td>
<td>min</td>
<td>50-150 μm</td>
<td>~1-2 mm/s (clutter filter limited)</td>
<td>mm (Freq Dep)</td>
<td>Flowing RBCs (Intravascular)</td>
<td>A, F</td>
<td>$$</td>
<td></td>
</tr>
<tr>
<td>OCT (Doppler)</td>
<td>sec-min</td>
<td>1-5 μm</td>
<td>&lt; 1 mm/s (clutter filter limited)</td>
<td>1-2 mm</td>
<td>Flowing RBCs (Intravascular)</td>
<td>A, F</td>
<td>$</td>
<td></td>
</tr>
<tr>
<td>IVM</td>
<td>sec-hours</td>
<td>1-5 μm</td>
<td>Fluorescence Intensity</td>
<td>1 mm</td>
<td>Fluorescent , RBC</td>
<td>A, F, M</td>
<td>$$$</td>
<td></td>
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<tr>
<td>Bioluminescence</td>
<td>sec-min</td>
<td>60 μm-1 cm</td>
<td>~ 500 bioluminescent cells</td>
<td>cm</td>
<td>Luciferins</td>
<td>F, M</td>
<td>$$</td>
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</tr>
</tbody>
</table>

**Table 1.3** – Summary of important attributes of pre-clinical imaging modalities. Note that increased temporal resolution can result in decreased spatial resolution for most imaging modalities, and vice versa. Both types of resolutions are also affected when imaging 3D volumes as opposed to single 2D planes. (A – anatomical; F – functional; M- molecular)\textsuperscript{102–104}.
microbubbles to separate contrast agent scattering from tissue scattering\textsuperscript{101}. However, obtaining images of whole 3D tumour volumes remains a complex task, requiring a constant microbubble infusion through the whole 3D scan, or the use of a 3D matrix array transducer. Furthermore, microbubbles remain expensive contrast agents and are administered intravenously, which is difficult to implement in longitudinal studies requiring daily imaging. This also adds a few levels of complexity in studies involving a large number of subjects (animals).

1.5 Three-Dimensional High-Frequency Power Doppler Ultrasound for Assessing Vascular Targeting Strategies

High-frequency ultrasound (> 20 MHz) is an ideal and relatively inexpensive imaging modality for pre-clinical assessment of tumour vasculature in animal models. It is capable of yielding enhanced structural resolution and enhanced Doppler flow signal detection (see Figure 1.8). Its superior resolution over common clinical-frequency ultrasound devices (1-10 MHz) comes at the expense of image depth, which is less of an issue when imaging superficial xenograft tumours. Doppler based techniques and contrast enhanced ultrasound are now commonly used for assessing tumour responses to therapy\textsuperscript{105}. Doppler techniques rely on moving red blood cells to yield flow velocity estimates and tumour perfusion. However, techniques that allow for velocity estimation often have poor resolution and are more susceptible to aliasing artefacts\textsuperscript{106}.

Power Doppler (an extension of Doppler based methods) is capable of overcoming some of the limitations encountered with color flow ultrasound\textsuperscript{99,107–112}. It works by measuring the total integrated power of all shifted Doppler frequencies as opposed to an average estimate of shifted Doppler frequencies as in color flow imaging. The Doppler power associated with the Doppler frequency is dependent on the number of scatterers present (in this case, red blood cells). At high frequencies (> 100 - 20 MHz), power Doppler ultrasound detects blood flow velocities down to 1-2 mm/s and vessel sizes of 30-100 µm\textsuperscript{107,111}. For instance, a 25 MHz center frequency transducer can theoretically resolve vessels down to 200-150 µm and detect flow in vessels as small as 50 µm. A summary of some ultrasound-based vascular and blood flow imaging methods are presented in Table 1.4, and an overview of power Doppler is given in greater detail in Appendix A.
Compared to conventional clinical ultrasound frequency ranges, high-frequency power Doppler ultrasound has an increased resolution and is more sensitive to slow flow in small vessels. It is ideal in longitudinal studies involving a large number of animal-subjects, yielding information about vascular density, blood vessel sizes, vascular distribution through the tumour and vessel branching \(^{10,99,113-116}\). Of special interest is the vascularity index (VI), which is computed by obtaining the volume covered by all power Doppler signal over the total tumour volume:

\[
VI = \frac{\text{Power Doppler Signal}}{\text{Tumour Volume}} \times 100\%
\]

Three-dimensional, high frequency power Doppler ultrasound’s main limitation is that it is unable to detect the smaller blood vessels of the tumour microcirculation. On the other hand, changes in the smaller capillaries of the tumour (i.e. pruning dead-ends) will affect the overall microcirculation, and may then be detectable in the signal from the larger vessels. A number of studies have used power Doppler ultrasound to assess various aspects of tumour vascular response to several therapies \(^{99,107,117,118}\). Finally, ultrasound-based flow techniques have predominantly been used to study treatments that induce a vascular shut-down. As the technology continues to mature, and as computational power increases, there is no doubt that new and novel ways to process power Doppler data will be introduced, eventually permitting a comprehensive and rapid assessment of tumour vascular response to vascular targeting strategies (VTS), including vascular normalization.
Figure 1.8 – A representation of Doppler signal from a single voxel at low and high frequency ultrasound. At low frequencies, the clutter signal covers a large portion of the true blood signal, while at high-frequencies, true Doppler signal stands out more from clutter signal. This can be attributed to the different frequency-dependent scaling of blood scattering and tissue scattering.
<table>
<thead>
<tr>
<th></th>
<th>CE-US</th>
<th>Color Flow Doppler</th>
<th>Power Doppler</th>
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<td>2D/3D</td>
<td>2D – Limited 3D</td>
<td>2D/3D</td>
<td>2D/3D</td>
</tr>
<tr>
<td>Acquisition Speed</td>
<td>Time to perfusion</td>
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<td>2D &lt; 1min</td>
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<tr>
<td></td>
<td>3D ~ 8-15 min</td>
<td>3D ~ 8-15 min</td>
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</tr>
<tr>
<td>Flow Angle Sensitive</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Artefacts</td>
<td>Bioeffects</td>
<td>Aliasing</td>
<td>Tissue artefacts</td>
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<td></td>
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<td>Random noise</td>
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<td>Tissue artefacts</td>
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<td>Microbubbles</td>
<td>RBCs</td>
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<td>Longitudinal Studies</td>
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<td>Yes</td>
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<tr>
<td></td>
<td>Complex (catheter)</td>
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<tr>
<td>Flow Measurement</td>
<td>Tumour perfusion relative to baseline</td>
<td>Single vessel velocity &gt; 2 mm/s</td>
<td>Flow presence ~ 1 mm/s</td>
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<tr>
<td></td>
<td></td>
<td>Functional</td>
<td>Functional</td>
</tr>
</tbody>
</table>

**Table 1.4** – Various properties of ultrasound-based vascular and blood flow-imaging methods.
1.6 Thesis Outline

The aim of the work presented in this thesis is to study the role of blood vessels in treatment response, and to examine novel strategic methods of targeting tumour vasculature in radiation therapy delivery. The guiding hypothesis of this investigation is that tumour response to cancer therapies is regulated by its vasculature, and that radiation based vascular targeting strategies can enhance overall treatment response. Experiments were conducted in vitro, on cells, and in vivo, on rodents, to study the regulating properties of tumour vasculature in overall cancer treatment response. Endothelial cell response to radiation therapy and vascular organization was modulated using pharmacological and biophysical agents. Treatment response was assessed using three-dimensional high-frequency power Doppler ultrasound, tumour growth assays, clonogenic assays and immunohistochemistry staining. The specific objectives of this work were:

1. To study radiation-based vascular effects in a human xenograft cell line (breast cancer).
2. To determine the dose-dependent response of tumour vasculature to radiation therapy
3. To determine if there is a dose-dependent link between tumour blood vessel destruction and tumour cell death as a response to radiation therapy.
4. To determine if Sunitinib can act as an anti-angiogenic, tumour radiosensitizing agent, and to investigate its mechanism of action.
5. To investigate the temporal and spatial response of endothelial vs. tumour cells in treatment response.
6. To determine the efficacy of biophysical VDAs in a highly aggressive and highly perfused cancer cell line.
7. To conduct preliminary experiments combining biophysical VDAs with pharmacological VTAs. Specifically, to use an anti-Dll4, angiogenesis-deregulating, agent as a maintenance therapy following vascular destruction.

In the second chapter, we directly address aims 1-4. A vascular protecting agent is used to modulate radiation-based endothelial cell damage, subsequently permitting the study of tumour response as a function of the dose-dependent vascular response. We further investigated
Sunitinib as a vascular targeting agent, where we found evidence that it may normalize tumour vasculature, leading to enhanced tumour oxygenation and tumour response to single dose radiation therapy. In the third chapter of this thesis, we study in vitro isolated endothelial cells and tumour cells, in order to address aims 4 and 5. These studies allow us to gain a better understanding of the resulting effects observed in experiments conducted in chapter two of this thesis. Finally, we address aims 6 and 7 in chapter four, where experiments are conducted to first study ultrasound-stimulated microbubble (USMB) therapy in conjunction with radiation in a highly aggressive colon cancer cell line. We then report on combining USMB therapy with a Dll4 monochlonal antibody as a vascular maintenance therapy, preventing functional vascular rebounds following vascular disruption.
Chapter 2
Dose-Dependent Response of Tumour Vasculature to Radiation Therapy and Sunitinib Depicted by Three-Dimensional High-Frequency Power Doppler Ultrasound

"Rather than being an interpreter, the scientist who embraces a new paradigm is like the man wearing inverting lenses."
- Thomas Kuhn

2.1 Overview

In this chapter, results from an investigation of the relative contributions of direct cell killing by radiation versus tumour cell death due to radiation effects on the vasculature are presented. We also examine Sunitinib’s mechanism of action as a tumour radiosensitizer. To address these, MDA-MB-231 xenografts were treated with radiation doses of 2 to 16 Gy alone, or in combination with bFGF (endothelial radio-protector) or Sunitinib as pharmacological modulators of the vasculature. Sunitinib was orally administered for two weeks at 30 mg/kg before radiotherapy; bFGF was intravenously injected 1 hour prior to irradiation. Three-dimensional high-frequency power Doppler ultrasound was used to assess relative changes in tumour vasculature. Immunohistochemistry and tumour growth assays were used to quantify tumour response. Significant reductions in power Doppler signal of up to 50% were observed for 8 and 16 Gy treatments, along with a dose-dependent increase in cell death. No significant change in power Doppler signal and minimal tumour cell death were noted for tumours treated with radiation and bFGF. Treatments where Sunitinib was combined with radiation demonstrated a significant increase in flow signal at doses greater than 8 Gy. This was accompanied with a significant increase in cell death when compared to radiation or Sunitinib alone. We confirm that tumour response to high doses of radiation is regulated by tumour vasculature. We also posit that the response observed when radiation is combined with Sunitinib is linked to a vascular “normalization” effect.
2.2 Background

Radiation therapy is a primary therapeutic modality in cancer treatment. Although it is well recognized that radiation acts by damaging DNA in tumour cells, recent evidence suggests that host-derived endothelial cells present in tumours are linked to overall tumour response to radiotherapy. Studies have suggested that single large doses of radiation (> 8 Gy) primarily damage the microvasculature within hours of treatment via an ASMase-dependent pathway\(^2,29,31,35\). This is followed by vascular destruction and subsequent secondary tumour cell death, and is postulated to be an important mechanism of radiation-induced tumour kill \textit{in vivo}. Standard 2 Gy fractionated radiation may however elicit other effects due to hypoxia, reperfusion and reactive oxygen species formation \(^{48,49,119}\); these often include an increased tumour microenvironment heterogeneity associated with poor prognosis and treatment response \(^{25,26}\).

Rapid endothelial apoptosis due to high radiation doses has been linked to a 20-fold enrichment of the ASMase enzyme in endothelial membranes compared to epithelial and tumour cells. High doses of radiotherapy (> 8 – 10 Gy) are suggested to cause substantial damage to the endothelial cell’s membrane, leading to extensive hydrolyzation by the ASMase enzyme, in turn releasing ceramide to signal for apoptosis. At high radiation doses, endothelial cells can undergo apoptosis through two pathways: by DNA damage, or via a ceramide-signalling apoptosis pathway \(^{29,69,120–124}\). At lower radiation doses however (< 6 - 8 Gy), it is speculated that insufficient ceramide is released to induce rapid endothelial cell death. Conventional radiation doses are believed not to activate the ceramide-dependent apoptosis pathway. Basic fibroblast growth factor (bFGF) has been shown to inhibit ceramide-dependent messaging for apoptosis \(^{120,124–128}\). In comparison, epithelial and tumour cells are predominantly damaged through direct or indirect DNA damage, and undergo cell death or senescence only when they have accumulated adequate damage \(^{26,129,130}\). Clinically approved anti-angiogenic agents, such as Sunitinib, have recently been demonstrated to yield a synergistic tumour response when combined with radiation therapy \(^{59,131}\). This observation is potentially due to enhanced vascular radiosensitization following anti-angiogenic therapy. It is possible that such agents could be used to lower a required radiation dose to cause vascular destruction effects similar to that observed at high radiation doses.

Sunitinib is a small molecule tyrosine-kinase inhibitor, which prevents the activation of a wide-spectrum of receptors including VEGF receptors (PDGFR\(\alpha\), PDGFR\(\beta\), VEGFR1, VEGFR2, VEGFR3, FLT3, CSF-1R, RET). It acts by inhibiting downstream signalling pathways
rather than binding directly to VEGF \(^{66,132-134}\), and is currently one of the few FDA approved anti-angiogenic agents for the treatment of renal cell carcinoma and imatinib-resistant gastro-intestinal stromal tumours. Nonetheless, there are still questions in regards to how this agent should be administered, and whether it should be used alone or in combination with other forms of therapies (i.e. chemotherapy, radiation therapy) in order to cause additive or synergistic tumour responses. Furthermore, Sunitinib has been reported to induce a multitude of effects on tumour blood vessels and tumour cells, including \textit{in vivo} and \textit{in vitro} radiosensitization in addition to vascular ‘normalization’ effects \(^{18,59,60,66,68,133,135-138}\). Studies by Cuneo \textit{et al.} \(^{59}\) have shown that tumour cells can be sensitized to ionizing radiation at low doses given as single fractions when pre-treated with Sunitinib. Their work further demonstrated that combining fractionated radiation therapy with Sunitinib can yield a synergistic response. Recently, Yoon \textit{et al.} \(^{60}\) demonstrated that treating with Sunitinib before and after two doses of 10 Gy radiation yielded a synergistic tumour response demonstrated histologically, and a synergistic tumour growth delay. Nevertheless, the mechanism of synergy with radiotherapy remains unknown. Speculations indicate that it may be caused by pre-irradiation vascular normalization resulting in increased radiosensitivity of the whole tumour due to enhanced oxygenation. Alternative models include Sunitinib-induced pre-irradiation endothelial cell radiosensitization leading to complete vascular collapse or destruction at the time of radiotherapy, and subsequent secondary enhanced cell death in the tumour.

In this work, we have used three-dimensional high-frequency power Doppler ultrasound to investigate tumour vascular response to therapy, and complementary immunohistochemistry to assess cell death. High-frequency ultrasound is an inexpensive imaging modality for pre-clinical assessment of tumour vasculature in animal models \(^{107,111,139}\). At clinical frequencies (< 15 MHz), power Doppler ultrasound yields greater flow detection and resolution than most other Doppler based techniques \(^{107,111,140,141}\). At high frequencies (> 20 MHz), ultrasound yields enhanced structural resolution and enhanced power Doppler signal detection capable of detecting blood flow down to 1-2 mm/s and vessel sizes of 50-150 \(\mu\)m. It is well suited for longitudinal studies involving large numbers of animal-subjects \(^{113,117,142}\). When used in three-dimensions, power Doppler ultrasound measures flow signal from the whole tumour volume as opposed to single two-dimensional planes, a limiting factor often encountered in many imaging modalities and histopathology. High-frequency power Doppler ultrasound’s main limitation is that it is
unable to detect the smaller blood vessels of the tumour microcirculation (which can be as small as 5 µm in diameter). On the other hand, changes in smaller capillaries are often reflected in detectable blood flow signal in the tumour microcirculation. A careful observation of the power Doppler flow signal allows detecting such subtle changes in response to anti-angiogenic agents and vascular targeting strategies.

Here, we investigate the relative contributions of direct cell killing by radiation versus tumour cell death due to radiation effects on the vasculature 24 hours after irradiation. We also examine the mechanism of action when Sunitinib is combined with radiation. Experiments were conducted in severe combined immune-deficient (SCID) mice bearing xenografts of an aggressive breast cancer cell line, MDA-MB-231. Tumours were treated with radiation doses of 2 to 16 Gy alone, in combination with basic fibroblast growth factor (bFGF), or Sunitinib. Basic fibroblast growth factor is a polypeptide produced by several cells, fibroblasts and endothelial cells and is one of the putative factors inducing angiogenic and stromal response in hosts. It is involved in mitogenesis, angiogenesis, cellular differentiation and tissue repair, and has been demonstrated to protect endothelial cells from lethal effects after exposure to clinically relevant radiation doses.

Our rationale in using bFGF as an endothelial radio-protector is to reduce rapid endothelial cell death, which occurs predominantly via a ceramide signalling pathway after high-radiation doses. This would then allow us to confirm the role of blood vessels in regulating tumour response to radiotherapy and to better understand the relative contribution of direct cell killing by radiation versus tumour cell death due to radiation effects on the endothelium.

Experiments conducted indicate a decrease in power Doppler flow signal of up to 50% when tumours are treated with 8 or 16 Gy of radiation. A reciprocal dose-dependent increase of tumour cell death is also observed. Animals pre-treated with bFGF demonstrated minimal change in power Doppler flow signal and minimal amounts of tumour cell death at high doses of radiation therapy. This suggests that tumour response is directly linked to the tumour vascular response at such doses. Treatments where Sunitinib is combined with radiation demonstrate no significant change in flow signal at doses lower than 8 Gy. However, in contrast to radiation doses equal to or greater than 8 Gy administered alone, where we observed a decrease in power Doppler signal, combining such doses with Sunitinib yielded a significant power Doppler signal increase. An overall increase in dose-dependent cell death is also observed when compared to
Sunitinib alone or radiation alone. We suspect that Sunitinib may be inducing vascular ‘normalization’, leading to enhanced oxygenation and enhanced overall tumour response.

2.3 Material and Methods

Animal Preparation

All animal experiments presented in this work were conducted in compliance with internationally recognized guidelines specified in protocols approved by the Sunnybrook Health Science Centre Institutional Animal Care Committee. MDA-MB-231 breast cancer cells were cultured in RPMI 1600 culture medium (ATCC, Manassas, VA), 5 % fetal bovine serum (FBS) with antibiotics (penicillin and streptomycin: Life Technologies, Grand Island, NY) to full confluence and $1 \times 10^6$ cells were injected subcutaneously into the hind leg of SCID mice (Charles River Laboratories International, Wilmington, MA). Tumours were grown for a period of three weeks, reaching an average of 200 mm$^3$ before experimentation. Five animals per experimental condition were used (Figure 2.1).

Acute Treatment Response

Each experimental condition was comprised a single dose of ionizing radiation therapy alone, or combined with Sunitinib (Pfizer, New York, NY) or bFGF (R&D Systems, Minneapolis, MN). Animals bearing MDA-MB-231 breast cancer xenografts were treated with single radiation doses of 0, 2, 4, 8 or 16 Gy alone, or in combination with bFGF one hour before irradiation. Another group was given Sunitinib daily for 14 days prior to radiation delivery at a dose of 30 mg/kg prior to irradiation. A separate cohort of tumours was left untreated during Sunitinib administration in order to assess clonogenicity and tumour growth during that period. Similar Sunitinib doses have been demonstrated to cause minimal tumour damage while inducing a synergistic effect when combined with radiation therapy$^{60,131,145}$. Mice treated with the vascular protecting agent bFGF received an intravenous injection of 0.45 µg/ml one hour before irradiation$^{120,124,125}$. Radiation therapy was administered with a Faxitron cabinet irradiator (Faxitron Bioptics, Lincolnshire, IL) using X-rays at an energy of 160 kVp, an SSD of 35 cm, and a dose rate of 200 cGy/min. Animals were anesthetised with ketamine (100mg/kg), Xylazine (5mg/kg) and acepromazine (1mg/kg) before imaging and irradiation. For radiation treatment,
the body of each animal was covered with a lead shielding 3 mm thick, exposing only the tumour.

**Tumour Growth Assessment**

Tumour growth assessment was performed with another set of animals. An additional subset of animals pre-treated with Sunitinib followed by radiation, had continued Sunitinib administration 3 times weekly up to 30 days after irradiation as maintenance therapy. Tumours were measured twice weekly with a calliper up to 30 days after single dose radiation therapy, or until they reached a diameter of 17 mm (end point).

**Ultrasound Imaging**

All animals were imaged with three-dimensional high-frequency ultrasound immediately before treatment and 24 hours after treatment. Data was acquired using a Vevo 770 high-frequency ultrasound imaging device (Visualsonics, Toronto, ON) with a 25 MHz center frequency transducer (RMV-710B: 70 μm axial resolution, 140 μm lateral resolution, focal length of 15 mm). A motorized scan stage (Visualsonics, Toronto, ON) was used to acquire 3D B-mode images and power Doppler flow data, at a step size of 0.1 mm. Power Doppler data was collected with the following optimized settings: a clutter-filter cut-off of 2 mm/s, a scan speed of 2 mm/s, a pulse repetition frequency of 5 KHz, a power Doppler gain of 20 dB and a frame rate of 10 fps). Power Doppler image analysis was conducted using in-house developed software in MATLAB (The MathWorks, Natick, MA). The vascularity index (VI) was computed from power Doppler images by obtaining the volume of all colored objects (colored pixels) over the volume of the selected ROIs. A relative vascularity index was used to assess overall vascular response to therapy, computed as follows: \((\text{VI}_{24 \text{ Hours}} / \text{VI}_{0 \text{ Hours}}) \times 100\).

**Histological Analysis**

Mice were sacrificed 24 hours after irradiation for immunohistochemistry and clonogenic assays. Cross sections of tumour xenografts were stained for DNA breaks using *in situ* end labelling (ISEL) stain as a cell death marker and cluster of differentiation (CD31) (Santa Cruz Biotechnology, Santa Cruz, CA) to evaluate vascular density. A subset of animals treated with Sunitinib only \((n = 5)\) was stained for \(\alpha\)-smooth muscle actin \((\alpha\text{-SMA}; \text{pericyte cell marker})\) and
Figure 2.1 – Overview of experiment. A) Tumour cells are injected and allowed to grow for an average of 21 days. Animals receiving radiation or radiation + bFGF are imaged, treated and imaged again 24 hours later, immediately before sacrifice. Sunitinib treated animals are gavaged with the drug from 14 days before imaging and radiation delivery. B) Imaging uses a custom set-up, where the hind leg of animals is emerged in a water bath to allow for three-dimensional data collection.
carbonic anhydrate 9 (CA9). A percentage of cell death was evaluated for each of the tumour cross-sections stained with ISEL. Similarly, a percentage of hypoxia was evaluated for each of the tumour cross-sections stained with CA9. Pericyte coverage was qualitatively assessed with α-SMA. The vascular density was computed from images of CD31 stained tumour cross sections. Three cross-sections per tumour were stained. For each of the cross sections, 4 fields of view (FOV) were analyzed with a 10X objective lens to compute the density of vasculature for a total of 12 FOVs per tumour, using automated software. To do so, we have developed a toolbox in Matlab which distinguishes the brown stained regions (CD31 staining of endothelial cells) in the tumour from the background stain (other cells) within a user selected ROI and computes the vascular density per chosen ROI.

**Statistical Analysis**

Data is presented as mean ± standard error of the mean (SED). Quantified VI, ISEL, CA9 and CD31 staining, colony counts and tumour volume changes were evaluated for statistical significance using a Mann-Whitney test (two-tailed, assuming unequal variances; α = 0.05* or α = 0.01**). Each treatment condition was compared directly to the 0 Gy control condition. Statistical tests were conducted using Prism (GraphPad Software version 5, La Jolla, CA).

### 2.4 Results

*Power Doppler Ultrasound and CD31 Immunohistochemistry Analysis*

Power Doppler data of *in vivo* tumours before and after treatment indicated that single large doses of radiation equal to or greater than 8 Gy caused a significant decrease in the flow signal. Figure 2.2 presents the maximum intensity projection of representative tumour volumes captured with 3D high-frequency power Doppler ultrasound. In figure 2.2B, the relative change in VI at 24 hours for a range of radiation doses is exhibited for all conditions. The data demonstrates no significant changes detected with power Doppler ultrasound for the 0, 2 and 4 Gy conditions, while a decrease of approximately 50% in relative detectable flow for the 8 Gy and 16 Gy (p < 0.01) conditions was observed. Experimental conditions where radiation was used in combination with bFGF or Sunitinib resulted in different effects from those using radiation alone. As anticipated, bFGF acted as a vascular protecting agent, and caused only minimal deviations in the relative VI (no drop in the power Doppler flow signal) when combined
with 0, 2, 4, 8 or 16 Gy radiation therapy. Sunitinib treatments caused a slight increase in the VI at 2 and 4 Gy, while a significant increase in VI at radiation doses equal or greater than 8 Gy was observed.

Power Doppler results were supported with CD31 micro-vascular density analysis, which demonstrated an acute significant decrease in the micro-vascular density at doses greater than 8 Gy (Figure 2.3) for radiation only conditions. Similarly, no vascular decrease was observed for 8 Gy when bFGF was administered before irradiation. However, Sunitinib treatments caused an overall decrease in the micro-vascular density in tumour cross-sections, which was minimally affected by the administration of radiation therapy.

**Cell Death Assessment using ISEL Staining**

Representative images of ISEL stained tumour cross-sections and their quantification are presented in figure 2.4. A dose dependent increase in staining was observed for radiation-only conditions. The observed increase was determined to be significant past the 8 Gy treatment condition (p < 0.05). Quantified ISEL staining in animals pre-treated with bFGF was consistent across all radiation conditions, indicating minimal tumour cell death. Sunitinib on its own appeared to cause cell death (p < 0.05), which was significantly enhanced when combined with radiation therapy compared to radiation or Sunitinib alone (p < 0.01). Despite the non-significant VI changes for Sunitinib treated conditions at low doses, a statistically significant increase in cell death was observed for all radiation doses combined with Sunitinib versus radiation alone.

**Tumour Growth Assessment**

Figure 2.5 displays tumour growth curve of treated tumours for up to 30 days. The percent change in tumour volume 15 days after radiation therapy is plotted in figure 2.6. Data indicate that tumour growth is significantly slowed down when treated with a single large dose of 8 Gy (p < 0.05). Pre-treating animals with bFGF however seemed to protect tumours from the effect of radiation therapy, causing no significant slowdown in tumour growth. Administering Sunitinib for two weeks prior to irradiation caused a slow-down in tumour growth (see curve before arrow in bottom left graph). The average tumour volume was 276 mm$^3$ following 14 days of Sunitinib administration. Non-treated control tumours left to grow in parallel with those receiving Sunitinib reached an average tumour volume of 630 mm$^3$. We have also noted that
Figure 2.2 - A) Representative maximum intensity projections of a tumour volume at 0 hours and 24 hours after treatment with 16 Gy radiation. Data was obtained with 3D high-frequency ultrasound. A decrease in total power Doppler flow signal (blood volume detected) can be observed 24 hours after treatment. Red color represents the lowest power Doppler intensity (15 dB) while yellow represents the highest power Doppler intensity (40 dB). The scale bar represents 2 mm. B) Relative change in VI at 24 hours for a range of radiation doses (0, 2, 4, 8 and 16 Gy) alone, in combination with bFGF or Sunitinib. Results indicate that there is a significant decrease in power Doppler flow signal only when tumours are treated with single doses of 8 Gy and 16 Gy. This signal decrease is almost 50%. No other statistically significant differences (compared to 0 Gy control) are observed. However, tumours pre-treated with bFGF appeared to be completely unaffected by these radiation doses. Furthermore, pre-treating with Sunitinib also appeared to cause no significant change in VI at radiation doses lower than 8 Gy, although a significant increase in flow signal was observed in animals treated with 8 or 16 Gy (p < 0.05). Each experimental condition is representative of n=7-9 animals. Error bars represent standard error. Statistically significant is indicated with * at p ≤ 0.05 or ** at p ≤ 0.01.
Figure 2.3 - A) Representative images of tumour sections (treated with 0, 2 or 8 Gy alone, or in combination with Sunitinib) stained with CD31. We observe diminishing amounts of vascular staining with single large doses of 8 Gy, in agreement with our power Doppler data. Sunitinib changes the appearance of the blood vessels causing them to be thinner and more similar to normal vasculature. A decrease in vascular density is also observed. Combining Sunitinib and 8 Gy radiation does not cause an apparent qualitative effect. The scale bar represents 200 µm. B) Quantified vascular density from CD31 staining of tumours treated with 0, 2 or 8 Gy alone, or in combination with bFGF or Sunitinib. CD31 results confirm that a single large dose of radiation (8 Gy) does impact the tumour vasculature by decreasing the vascular density. The decrease in vasculature caused by Sunitinib alone however does not change with radiation therapy, consistent with the average VI obtained from power Doppler data. Furthermore, bFGF seemed to also protect the vasculature of the tumour, evidenced by minimally deviating the vascular density measurements for all radiation doses. Error bars represent standard error. Statistically significant are indicated with * at p ≤ 0.05 or ** at p ≤ 0.01.
Figure 2.4 - A) Representative images of tumour sections (treated with 0, 4 or 16 Gy alone, or in combination with Sunitinib or bFGF) stained with in situ end labelling (ISEL) for cell death. We observed increasing amounts of cell death with increasing radiation doses. There appears to be lesser amounts of ISEL staining when 16 Gy radiation is combined with bFGF than 16 Gy alone. Treatments with Sunitinib alone demonstrate cell death staining equivalent to 16 Gy alone. However, combining Sunitinib with radiation appears to enhance cell death significantly. The scale bar represents 1mm. B) Quantified ISEL staining data for all 15 treatment conditions. A dose dependent increase in cell death was observed for radiation only conditions. This was significant at doses greater than 8 Gy (p < 0.05). This effect did not occur in bFGF treated animals. Sunitinib treatment caused the most significant amount of cell death when combined with radiation therapy, covering at times more than 40-50% of the tumour cross section at doses greater than 8 Gy. Error bars represent standard error. Statistically significant are indicated with * at p ≤ 0.05 or ** at p ≤ 0.01.
discontinuation of Sunitinib caused tumours to immediately begin growing with a similar trend to control tumours.

Vascular rebounds have recently been reported in literature. A growth delay was observed when animals were treated with 8 Gy and 2 Gy radiation therapy following Sunitinib treatment; this was significant only for the 8 Gy condition (p < 0.05) at 15 days (Figure 2.6). Continuing the administration of Sunitinib post-irradiation had the most drastic effect. Here, a significant slowdown was achieved at 2 Gy (p < 0.05) at 15 days after radiotherapy (Figure 2.6), while an eventual regrowth was observed (Figure 2.5). From figure 2.6, no tumour growth was observed 15 days after irradiation when 8 Gy was administered in conjunction with Sunitinib; similar observations were made when Sunitinib was continued after 8 Gy. However, in figure 2.5, comparing the Sunitinib and 8 Gy condition to the same with continued Sunitinib administration, we have found that there is a greater anti-growth effect when Sunitinib is continued following radiotherapy. Here, tumour growth was observed following Sunitinib and 8 Gy (at approximately day 30), while no tumour growth was observed when Sunitinib was continued as maintenance therapy for the duration of the tumour growth experiment.

**Pericyte and Tumour Oxygenation Assessment**

Figure 2.7 displays representative images of CD31, α-SMA and CA9 staining (A) and average quantification of CA9 staining in control and Sunitinib treated animals (B). Images are of the exact same blood vessel stained with CD31 and α-SMA in control and Sunitinib conditions. A qualitative observation of the α-SMA in control animals indicates abnormalities in perivascular cell morphology in our MDA-MB-231 tumour xenografts, this includes irregular arrangement and loose coverage of blood vessels. In Sunitinib treated animals, pericyte coverage appears to be more ‘normal’ – a more circumferential and tighter coupling to tumour blood vessels (as marked by CD31). Quantified CA9 staining of Sunitinib and control tumour cross-sections results in a decrease in the average staining in the Sunitinib treated animals.

**2.5 Discussion**

This study aimed to investigate the acute response of tumours in relation to tumour blood vessel responses to a single radiation dose. We modulated the tumour vascular response to ionizing radiation by administering or inhibiting growth factors involved in endothelial cell survival. This permitted the assessment of the relative contribution of direct tumour cell killing
Figure 2.5 - Long-term tumour growth response to radiation alone, or in combination with bFGF or Sunitinib. Conditions include radiation at 0, 2 or 8 Gy alone, or in combination with bFGF or Sunitinib. A supplementary condition, where Sunitinib is continued following irradiation as maintenance therapy, is also presented. Sunitinib maintenance therapy was administered three times weekly at 30 mg/kg following the initial 14 day dose and radiation treatment delivery. We find a growth delay when animals are treated with 8 Gy radiation therapy alone (top left graph). However, this growth delay was not observed for animals pre-treated with bFGF (top right graph). Treatments where bFGF is administered alone demonstrate an accelerated tumour growth compared to control animals. We noted that Sunitinib alone caused a slow-down in tumour growth (see curve before arrow in bottom left graph), and that the discontinuation of Sunitinib caused tumours to immediately begin growing with a similar trend to control tumours. Unlike radiation alone however, administering 2 Gy following Sunitinib therapy caused a significant slow-down of tumour growth. Administering a single 8 Gy dose following radiotherapy blocked tumour growth for nearly 15 days. The use of Sunitinib as a maintenance therapy greatly enhanced tumour growth delay for all three radiation conditions. Furthermore, we observed no tumour growth for the 8 Gy condition. Black arrow indicates day of radiation administration. Radiation and Radiation + bFGF conditions have a baseline of 8 days before radiation treatment. Sunitinib conditions have a baseline of 16 days (which include 2 days of baseline followed by 14 days of Sunitinib administration) followed by irradiation on the 16th day.
Figure 2.6 - Percent change of tumour growth 15 days after radiation therapy administration. Conditions include radiation at 0, 2 or 8 Gy alone, or in combination with bFGF or Sunitinib. An extra condition is added to this growth assay, where Sunitinib administration is continued (three times weekly) after the initial 14 day dose and radiation therapy. Results indicate that Sunitinib maintenance therapy seemed to have the most effect on tumour growth for all three radiation doses. Tumours treated with Sunitinib and 8 Gy alone, or through maintenance therapy, caused a near complete cessation of tumour growth over the 15 day period. On the other hand, combining Sunitinib with 2 Gy radiotherapy seemed to slow down tumour growth by half the time. Finally, bFGF seemed to protect tumours from radiotherapy, allowing for continued tumour growth. Error bars represent standard error. Tumours treated with Sunitinib for two weeks had their tumour volumes remain at an average of 250 mm$^3$, while non-treated control tumours grew by a factor of 2 (see supplementary data). Statistically significant are indicated with * at p ≤ 0.05 or ** at p ≤ 0.01.
Figure 2.7 - A) From left to right: CD31, α-SMA and CA9 staining of Sunitinib treated and control tumours. Arrows indicate the same blood vessel as delineated with CD31 staining in treated vs. control conditions in order to assess α-SMA coverage. We observe an abnormal arrangement of the pericyte coverage in control animals, which appears loose and irregular. Contrary to this, Sunitinib treated animals appear to have a more ‘normal’-like pericyte coverage, and there is an increase in the amount of micro-vascular coverage. B) CA9 quantification indicates an increase in oxygenation (decrease in CA9 hypoxia staining; p < 0.05) in Sunitinib treated animals. These findings indicate that our treatments may be causing an effect reminiscent of vascular normalization. Scale bar is 15 µm in CD31 & α-SMA and 1 mm in CA9 image.
by radiation versus secondary death due to radiation effects on tumour vasculature. We further aimed to investigate whether Sunitinib, an anti-angiogenic agent, can induce tumour radiosensitization, and the mechanism by which it may be doing so. Tumour vascular response was assessed using previously established high-frequency 3D power Doppler ultrasound methods, while tumour response was assessed using DNA break immunohistochemistry staining (ISEL), and assessment of tumour growth delay. Vascular density measurements obtained from CD31 stained tumour cross-sections were used to complement power Doppler measurements. CA9 and α-SMA staining were used to assess Sunitinib’s potential mechanism of tumour radiosensitization.

Power Doppler results for tumours treated with radiation alone were in agreement with findings by Garcia-Barros et al. 29, where single large doses of radiation had a vascular effect here causing a significant decrease in detectable power Doppler flow signal (Figure 2.2). This led to a dose dependent increase of cell death as reflected in ISEL staining (Figure 2.4). As anticipated, the administration of bFGF one hour before irradiation prevented a decrease in power Doppler flow signal and a subsequent increase in tumour cell death 24 hours post-therapy. Studies have shown that bFGF can inhibit effects of radiation on endothelial cells if administered into the blood circulation of animals up to an hour before radiation delivery 35,125. This in turn hinders tumour cell death associated with vascular destruction. Patches of cell death were noted in ISEL stained tumour cross-sections when bFGF was combined with radiation. However, a dose-dependent increase, such as the one observed for radiation alone, was not observed. These results, supported by tumour growth assays, suggest that the blood vessel response to radiation may be essential for increased acute tumour response to radiation, as assessed 24 hours after therapy. Taken together, our data is in general agreement with the paradigm described by Fuks et al. 2, which emphasizes the importance of rapid vascular destruction immediately after a single large doses of radiation (> 8 Gy) for enhanced tumour cell kill.

Our results suggest that Sunitinib, at the administered dose, may be normalizing tumour blood vessels. We found and increased acute response (percent cell death) of tumours to radiation therapy, while tumour blood vessels appeared to be more resistant to single doses of radiation after two weeks of Sunitinib treatment. In fact, we noted an increase in power Doppler flow signal at doses equal to or greater than 8 Gy when these were delivered in conjunction with radiation therapy. These results are potentially linked to a vascular normalization process, as
described by Jain \(^{76,94}\), which leads to enhanced tumour oxygenation and a potential pruning of the more abnormal and immature tumour blood vessels. Whereas single large doses of radiation destroy the abnormal vasculature of tumours as observed in figure 2.2 and 2.3, treating with Sunitinib for up to two weeks may have permitted the tumour vessels to become more “normal-like”, yielding better-oxygenated and radiosensitized tumour cells. Since Sunitinib exhibits little effects on established blood vessels \textit{in vivo}, as suggested by Osusky \textit{et al.} \(^{133}\), it may also be that it radiosensitizes immature endothelial cells, leading to capillary destruction after radiation therapy, thereby enhancing flow in larger, more radio-resistant, tumour blood vessels as observed in quantified power Doppler results. Normal micro-vasculature has been shown to be more resistant to radiation therapy, while smaller (and often immature/proliferating) capillaries have been shown to be more sensitive to radiation than arterioles and venules \(^{147-150}\). α-SMA staining suggests that vascular normalization may be taking place while quantification of CA9 staining supports a potential increase in oxygenation levels in the tumour.

The enhanced cell death observed in ISEL stained tumour cross-sections when radiation is combined with Sunitinib is potentially related to enhanced oxygenation conditions throughout the tumour. Tumour growth was also delayed for these treatment conditions and was further enhanced if Sunitinib treatment was maintained after radiation therapy. However, further investigations will be required to better understand the long-term response of tumours after Sunitinib and radiation combined. Such results indicate that a relatively small dose of Sunitinib, such as the one used in this study, administered before irradiation, will likely enhance tumour response to therapy. However, the process by which tumour response is enhanced is not directly linked to a vascular radiosensitization effect. Our current data suggests that the enhanced tumour response may involve a vascular normalization process caused by Sunitinib. Further experimentation will be required to confirm this.

Although the stroma of SCID mice has been described in the past to be more sensitive to radiation effects \(^{53,54}\), recent studies suggest that SCID mice also undergo a similar vascular response to radiation at doses greater than 8 Gy. This vascular response was subsequently linked to overall tumour response \(^{31}\). Experiments we have conducted with nude mice indicate a similar VI decrease in response to single large doses of radiation treatment. Potential designs for clinical trials of radiotherapy combined with anti-angiogenic agents make mention of the potential use of Sunitinib specifically, and suggest that there is a growing interest in bringing this type of
combination into clinical use\textsuperscript{151}. As blood vessels are common to different tumour types and play an important role in the growth and survival of tumour cells, it makes them an ideal target for cancer therapies. The work here forms a basis for such experimentation in concert with radiation.
Chapter 3

*In vitro* and *in vivo* Comparison of Tumour and Endothelial Cell Response in Radiation-Based Vascular Targeting Strategies

"Science may be described as the art of systematic over-simplification."
- Karl Popper

### 3.1 Overview

In this chapter, we present results of an investigation into the temporal response of tumour cells versus endothelial cells to radiation and anti-angiogenics. Our aim is to further study the role of endothelial cells as tumour response regulators by investigating each of these tumour components separately *in vitro*. We have conducted experiments to discern the effects of radiation combined with the anti-angiogenic Sunitinib on endothelial (HUVEC) and tumour (MDA-MB-231) cells, and further compared findings to results obtained *in vivo*. In vitro and in vivo treatments consisted of single dose radiation therapy of 2, 4, 8 or 16 Gy administered alone or in combination with bFGF or Sunitinib. In vitro, in situ end labeling (ISEL) was used to assess 24-hour apoptotic cell death, and clonogenic assays were used to assess long-term response. In vivo MDA-MB-231 tumour were grown in CB-17 SCID mice. The vascular marker CD31 was used to assess 24-hour acute response while tumour clonogenic assays were used to assess long-term tumour cell viability following treatments. In *in vitro* studies, we observed an enhanced endothelial cell response to radiation doses of 8 and 16 Gy when compared to tumour cells. Administering Sunitinib alone significantly increased HUVEC cell death, while having modest additive effects when combined with radiation. Sunitinib also increased tumour cell death when combined with 8 and 16 Gy radiation doses. In comparison, we found that the clonogenic response of *in vivo* treated tumour cells more closely resembled that of *in vitro* treated endothelial cells than in vitro treated tumour cells. Our results indicate that the endothelium is an important regulator of tumour response to radiotherapy, and that Sunitinib can enhance tumour radiosensitivity.
3.2 Background

Recent studies suggest that tumour stroma plays an integral role in regulating tumour response to radiation therapy\(^2,4,2,70,152,153\), challenging the canonical notion that tumour response is primarily dependent on the inherent radiosensitivity of its clonogenic cells. Furthermore, observed differences in tumour cell radiosensitivity \textit{in vitro} and \textit{in vivo} have been linked to the presence of host derived supporting cells \textit{in vivo}, which include endothelial cells\(^29,31,53,54,119,154–157\). Tumour endothelial cells have been demonstrated to undergo radiation-induced apoptosis faster (< 6 – 20 hours after treatment) than most other cancer cell lines\(^2,29–31,120,128\). This has been linked to a 20-fold enrichment of the ASMase enzyme in endothelial cell membranes compared to epithelial and tumour cells. High doses of radiotherapy (> 8 – 10 Gy) are suggested to cause substantial damage to the endothelial cell membrane, leading to extensive hydrolyzation by the ASMase enzyme, in turn releasing ceramide, which signals apoptosis. At high radiation doses, endothelial cells can undergo apoptosis through two pathways: by DNA damage, or via a ceramide-signaling apoptosis pathway\(^29,69,120–124\). At lower radiation doses however (< 6 - 8 Gy), it is speculated that insufficient ceramide is released to induce rapid endothelial cell death. Conventional radiation doses of 1.8-2 Gy are believed not to activate the ceramide-dependent apoptosis pathway. Basic fibroblast growth factor (bFGF) has been demonstrated to inhibit ceramide-dependent apoptosis messaging\(^120,124–127\), thus protecting endothelial cells from radiation induced cell death. Separate from endothelial effects, it is believed that epithelial and tumour cells are predominantly damaged through direct or indirect DNA damage, and undergo cell death or senescence only when they have accumulated sufficient DNA damage\(^26,129,130\).

As stated above, enhanced radiation effects associated with rapid endothelial apoptosis have predominantly been observed at high radiation doses. It has been hypothesized that vascular targeting agents, which can target endothelial cells in a number of ways, can also radiosensitize whole tumours by altering the tumour micro-vasculature, the tumour microenvironment, or by directly radiosensitizing endothelial cells to clinical doses of radiotherapy\(^45,66,78–80,131,136–138,158–160\). Small-molecule receptor inhibitors such as Sunitinib have been suggested as radiosensitizing agents. Sunitinib is a small molecule tyrosine-kinase inhibitor (TKI), which prevents the activation of a wide-spectrum of receptors including VEGF receptors (PDGFR\(\alpha\), PDGFR\(\beta\), VEGFR1, VEGFR2, VEGFR3, FLT3, CSF-1R, RET). It acts by inhibiting downstream signaling pathways rather than binding directly to VEGF, and is one of the few FDA approved anti-
angiogenic agents for the treatment of renal cell carcinoma and gastro-intestinal stromal tumours \(^{66,132–134}\). Nevertheless, there remain unanswered questions in regards to how this type of agent should be administered, and whether it should be used alone or in combination with other forms of therapies (i.e. chemotherapy, radiation therapy) in order to optimize tumour responses.

On its own, Sunitinib has been reported to impede endothelial cell migration, tubule formation and blood vessel formation. It also impedes the induction of apoptosis in less mature endothelial cells, while having minimal effects on existing mature endothelial cells \(^{133}\). Combined with radiation, Sunitinib has been demonstrated to moderately enhance endothelial radiosensitivity \(in\,vitro\) and diminish micro-vascular densities with \(in\,vivo\) xenograft models \(^{61,66,160}\). Sunitinib has also been reported to induce a vascular remodelling process known as vascular ‘normalization’ \(^{60,146,161–165}\). It has further been shown to induce \(in\,vivo\) and \(in\,vitro\) radiosensitization (for a limited number of single and fractionated radiation doses) of a range of cancer cell lines, including endothelial cells \(^{18,57,59–61,131,166–168}\). Nevertheless, the mechanism of synergy in such therapeutic combinations, and how it changes with radiation, remains unclear and controversial \(^{145,169,170}\). Potential mechanisms include vascular normalization resulting in increased radiosensitivity of the whole tumour due to enhanced oxygenation, and/or endothelial cell radiosensitization leading to vascular destruction an secondary tumour cell death at the time of radiation delivery. A number of parameters that may affect the mechanism by which this therapeutic combination acts still remain to be investigated. These parameters include: radiation dose, radiation fractions, drug dose and scheduling, amongst a number of other parameters \(^{61,171,172}\).

The primary objective of this study is to understand how endothelial and tumour cells respond to a range of radiation doses alone, or in combination with vascular targeting therapies, and to compare the responses of each of these cell lines. It is hypothesized that endothelial cells have a different dose dependent response to radiation therapy than tumour cells and that vascular targeting agents can alter endothelial cell response to single radiation doses. It is posited that the altered endothelial response directly affects tumour cell response to radiotherapy. In order to address this, we have investigated radiation responses \(in\,vitro\), comparing endothelial cells versus breast cancer cells to a range of radiation doses between 0 - 16 Gy. Sunitinib was used as a vascular targeting agent and investigated as a potential tumour radiosensitizer. Additionally, bFGF was used to modulate endothelial responses to radiotherapy, and as a comparative
condition to radiation-Sunitinib combination therapy. Experiments assessed the acute (24 hour cell death) and long-term response (clonogenic assay) of HUVEC cells compared to MDA-MB-231 cells. Results were compared to *in vivo* MDA-MB-231 tumour xenograft experiments.

Results indicated an enhanced endothelial cell response to radiation therapy at doses greater than 8 Gy in comparison to breast cancer tumour cells. The use of bFGF resulted in minimized endothelial damage after radiation therapy, while having minimal effects on MDA-MB-231 cells. Sunitinib caused a significant endothelial response when administered alone, while having modest additive effects when combined with radiation. The combination of Sunitinib and radiation had minimal effects on MDA-MB-231 breast cancer cells. In animal studies, results indicated that Sunitinib acts as a tumour radiosensitizer and that tumour responses at higher doses of radiation alone are linked to endothelial cell responses to radiotherapy.

### 3.3 Methods and Materials

**Cell Culture**

MDA-MB-231 breast cancer cells were cultured in RPMI 1600 culture medium (ATCC, Manassas, VA), 5 % fetal bovine serum (FBS) with antibiotics (penicillin and streptomycin: Life Technologies, Grand Island, NY) to 80-90% confluence. Human umbilical vein endothelial cells (HUVEC; ATCC, Manassas, VA) were cultured using endothelial cell basal medium-2 kit from Lonza (Walkersville, MD, USA). Both cell lines were allowed to undergo two passages before plating into treatment dishes and drug/radiation exposure. Passages were conducted following trypsinization using 0.05% trypsin EDTA (GIBCO; Invitrogen, Carlsbad, CA). Each cell line was treated with 15 different conditions: radiation alone at 0, 2, 4, 8 or 16 Gy, or in combination with bFGF, or with Sunitinib. On the day before treatment administration, 2 x 10^5 cells were plated in chamber-slides (Chamber Slides; Lab-Tek, Rochester, NY), for a total of 30 chamber-slides, 15 for MDA-MB-231 cells and 15 for HUVEC cells. Cells were also plated in petri dishes for clonogenic assays at 3 different cell concentrations accordingly (10^1, 10^2 and 10^3), for each of the treatment conditions.
**Treatment**

Each treatment condition was comprised of a single dose of ionizing radiation therapy delivered alone, or combined with Sunitinib (Pfizer, New York, NY) or bFGF (R&D Systems, Minneapolis, MN). Cells plated on the day before experimentation received single doses of radiotherapy of 0, 2, 4, 8 or 16 Gy alone, or in combination with bFGF or Sunitinib, delivered to plates at 0.75 ng/ml and 398.5 ng/ml, respectively, one hour before irradiation; concentrations were based on the literature used in vivo for maximal effect. Cells were washed with PBS immediately before irradiation to remove bFGF or Sunitinib, and re-suspended in their respective media as described in the cell culture section. Radiation therapy was administered using a Faxitron cabinet irradiator (Faxitron Bioptics, Lincolnshire, IL) with 160 kVp X-rays at an SSD of 35 cm, and a dose rate of 200 cGy/min.

**ISEL Assay**

After radiation therapy, cells in chamber slides were washed again with PBS, re-suspended in their respective medium and left overnight in an incubator at 37°C. The following day (at 24 hours after irradiation), cells were fixed in 10 % formalin for 1 hour at room temperature and left in 70% ethanol until staining. Cells were then stained for DNA breaks using in situ end labeling (ISEL) stain as a cell death marker (Santa Cruz Biotechnology, Santa Cruz, CA). Once staining was complete, the total number of cells and the number of ISEL positive cells (nuclei brown staining) were counted for 8-10 fields of view (FOV) in the center of each slide at 10 X magnification using an upright microscopy (Carl Zeiss, Göttingen, Germany). The total number of cells and the number of ISEL positive cells (nuclei brown staining) were counted. A percent apoptotic index was determined by dividing the number of dead cells by the total number of cells in each FOV, and averaged over all 10-15 FOVs per condition. All treatment condition data were normalized to respective controls.

**Clonogenic Assay**

For clonogenic assays, cells plated in petri dishes were washed after irradiation and suspended in their respective medium. Dishes were left in an incubator for colony growth at 37°C for approximately 2 weeks. Cells were then fixed, stained and counted. All plating efficiencies were normalized to control cells.
In vivo Animal Experiments

All animal experiments presented in this work were conducted in compliance with internationally recognized guidelines specified in protocols approved by the Sunnybrook Health Science Centre Institutional Animal Care Committee. MDA-MB-231 breast cancer cells were cultured as described above for full confluence and $1 \times 10^6$ cells were injected subcutaneously into female CB-17 SCID mice (Charles River Laboratories International, Wilmington, MA). Tumours were grown in one hind leg of each mouse for a period of three weeks, reaching an average volume of 200 mm$^3$ before experimentation. Five animals per experimental condition were used.

Animals bearing MDA-MB-231 breast cancer xenografts were treated with single radiation doses of 0, 2, 4, 8 or 16 Gy alone, or in combination with bFGF (0.45 µg/ml) given one hour before irradiation. Another group was given Sunitinib daily for 14 days prior to radiation delivery at a dose of 30 mg/kg. Radiation was delivered as described above. Mice were euthanized 24 hours after irradiation for immunohistochemistry and clonogenic assays. Tumour xenograft cross-sections were stained for tumour blood vessels using a cluster of differentiation-31 (CD31) antibody stain (Santa Cruz Biotechnology, Santa Cruz, CA) and standard hematoxylin and eosin (H&E). Three cross-sections per tumour were stained for both CD31 and H&E. Tumour cross-sections stained with CD31 were quantified for tumour micro-vascular density (MVD). Stained tissue section slides were digitized using a Mirax slide scanner (Carl Zeiss, Göttingen, Germany) after preparation. For each of the CD31 stained tumour cross-sections, 4 fields of view (FOV) were analyzed at 10X magnification to compute the density of vasculature for a total of 12 FOVs per tumour; the MVD was obtained as described in literature. For in vivo tumour clonogenic assays, cells from 18 tumours (n = 3 per treatment condition) were disaggregated, suspended in culture medium and plated into petri dishes for colony formation. Cells were fixed, stained and counted two weeks after plating. All plating efficiencies were normalized to the 0 Gy condition.

Statistical Analysis

All comparisons of quantified in vitro and in vivo cell death, CD31 MVD and colony counts were evaluated for statistical significance using a Mann-Whitney test (two-tailed, assuming unequal variances; $\alpha < 0.05 (*)$, 0.01 (**) or 0.001 (***)). Each treatment condition
was compared directly to the 0 Gy control condition, or as specified in the text. For clonogenic assays, radiation and bFGF or Sunitinib combination treatment conditions were tested against the equivalent irradiation dose in radiation only conditions. Statistical tests were conducted using Prism (GraphPad Software version 5, La Jolla, CA).

### 3.4 Results

The *in vitro* dose-dependent radiation response of tumour and endothelial cells, treated with radiation alone or in combination with bFGF or Sunitinib, was assessed. Experiments evaluated the acute (24 hour cell death) and long-term viability (clonogenic assay) of HUVEC and MDA-MB-231 cells. Findings were then compared to *in vivo* treated MDA-MB-231 xenograft clonogenic assays (long-term response) and CD31 staining (acute response). Figure 3.1 displays representative images of MDA-MB-231 and HUVEC radiation treated cells.

The number of dead cells 24 hours after a single dose of radiation therapy, as a percentage of all cells in a single FOV, is plotted in figure 3.2. Quantitative analysis of ISEL staining revealed that a single dose of 16 Gy caused the most significant amounts of acute HUVEC cell death, followed by the 8 Gy dose (*P* < 0.001). Almost no ISEL staining was observed in doses lower than 8 Gy. The addition of bFGF before high-dose radiotherapy protected endothelial cells from rapid 24-hour cell death. In HUVEC cells treated with Sunitinib alone, we observed significant acute cell death by 24 hours (increase of 9.4 %). Combining Sunitinib with radiation caused a moderate dose-dependent increase in acute cell death (2 Gy – 10.4 %; 4 Gy – 14 %; 8 Gy – 19.13 %; 16 Gy – 29.1 %). All were statistically significant when compared to control HUVEC cells (*P* < 0.001). However, combining Sunitinib with 2 or 4 Gy radiation did not significantly increase cell death in comparison to Sunitinib alone. We did, however, find a significant increase in cell death when Sunitinib was combined with 8 or 16 Gy, in comparison to Sunitinib alone; this effect was less substantial than radiation administered alone at doses greater than 8 Gy. In comparison, minimal (less than 7 %) amounts of cell death were observed for MDA-MB-231 cells treated with radiation alone, or in combination with bFGF, for all radiation doses. Sunitinib significantly elevated the ISEL stained cell death count of tumour cells following radiotherapy to an average of ~ 15% for radiation doses greater than 8 Gy (*P* < 0.05 at 8 Gy and *P* < 0.01 at 16 Gy).
Figure 3.1 - Representative images of ISEL stained MDA-MB-231 cells (top) and HUVEC cells (bottom). MDA-MB-231 cells have minimal response to radiation therapy. 16 Gy treated MDA-MB-231 cells have slight staining, these are deemed viable when compared to cells undergoing apoptosis in the bottom panel. One can further observe enlargement of MDA-MB-231 cells after radiotherapy, likely associated with mitotic arrest. HUVEC cells respond by 24 hours to 16 Gy irradiation. We can observe in the bottom right-hand image that many cells have stained nuclei after the single 16 Gy dose administration. Images were taken at 40 X magnification. Scale bar is 10 µm.
Figure 3.2 - Quantified ISEL stained cells as a percentage of the total number of cells in each FOV taken at 20 x magnification. Each bar is an average of 8-10 FOVs. Top panel (A) is of MDA-MB-231 cells; bottom panel (B) is of HUVEC cells. The first column is of cells treated with radiation alone, the second column is of cells pre-treated with bFGF followed by radiotherapy and the third column is of cells pre-treated with Sunitinib (SU) followed by radiotherapy. While minimal cell death is observed in all treatment conditions for MDA-MB-231 cells, apparent amounts of cell death were quantified in HUVEC cells at 8 and 16 Gy and for all radiation conditions when combined with Sunitinib. For Sunitinib alone, we observed significant cell death by 24 hours. Combining Sunitinib with radiation caused a moderate dose-dependent increase in cell death. Statistical significance is indicated for treatment condition compared to the control condition ($\alpha < 0.05$ (*), 0.01 (**) or 0.001 (***)). Error bars represent standard error.
Colony counts for MDA-MB-231 and HUVEC cells exposed to 2-16 Gy are presented in figure 3.3. Respective plating efficiencies are presented in figure 3.6. From these results, we observed that administering bFGF before radiation treatment minimized the effects of 8 and 16 Gy doses on HUVEC cells significantly (P < 0.05). This was not the case with MDA-MB-231 cells. Sunitinib had minimal effects on the formation of MDA-MB-231 colonies, nor their plating efficiency. It also did not decrease HUVEC colony formation. In contrast, a decrease in HUVEC plating efficiency was noted when Sunitinib was used. For example, for MDA-MB-231 cells, the normalized surviving fractions were 5.7 %, 4.9 % and 7.9 % for 8 Gy alone, 8 Gy with Sunitinib and 8 Gy with bFGF, respectively. In contrast, corresponding endothelial cell clonogenic surviving fractions were 6.7 %, 8.4 % and 12 % respectively. These in vitro results suggest that Sunitinib minimally affects the long-term radiation response of HUVEC and MDA-MB-231 cells. However, we note that Sunitinib does affect the plating efficiency of HUVEC cells (Figure 3.6), and not that of MDA-MB-231 cells.

For in vivo treatments, results from histological staining of tumour cross-sections and colony formation assays of MDA-MB-231 cells are presented in figures 3.4 and 3.5. After treatment administration of radiation alone, or in combination with bFGF or Sunitinib, animals were sacrificed and tumours were processed for immunohistochemistry (H&E and CD31) staining or clonogenic assay as described above. In H&E stained tumour cross-sections, we noted the most necrotic regions in tumours treated with Sunitinib and radiotherapy, while Sunitinib alone caused minimal tumour necrosis (Figure 3.4).

Clonogenic assay results (Figure 3.5) indicated a dose dependent response to radiation doses. However, administering bFGF one hour before irradiation decreased the effects of high-dose radiation on tumour cell colony formation at 8 and 16 Gy (P < 0.05). Sunitinib alone caused a decrease in colony formation plating efficiencies compared to control animals (see in vivo tumour plating efficiencies: Figure 3.6). On the other hand, in-advance treatment with Sunitinib when combined with radiotherapy caused a decrease in colony formation at doses greater than 4 Gy. The quantified acute MVD from CD31 staining of tumour cross-sections significantly decreased (P < 0.01) at doses greater than 8 Gy; for instance, at 16 Gy, it decreased by ~ 75 % compared to untreated animals. In contrast, a minimal MVD decrease following radiation was observed when tumours were pre-treated with bFGF. The use of Sunitinib in conjunction with radiotherapy enhanced overall tumour response for all radiation doses, but only moderately.
Figure 3.3 - Normalized colony counts from clonogenic assay experiments of MDA-MB-231 (A) and HUVEC (B) cells. Cells were plated 24 hours before irradiation and left to form colonies for 14 days post-radiation. We observe that bFGF and Sunitinib had minimal effects on the dose-dependent response of MDA-MB-231 cells. In HUVEC cells, bFGF acted as a radio-protector at 8 and 16 Gy. Sunitinib had no effects on the dose-dependent response of HUVEC cells. Plating efficiencies are shown in Figure 3.6. Error bars represent standard error.
Figure 3.4 - CD31 (A) and H&E (B) staining of representative tumour cross-sections. In (A), while tumour vasculature is dense in MDA-MB-231 tumours, we note a decrease in vascular density when tumours are treated with 8 Gy. Treatment with Sunitinib diminishes the overall tumour vascular density, causing these blood vessels to appear less tortuous and less chaotic. It appears however that the administration of 8 Gy radiotherapy to tumours already treated with Sunitinib minimally alters the acute vascular density. In panel (B), representative H&E stained tumour cross sections are shown; we have outlined in yellow regions of extreme cell death in the tumour as observed at higher magnifications. Our results indicate that the greatest amount of cell death occurs in tumours treated with the combination of radiation and Sunitinib. Scale bar for CD31 is 500 µm, and 1 mm for H&E.
Figure 3.5 - Quantified results from colony formation assays (A) and CD31 staining of tumour cross-sections (B) of MDA-MB-231 tumours treated *in vivo*. After treatment administration of radiation alone, or in combination with bFGF or Sunitinib, animals were sacrificed and tumours were processed for histological staining or clonogenic assay as described in the methods section. Results indicate a dose dependent decrease in colony formation for cells treated with radiation alone. The administration of bFGF before radiotherapy caused tumour cells to be less responsive to ionizing radiation. On the other hand, pre-treatment with Sunitinib caused a decrease in colony formations at all radiation doses. CD31 staining demonstrated minimal MVD decrease at 2 and 4 Gy, and a significant decrease at 8 and 16 Gy. This was not observed when tumours were treated with bFGF before irradiation. Sunitinib decreased the overall MVD of tumours, however, no further decrease in MVD was observed when combined with radiotherapy. Statistical significance is indicated for treatment condition compared to control condition ($\alpha < 0.05$ (*), 0.01 (**) or 0.001 (***)). Plating efficiencies for *in vivo* tumour clonogenic assays are shown in Figure 3.6. Error bars represent standard error.
Figure 3.6 - Quantified plating efficiencies for *in vitro* clonogenic assay (MDA-MB-231 and HUVEC) and *in vivo* tumour clonogenic assays. The first group of columns is for MDA-MB-231 tumour cells, the second group is for HUVEC endothelial cells, and the third group is for MDA-MB-231 tumour cells treated *in vivo* as tumours. We note that the average control plating efficiencies was always 1 for all three groups. From these, we observed a decrease in plating efficiency when MDA-MB-231 cells were treated with bFGF. This may be linked to reports that have suggested that bFGF decreases MDA-MB-231 aggressiveness. Contrary to this, bFGF slightly increased HUVEC cell growth, while having minimal effects on *in vivo* treated MDA-MB-231 tumour cells. Sunitinib had no effects on MDA-MB-231 cells, while decreasing the plating efficiencies of both HUVEC and *in vivo* treated MDA-MB-231 cells. Error bars represent standard error.
decreased the MVD when compared to control animals. An average MVD decrease of 45% – 50% was noted for all Sunitinib treated animals in comparison to control animals; the MVD did not decrease further after single dose radiotherapy.

3.5 Discussion

We have investigated the acute and long-term effects of single dose radiation on tumour and endothelial cells in vitro. We have further conducted in vivo animal experiments to compare to our in vitro results. The aim of this work was to determine the relative temporal responses of tumour cancer cells and endothelial cells in vitro, in order to better understand which of the two tumour components predominantly determines tumour radiation-response in vivo. Endothelial cell radiation-responses were altered using pharmacological modulators of endothelial radiosensitivity in vitro and in vivo. Experiments were carried out using bFGF as an endothelial radio-protector to determine the extent of vascular-driven tumour response to a range of radiation doses. Sunitinib was used as a vascular targeting agent and investigated as a potential tumour radiosensitizer. Experiments with this anti-angiogenic agent aimed to better understand its effects when combined with a number of radiation doses in vitro and in vivo.

Results were consistent with previous findings that endothelial cells respond rapidly to single high doses of radiotherapy above 8 Gy \(^2,29,175\). Cultured endothelial cells underwent cell death within 24 hours when exposed to single radiation doses of 8 Gy or 16 Gy. The percentage of cells staining for ISEL, 24 hours after irradiation, were found to be 21 % and 60 %, respectively. In contrast, minimal cell death was observed when HUVEC cells were exposed to bFGF before radiotherapy, which is consistent with previous studies demonstrating the efficacy of bFGF as a biological radio-protector. Studies have since demonstrated that this fibroblast growth factor has radio-protecting properties; it has been suggested to play a role in mitigating the initiation of a ceramide-signaling endothelial cell apoptosis pathway \(^35,37,39,120,125\). Contrary to HUVEC cells, MDA-MB-231 cells were minimally apoptotic 24 hours after radiotherapy. We did find that some MDA-MB-231 cells had morphological changes, likely related to radiation–induced mitotic arrest. As anticipated, bFGF had minimal effects on the acute cell death of MDA-MD-231 cells.

The exposure of HUVEC cells to Sunitinib alone in vitro yielded a significantly elevated apoptotic index of 9% by 24 hours. Combining Sunitinib with radiation did enhance cell death in
comparison to control animals. The dose-dependent response was however moderate, especially when compared to 8 and 16 Gy alone. We interpret this to indicate that Sunitinib moderately radiosensitizes endothelial cells. It may be that Sunitinib rapidly prunes (vascular remodelling involving regression of unnecessary/superfluous blood vessels) the less mature/proliferating, more radiosensitive endothelial cells. Sunitinib has been reported to have biophysical effects on endothelial cells, such as anti-proliferation and anti-migration effects, as well as causing the activation of rapid apoptotic pathways in less mature or proliferating cells. From our results, we posit that Sunitinib is having minimal effects on existing mature endothelial cells, while inducing apoptosis in less mature or proliferating ones. These results also suggest that cells that undergo cell death due to Sunitinib alone may be ones that are more sensitive to apoptosis by the ceramide pathway. This may then explain the lowered quantified apoptosis index when Sunitinib is combined with 8 or 16 Gy in comparison to delivering these doses alone. We anticipate that future in vitro studies would examine the rapid response of endothelial cells (< 24 hours) to the Sunitinib-radiation combination therapy and the involvement of the ceramide pathway in acute cell death.

Clonogenic assays were used to assess long-term viability of cells. In HUVEC cells, we noted a clonogenic difference between cells treated with and without bFGF at 8 and 16 Gy (Figure 3.3). This was in agreement with ISEL assays, and consistent with the interpretation that endothelial cells have an enhanced response at 8 and 16 Gy radiotherapy, and that bFGF can act to prevent these effects at high radiation doses. The addition of Sunitinib to the cell medium before irradiation did not increase HUVEC radiosensitivity, although it did decrease its plating efficiency. In the radiation treated MDA-MB-231 tumour cells, we found that Sunitinib had minimal effects on their clonogenic proliferation. Interpreting in vitro clonogenic assay and ISEL staining results together, our results suggest that Sunitinib does not directly radiosensitize MDA-MB-231 cells in the long-term, but that it may have some acute radiosensitizing effects at high-radiation doses. We also find that Sunitinib causes a rapid wave of acute HUVEC cell death (pruning), while minimally affecting HUVEC radiosensitivity. Finally, we have noted that in vitro treatment of MDA-MD-231 cells with bFGF decreases their plating efficiencies; this can be linked back to studies which have suggested that bFGF alters the malignancy of MDA-MB-231 cells.
Experiments conducted in vivo with MDA-MB-231 tumour xenografts demonstrated that the clonogenic response of tumour cells treated with Sunitinib and radiation is greater than those treated with radiation alone. The administration of bFGF before irradiation reduced the clonogenic response of tumour cells to all radiation doses; this result directly supports the notion that endothelial response to radiation therapy is an important regulator of in vivo tumour response. Overall, the colony formation trends observed in vivo are more similar to those of HUVEC cells in vitro than tumour cells in vitro. Similarly, the quantification of an average CD31 MVD revealed an acute reduction in tumour microvasculature at high radiation doses, suggesting a rapid endothelial cell death mechanism at these doses. Similarly, we qualitatively observed increased cell death in tumour cross-sections stained with H&E. These results parallel those observed in endothelial cells in vitro. This suggests that the effects of radiation on endothelial cells and blood vessels contributed to overall tumour response, specifically at the higher radiation doses, and that the tumour endothelium bed is an important regulator of tumour response to radiotherapy. This may be in part, due to the high radiosensitivity of endothelial cells at higher radiation doses as demonstrated in vitro or due to effects of radiation on endothelial cells in the milieu of tumour cells.

Our results further demonstrate that Sunitinib has the potential to enhance tumour radiation response; MVD quantification indicate an initial pruning of tumour micro-vasculature after Sunitinib treatment alone. Meanwhile, the administration of radiation after Sunitinib had minimal effects on the MVD. These results are similar to our in vitro acute cell death findings, which suggest that Sunitinib causes rapid endothelial cell death minimally enhanced with the addition of radiation therapy. We also observed increased areas of necrosis 24 hours after radiation in combination with Sunitinib (Figure 3.4). Finally, we found that clonogenic response of tumour cells treated with radiation and Sunitinib increased in comparison to radiation alone, while in vitro treatments of MDA-MB-231 cells demonstrated no Sunitinib-based radiation enhancement. This may be due to vascular re-modeling taking place in the tumour after Sunitinib treatment, in turn affecting the overall radiation response of tumour cells. These tumour radio-enhancement effects are potentially linked to some acute endothelial radiosensitization, as well as vascular ‘normalization’ effects such as described by Jain et al. Future in vitro and in vivo studies will aim to give insight on mechanisms behind targeting tumour vasculature and
maximizing tumour radiation response in tandem. Taking advantage of newly developed tumour and vascular imaging technologies could potentially facilitate such longitudinal studies.

Taken together, it is likely that the role of blood vessels in radiation response is complex, considering the intricacy of the tumour microenvironment. Nonetheless, our results support an ongoing paradigm shift from the accepted concepts of classic radiobiology advocating that tumour cells (and their innate radiosensitivity) as the primary regulator of tumour response to radiation therapy. This new paradigm assigns a more prominent role to the tumour’s endothelium and stroma in radiation response. The in vivo results presented here have been conducted in SCID mice. As discussed in the introduction to this thesis, there has been conflicting evidence regarding the use of these animals in experiments which investigate the relationship between vascular and tumour cell response\textsuperscript{29,30,33,50,51,53,54}. Recently published experiments have however demonstrated SCID animals are indeed a valid experimental model in the context of experiments such as those conducted here\textsuperscript{31}. In summary, our results support past findings suggesting that endothelial cells are important regulators of tumour response to radiation therapy at high radiation doses, but that conventional tumour cell DNA damage may prevail at lower radiation doses. We further give evidence that Sunitinib can alter endothelial radiosensitivity, while re-modeling the tumour vasculature to enhance tumour response to radiation therapy.
Chapter 4
Dll4-NOTCH Signalling Blockade Synergizes Combined Ultrasound-Stimulated Microbubble and Radiation Therapy
"The only principle that does not inhibit progress is: anything goes"
- Paul Feyerabend

4.1 Overview

Tumour vasculature acts as an essential lifeline for tumour growth, survival and facilitates metastatic spread. Novel vascular targeting strategies aiming to sustain vascular shutdown could potentially induce substantial damage, resulting in a significant tumour growth delay. We investigated the combination of two novel complementary vascular targeting agents with radiation therapy in a strategy aiming to sustain vascular disruption. Experiments were carried out with delta-like ligand 4 (Dll4) blockade (angiogenesis deregulator) treatment administered in combination with a radiation-based vascular destruction treatment in a highly aggressive well-perfused colon cancer tumour line. Tumours were treated with permutations of radiation, ultrasound-stimulated microbubbles (USMB) and Dll4 monoclonal antibody (mAb). Tumour vascular response was assessed with three-dimensional power Doppler ultrasound to measure active flow and immunohistochemistry. Tumour response was assessed with histochemical assays and measurements of tumour growth. Our results suggest a significant tumour response in animals treated with USMB combined with radiation, and Dll4 mAb, leading to a synergistic tumour growth delay of up to 24 days. This is likely linked to a rapid cell death within the tumour and a sustained tumour vascular shutdown. We conclude that the triple combination treatments cause a vascular shutdown followed by a sustained inhibition of angiogenesis and tumour cell death, leading to a rapid tumour vascular-based ‘collapse’ and a significant tumour growth delay.
4.2 Background

Tumours require activation of an angiogenic ‘switch’ to grow beyond 1-2 mm³ in volume. Vascular development in tumours occurs via the release of various endothelial growth factors and angiogenesis regulators (i.e. vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), delta-like ligand 4 (Dll4)), leading to neo-vascularization predominantly via a sprouting process reminiscent of embryonic development. Newly formed vessels provide a lifeline of nutrients and oxygen to permit further tumour growth. Anti-angiogenic drugs, originally designed to block the development of tumour vasculature, have become important cancer therapies. For some time, pharmacological anti-VEGF agents gained important ground in targeting angiogenesis, but were found to be less effective than originally anticipated due to tumour resistance mechanisms. These agents were shown to be useful when strategically combined with other anti-cancer therapies. Presently, pharmacological agents targeting different aspects of tumour angiogenesis are being developed and investigated in conjunction with various existing therapeutic modalities.

A variety of physiological and biological factors stimulate tumour vascular development (i.e. hypoxia). These stimuli act as external queues for angiogenic (i.e. VEGF, Dll4) molecular signalling. The Notch pathway has been demonstrated to regulate the sprouting of new vessels in tumours. The Notch ligand Dll4 was found to provide critical downstream regulatory signalling for initiating VEGF stimuli. This pathway has been identified as a promising cancer treatment target. Studies have demonstrated that inhibiting Dll4 signalling results in excessive but non-functional angiogenesis in tumour tissue. In addition, Dll4 blockade causes a significant tumour growth delay in various tumour cell lines. Studies have also demonstrated the potential of Dll4 blockade in combination with other cancer therapies. A recent study observed synergistic enhancement of tumour growth delay when a neutralizing Dll4 monoclonal antibody (mAb) was combined with radiation therapy.

Alongside the numerous pharmacological agents that have been developed to target angiogenesis, emerging biophysical vascular targeting agents, including ultrasound-stimulated microbubbles and gold nanoparticles, have also been demonstrated to increase conventional cancer treatment efficacy. These have been suggested to cause a rapid onset of tumour vascular shutdown and/or increased vessel wall permeability, acting to strategically enhance the
efficacy of cancer drugs or radiation therapy. More recently, Czarnota et al. demonstrated that ultrasound-stimulated microbubbles (USMB) enhances tumour cell death in prostate, bladder and breast cancer lines implanted into rodents. They posited that enhancement of radiation response was linked to the biophysical membrane damage of tumour endothelial cells associated with microbubble stimulation, subsequently leading to ceramide signalling and a vascular shutdown due to endothelial cell death. The observations are potentially linked to recently reported, asmase-dependent, acute endothelial apoptosis following single large doses of radiation.

Here, we investigatedDll4-blockade following combined ultrasound-microbubble and radiation-based vascular destruction in a highly aggressive and well-perfused colon cancer line. Tumours were grown in the hind leg of mice and treated with permutations of radiation, USMB and Dll4 monoclonal antibody (mAb). Animals were imaged using three-dimensional power Doppler ultrasound to investigate changes in tumour perfusion with treatment (Figure 4.2A).

Three-dimensional high-frequency power Doppler ultrasound is an imaging modality well suited for pre-clinical assessment of tumour vasculature in animal models. It yields high-resolution structural information and enhanced Doppler flow signal detection, and is ideal for imaging xenograft tumours. In addition, its facile use makes it suitable for longitudinal studies, where relative changes in vasculature can be assessed. Doppler results were complemented with vascular densities determined from CD31 staining of tumour cross-sections. Tumour response to Dll4 mAb, ultrasound-microbubbles and radiation combination therapy was also assessed with measurements of immunohistochemistry and tumour growth assays.

The results indicate that Dll4 blockade followed by USMB and radiation produces a synergistic tumour growth delay greater than when radiation is combined with Dll4 mAb alone. These findings also suggest that USMB and radiation act to acutely shut down tumour vasculature, as in other types of tumours, but that due to the aggressive nature of the colon cancer cell line, a rapid tumour rebound follows. Treatment with Dll4 mAb then deregulates neovascularization, leading to minimally functional tumour vasculature and a sustained tumour vascular ‘collapse’. Thus, the combination of DLL4 blockade with USMB and radiation is a novel, highly effective approach to delay tumour growth through biological and biophysical targeting of the tumour vasculature (Figure 4.2B).
Figure 4.1 – A) NOTCH-1 angiogenesis regulating pathway. Dll4 signalling originates from the tip cell and regulates stalk cells in sprouting vessel 73,179,180,182. B) Effects of increasing or decreasing Dll4 signalling 180.
Figure 4.2 - A) Schematic overview of experiments. Tumours were left to grow for 9 - 14 days. Animals were then imaged and treated accordingly. Follow up imaging took place at 24 hours and 7 days. Animals treated with Dll4 mAb continued receiving the agent until sacrifice. B) Potential model for vascular strategy. While USMB + XRT is likely causing a rapid vascular shutdown, the aggressive nature of the cell line is inducing a rapid vascular rebound. In contrast, Dll4 mAb following USMB and XRT blocks functional vascular rebounds for a longer period of time.
4.3 Material and Methods

Xenograft Tumour Model

All animal experiments presented in this work were conducted in compliance with internationally recognized guidelines specified in protocols approved by the Sunnybrook Research Institute institutional animal care committee. All animal procedures were performed under anaesthesia, as described below, and all efforts were made to minimize suffering. For this study, up to 90 animals were randomly divided into 3 cohorts. Animals in cohort 1 were sacrificed at 24 hours; animals in cohort 2 were sacrificed at 7 days and animals in cohort 3 were kept for tumour growth assessment for up to 30 days. In order to initiate tumour growth, LS174T ($3 \times 10^6$) colon cancer cells were injected subcutaneously into the right hind leg of 6 to 7-week-old female athymic nude mice (Harlan, Indianapolis, IN). All tumours grew for 9-14 days to reach an average volume of 200 mm$^3$. An average of 5 animals were used per treatment condition for each of the treatment assessment methods (i.e. power Doppler, growth delay, immunohistochemistry).

Treatment Administration

Tumour-bearing animals were given one of six treatments: no treatment (CTRL), radiation (XRT), Dll4 mAb, XRT and Dll4 mAb, ultrasound-stimulated microbubbles (USMB) combined with XRT, or a triple combination of USMB, XRT and Dll4 mAb. Animals were anesthetised with isofluorane before imaging or for catheter insertion (when receiving USMB treatment). All animals receiving radiation therapy were treated with a single dose of 5 Gy ionizing radiation administered using a Faxitron cabinet irradiator (Faxitron Bioptics, Lincolnshire, IL), using X-rays at an energy of 160 kVp, an SSD of 35 cm, and a dose rate of 200 cGy/min. For radiation treatment, the body of each animal was covered with 3 mm thick lead shielding, exposing only the tumour. Animals receiving the Dll4 monoclonal antibody (mAb; MedImmune LLC, MD) were administered the mAb twice weekly, starting on the treatment day following imaging, at a dose of 5 mg/kg of body weight. Animals receiving radiation in conjunction with Dll4 mAb, received the initial anti-Dll4 mAb treatment 1 hour after irradiation. Dll4 mAb treatment was continued in animals until sacrifice (at 24 hours or 7 days), or until a tumour volume reached at least double the original volume. A schematic of the treatment delivery and imaging schedule is presented in figure 4.2A.
For animals treated with USMB, Definity microbubbles (perfluoropropane gas/liposome shell, Lantheus Medical Imaging, N. Billerica, MA) were prepared using a Vialmix device (Lantheus Medical Imaging) and administered through a tail vein catheter at a volume concentration of 3% v/v (1.08 x 10^9 microbubbles in 90 µL) as described in 91. Animals were then immersed in a water bath at 37°C in order to be exposed to ultrasound. The ultrasound therapy system involved a micro-positioning system, waveform generator (AWG520, Tektronix, Beaverton, OR), power amplifier with pulser/receiver (RPR4000, Ritec, Rochester, NY), and a digital acquisition system (CC103, Agilent, Monroe, NY). Animals were exposed within the half maximum peak of the acoustic signal (-6 dB beam width of 31 mm and depth of field greater than 2 cm) using a 16-cycles tone burst at 500 kHz center frequency with a 2.85 cm unfocused planar ultrasound transducer (ILO509HP, Valpey Fisher, Hopkinton, MA). Sonification used a 3 kHz pulse repetition frequency for 50 ms at a time with a peak negative pressure set to 570 kPa, corresponding to a mechanical index (MI) of 0.8. An intermittent 1950 ms period between sonifications was employed to minimize biological heating in the tissue during ultrasound exposures. The total insonification time was 750 ms over 5 minutes for an overall duty cycle of 0.25% 91. Tumours were irradiated with a 5 Gy dose of ionizing radiation immediately after USMB treatment.

**High-Frequency Ultrasound Power Doppler Imaging**

All animals were imaged with three-dimensional high-frequency ultrasound immediately before treatment and 24 hours after treatment. Data was acquired using a Vevo 770 high-frequency ultrasound-imaging device (Visualsonics, Toronto, ON) with a 30 MHz center frequency transducer (RMV-707: 55 µm axial resolution, 115 µm lateral resolution, focal length of 12.7 mm). A motorized scan stage (Visualsonics, Toronto, ON) was used to acquire 3D B-mode images and power Doppler flow data, at a step size of 0.2 mm. Power Doppler data was collected with the following settings: a clutter-filter cut-off of 1-2 mm/s, a scan speed of 1 mm/s, a pulse repetition frequency of 5 KHz, a power Doppler gain of 20 dB and a frame rate of < 10 fps. Power Doppler image analysis was conducted using in-house developed software in MATLAB (The MathWorks, Natick, MA). The vascularity index (VI) was computed from power Doppler images by obtaining the volume of all coloured objects (coloured pixels) over the volume of the selected ROIs. A relative vascularity index was used to assess overall vascular response to therapy, computed as follows: (VI_{24 Hours} / VI_{0 Hours}) x 100.
**Tumour Growth Assessment**

Tumour growth was assessed in an average of 5 animals per treatment condition. Tumour volume was determined by calliper measurements performed every 2 – 4 days and calculated by using the modified ellipsoid formula (volume = length × width²/2). Tumour volumes were normalized to their starting volume and tumour growth delay was calculated by subtracting the average number of days for treatment groups to reach two times their starting volume from that of the control group.

**Statistical Analysis**

Quantified relative VI, ISEL and CD31 staining and tumour growth delays, were evaluated for statistical significance using a Mann-Whitney test (two-tailed, assuming unequal variances; α = 0.05). Each treatment condition was compared directly to the 0 Gy control condition. Statistical tests were conducted using Prism (GraphPad Software version 5, La Jolla, CA).

**4.4 Results**

We conducted experiments to assess the efficacy of vascular targeting strategies, where USMB, radiation and anti-Dll4 mAb treatments were combined to disrupt tumour vasculature and preserve vascular response in order to maximize overall tumour response (Figure 4.2B). Tumour vasculature response was assessed with three-dimensional high-frequency power Doppler ultrasound at 24 hours and 7 days after treatment, relative to baseline. Figure 4.3A displays representative images of three-dimensional power Doppler data at 24 hours after treatment for the control, Dll4 mAb, USMB + XRT and USMB + XRT + Dll4 mAb treatment conditions. A decrease in flow signal was apparent in tumours treated with the triple combination. Quantified power Doppler results at 24 hours and 7 days after treatment are presented for all treatment conditions in figure 4.3B and 4.3C. Minimal changes in the VI were observed for control animals at both times. We observed a non-significant increase in power Doppler signal related to blood flow at 24 hours, and 7 days following XRT. Animals receiving Dll4 mAb alone were observed to have a decrease in flow signal (VI) 24 hours after treatment (p < 0.05). In addition, treatment with Dll4 mAb alone caused the VI to drop further 7 days after therapy (p < 0.05). Treatments with USMB + XRT also caused a rapid (within 24 hours) and
Figure 4.3 – Power Doppler results. A) Representative images of three-dimensional maximum intensity projection of power Doppler signal at 24 hours post treatment. A decrease in blood flow signal was observed in the DLL4 mAb, USMB + XRT and the triple combination conditions. Quantified power Doppler at B) 24 hours and C) 7 days. A non-significant increase in power Doppler signal at 24 hours and 7 days following single dose of 5 Gy irradiation (XRT). Animals receiving DLL4 mAb alone had a decrease in flow signal (VI) 24 hours after treatment delivery; the VI dropped further 7 days after therapy. USMB + XRT also caused a rapid (24 hours) and significant decrease in power Doppler signal. However, the active blood volume returned to baseline levels by 7 days, indicating a short-lived microbubble-based vascular disruption. This result may be related to the aggressive nature of the colon cancer cell line used in this study. A rapid VI decrease of almost 60% was observed for XRT and DLL4 mAb as well as triple combination conditions. The VI drop persisted to 7 days in animals receiving the triple condition treatment. Error bars represent one standard error of the mean. Statistically significant changes are marked with * (p ≤ 0.05). The scale bar represents 2 mm.
significant (p < 0.05) decrease in power Doppler signal. However, the blood flow volume differences by power Doppler returned to baseline levels by 7 days, indicating a temporary, short-lived microbubble-based vascular disruption. A rapid VI decrease of almost 60% was observed for treatments with XRT and Dll4 mAb, as well as the triple combination conditions (p < 0.05). The VI drop persisted to 7 days in animals receiving the triple condition treatment and those receiving Dll4 alone (p < 0.05). In contrast, an increase of ~25% in the VI for XRT + Dll4 mAb treated animals indicates a potential reperfusion of the tumour.

Figure 4.4A displays representative H&E stained tumour cross-sections for each of the treatments. Data demonstrate cell death at 24 hours in the combined XRT and Dll4 mAb treatment, as well as the triple combination treatment condition. Cell death regions persisted in tumours at 7 days after treatment in both these conditions. Quantified cell death areas from ISEL stained tumour cross-sections are presented in Figures 4.4B and 4.4C. Significant (p < 0.05) cell death was observed in tumours treated with USMB + XRT + Dll4 mAb, while a non-significant increase in cell death is observed for animals treated with radiation combined with Dll4 mAb. At 7 days after initial treatment, we noted significant (p < 0.05) amounts of cell death in both the XRT + Dll4 mAb and the USMB + XRT + Dll4 mAb treatment conditions. Furthermore, unique tumour H&E morphology, consisting of multiple necrotic regions and leaked red blood cells, is observed in tumours receiving the triple combination at the time of the 30-day sacrifice (Figure 4.7).

Power Doppler and cell death results were complemented with results from CD31 staining. Representative CD31 immunohistochemistry is shown in figure 4.5A. By 24 hours after therapy (Figure 4.5B), we noted a non-significant, but near doubling of CD31 staining in animals treated with Dll4 mAb only. Meanwhile, we observed a significant decrease in CD31 staining in animals treated with USMB + XRT as well as those treated with the triple treatment conditions (p < 0.05). By 7 days after treatment (Figure 4.5B), we observed a significant increase in CD31 staining for all animals receiving Dll4 mAb treatment, whether alone or in combination with USMB and/or XRT. We also noted that animals treated with XRT alone, or in combination with USMB, had CD31 staining levels similar to control animals.

Figure 4.6A displays the normalized tumour growth curve for each of the treatment conditions. Whereas control animals doubled in volume in less than 5 days following treatment
initiation, we noted a delayed tumour growth in all other treatment conditions. Growth curves followed similar trends for the tumour growth treated with XRT alone, Dll4 mAb alone, and the USMB + XRT treatment. Tumours treated with XRT and continued Dll4 mAb therapy demonstrated a significant and synergistic tumour growth delay (see Figure 4.6B) of 14 days (p < 0.05). Triple combination treatments caused the greatest tumour growth delay, with a decrease in tumour size evident at 5 days after the start of treatment, followed by an interruption in growth lasting for nearly 15 days. An average tumour growth delay of 24 days was observed (p < 0.05) for the triple combination treatment.

4.5 Discussion

In this study, we set out to investigate the efficacy of strategically combining a biophysical, radiation-based, vascular disrupting treatment followed by an angiogenesis-deregulating agent. It was posited that this would increase treatment response (Figure 4.8). In order to maximize vascular destruction, ultrasound-stimulated microbubble therapy was used to first radiosensitize tumour endothelial cells, followed by a single dose of ionizing radiation (XRT) to induce rapid vascular effects. Dll4 mAb was subsequently administered as a ‘maintenance’ therapy. Different permutations of the three combined treatment modalities were also investigated. Results suggest a 1.4X synergistic tumour growth response in animals treated with one session of triple combination therapy, where a ~ 24 day tumour growth delay was achieved. This is likely linked to a sustained tumour vascular shutdown inducing cell death within the tumour. Anti-Dll4 mAb treatment then acts to maintain vascular shutdown. In comparison, combining radiation and Dll4 mAb caused a significant growth delay of ~ 14 days. No other significance was observed in growth delay assays. Tumours receiving radiation alone experienced the least tumour growth delay (~3 days), likely linked to conventional radiobiological DNA damage.

A decrease in tumour size was observed within 5 days following the triple combination treatment, demonstrating an acute response and suggesting a rapid tumour vascular-based ‘collapse’. We observed a power Doppler VI decrease of more than 50% at 24 hours in both XRT + Dll4 mAb and triple combination treatments, in comparison to radiation alone, where no decrease in the VI was observed. The VI decrease was only sustained to 7 days in the triple combination treatments. Tumour volume recession is likely associated with extensive vascular
**Figure 4.4** – Cell death results. A) Representative 24 hour H&E stained tumour cross-sections for specified treatment conditions at low magnification. The bottom row is of high-magnification images of representative H&E stained tumour cross-sections for the specified treatments. The top row scale bar represents 2 mm and the bottom row scale bar represents 50 µm. Quantified ISEL staining at B) 24 hours and C) 7 days after treatment. Significant (p ≤ 0.05) cell death was observed in tumours treated with USMB + XRT + Dll4 mAb, while a non-significant amount of cell death is observed for animals treated with XRT and Dll4 mAb. At 7 days after initial treatment, we noted significant (p ≤ 0.05) amounts of cell death in both the combined XRT + Dll4 mAb and USMB + XRT + Dll4 mAb treatment conditions. Statistical significance is indicated with * for p ≤ 0.05. Error bars represent standard error.
Figure 4.5 – CD31 staining results. A) Representative images of CD31 staining of tumour cross-sections. Images are for the four listed treatment conditions obtained at 24 hours and 7 days after therapy. The scale bar represents 100 µm. B) Normalized CD31 staining per ROI at 24 hours and C) at 7 days after treatment start. We noted a non-significant, but near doubling of CD31 staining in animals treated with Dll4 mAb only. There was a significant decrease in CD31 staining in animals treated with USMB + XRT as well as those treated with the triple treatment conditions. By 7 days after treatment, there was a significant increase in CD31 stained vessels for all animals receiving Dll4 mAb treatment, whether alone or in combination with USMB and/or XRT. Animals treated with XRT alone, or in combination with USMB, had quantified CD31 stained counts similar to control animals. Statistically significance is indicated with * indicating a \( p \leq 0.05 \). Error bars represent standard error.
Figure 4.6 – Tumour growth results. A) Normalized tumour growth curves. A delayed tumour growth in all treatment conditions was noted. Tumour growth curves followed similar trends for the XRT alone, the Dll4 mAb alone, and the USMB + XRT treatments. Combination triple treatment condition (USMB + XRT + Dll4 mAb) was observed to have the greatest response, with a decrease in tumour size at 5 days after treatment, followed by growth inhibition lasting for nearly 15 days. B) Quantified tumour growth delay to reach two times the starting volume. Treatments with XRT and continued Dll4 mAb experienced a significant synergistic tumour growth delay of 14 days. Meanwhile, the triple combination treatment (USMB + XRT + Dll4 mAb) caused a tumour growth delay of 24 days. Statistically significant differences in tumour growth delay are indicated with * for $p \leq 0.05$. Error bars represent standard error.
**Figure 4.7** - Representative H&E images of control, DLL4 mAb, USMB + XRT and USMB + XRT + DLL4 mAb at tumour growth assay end point (~30 days). Persisting tumour heterogeneity is observed in the triple combination H&E stained tumour cross-section. The scale bar represents 2 mm.
shutdown, leading to large regions of necrosis at the core of the tumour. We have also observed a significant amount of cell death 24 hours following this triple combination therapy, while no other significance was found in other treatment conditions. Here, ISEL-quantified cell death areas per tumour cross-section persisted in the long-term (beyond 24 hours) and reached nearly a mean of 30% of the tumour cross-section by 7 days following therapy. Radiation combined with Dll4 mAb also caused significant cell death 7 days following therapy, however tumour volumes in this condition were noted to be larger than tumour treated with the triple combination therapy. H&E stained images indicated a persisting heterogeneous and necrotic tumour microenvironment 30 days after therapy (Figure 4.7). These results clearly demonstrate the potency of combining USMB, radiation and Dll4 mAb above and beyond any therapy alone.

For animals treated with XRT + Dll4 mAb or USMB + XRT + Dll4 mAb, a significant rapid decrease in the vascular index was observed by 24 hours following therapy. However, we posit that the resulting vascular shutdown in some of these treatments is of a differing nature. In USMB + XRT + Dll4 mAb treated animals, endothelial cells likely undergo a rapid wave of apoptosis associated with a mechanical membrane perturbation due to microbubble mechanical effects resulting in ceramide release. Return of functional vessels in treated tumours is then suppressed by Dll4 mAb treatments. In contrast, treatments with XRT + Dll4 mAb are likely causing some endothelial cell death (as suggested in CD31 staining), leading to a temporary vascular shutdown. At 7 days following XRT + Dll4 mAb treatments, the vascular index appears to be returning to the baseline level, while the vascular index depression persists in the triple combination treated-animals.

Treatment with Dll4 mAb alone induced a similar response to those previously reported. As anticipated, the quantified CD31 staining increased after treatment initiation, while active blood flow, as assessed using power Doppler ultrasound, decreased. This is characteristic of Dll4 blockade, where excessive angiogenesis occurs, but paradoxically vascular perfusion decreases. In this study, shutdown of vascular function was sustained for up to 7 days after the start of Dll4 mAb treatment. However, a tumour growth delay similar to radiation alone was observed, indicating that Dll4 mAb based vascular shutdown is not sufficient to induce substantial tumour damage.
Recent investigations have demonstrated that pre-treating tumours with USMB can radiosensitize endothelial cells to radiation, subsequently increasing the overall response of tumours \(^9^1\). We have found that USMB treatment followed by XRT causes an acute and significant decrease in the VI, as measured with power Doppler ultrasound. Quantification of CD31 staining was in agreement with this, where a significant decrease in staining was observed at 24 hours. However, tumour reperfusion was observed at 7 days after treatment. Similar observations were recently reported \(^1^9^0\). This effect is likely linked to the aggressive nature of the LS174T colon cancer cell line. Generally, treatment response may be a function of tumour aggressiveness and vascular architecture.

Following XRT, we noted an increase in the power Doppler vascularity index at 7 days. A similar effect was noted in animals receiving XRT + Dll4 mAb. Observed variations are likely linked to response variations in animals. In addition, studies have indicated that small doses of radiation can promote tumour angiogenesis, or induce inflammatory effects resulting in increased tumour perfusion \(^4^6,^1^9^2,^1^9^3\). It is also possible that radiation may be pruning less mature endothelial cells, leading to an enhanced vascular architecture and increased blood flow \(^2^0\).

In summary, the results demonstrated an enhanced tumour response when ultrasound-stimulated microbubble therapy and XRT were combined with Dll4 mAb as a maintenance therapy. Our results clearly demonstrate that a synergistic effect enhances treatment response when USMB, radiation and Dll4 mAb are strategically combined. In the future, we anticipate that a yet greater treatment response can be achieved through the administration of multiple fractionated treatments. Our results are encouraging given the increasing use of single fraction radiation therapy. Such treatments are now commonly used for palliation with the aim of eliciting rapid anti-vascular effects, in turn minimizing bleeding in patients, arresting tumour growth and alleviating pain. The use of USMB and Dll4 mAb in combination with a single radiation fraction may enhance such effects. Results presented in this study also confirm the importance of Dll4 in regulating ongoing tumour angiogenesis and its potential use in combination with other novel cancer therapies. Finally, our results add to the paradigm that tumour blood vessels play an important role in tumour response to therapy and demonstrate a maximal tumour response when tumour vasculature is biophysically disrupted, followed by an angiogenesis deregulating ‘maintenance’ treatment.
Figure 4.8 – Illustration of posited USMB, XRT and Dll4 mAb effects on tumour endothelial cells. We posit that pre-treating tumours with ultrasound-activated microbubbles may first radiosensitize the cells via a biophysical process. Upon radiation delivery, endothelial cells would first undergo apoptosis, leading to tumour cell death. Continued delivery of an angiogenesis deregulator would prevent tumour reperfusion, serving as a maintenance therapy.
Chapter 5
Summary and Future Directions
"He who is fixed to a star does not change his mind."
- Leonardo da Vinci

5.1 Summary of Thesis Results

The aim of the work conducted for this thesis was to determine the regulatory role of tumour vasculature in tumour response to radiation therapy, and to subsequently investigate novel combinatory vascular targeting strategies involving radiation. Taken together, our investigations demonstrate the importance of tumour blood vessels as a target in radiation therapy. Results also reveal that vascular targeting strategies involving pharmacological and/or biophysical agents enhance tumour response to radiation therapy. The idea of combining vascular targeting agents with radiation therapy opens new and innovative avenues for delivering cancer therapy and in turn yields new clinical paradigms, which allocate a larger role to tumour blood vessels in treatment response. In summary, through the work presented here, we have demonstrated that:

1. Tumour vasculature is rapidly shut down within 24 hours after a single dose of radiation therapy greater than 8 Gy. Observations were made using high-frequency, three-dimensional power Doppler ultrasound. These results were complemented with CD31 vascular staining, where a decrease in the vascular density in tumour cross-sections was noted.

2. Acute tumour cell death, as assessed with ISEL staining, is at least in part regulated by tumour vasculature following single dose radiation therapy. This was demonstrated using bFGF as a vascular protecting agent to modulate vascular response to therapy. Effects on tumour cell colony formation and tumour growth were also minimal when radiation was used in conjunction with bFGF.

3. Endothelial cell death, as assessed with ISEL staining in chapter 3, occurs faster than tumour cell death in \textit{in vitro} settings, when treated with single high radiation doses. Similarly, the clonogenic response of endothelial cells was greater at high radiation doses, while minimal when cells were pre-treated with bFGF. We also found that the
clonogenic response of *in vivo* treated tumour cells more closely resembled that of *in vitro* treated endothelial cells than *in vitro* treated tumour cells.

4. Sunitinib, when delivered at a low-dose, may induce a vascular normalization process, subsequently enhancing oxygenation and tumour radiation response. Our results yield strong evidence that the tumour vasculature is pruned during Sunitinib delivery, henceforth yielding increased tumour cell death following radiation therapy, and an overall increased tumour response. *In vitro* results also support the pruning effect of Sunitinib and complement our *in vivo* results. Overall, we demonstrate the potential of vascular normalization strategies for enhancing radiation response.

5. In chapter 4, from power Doppler, tumour growth delay and immunohistochemistry results, we have found that combined USMB and radiation treatment is minimally effective in highly aggressive, well perfused tumour lines, such as the LS174T colon cancer line. Although a vascular effect is apparent immediately after treatment delivery, tumours quickly overcome this via rapid reperfusion. Past investigations have found this treatment combination to be more effective in prostate, breast and bladder cancer cell derived tumours.

6. The addition of a vascular targeting agent, specifically a Dll4 mAb, prevents tumour vascular rebounds – at least for a period - following XRT + USMB treatment delivery. Our results demonstrate a tumour vascular ‘collapse’ by 3-5 days after the start of this triple combination treatment. Tumour growth delay synergy was also observed in these treatments. All together, these results demonstrate the potential of vascular maintenance therapy following a vascular destructive therapy.

A summary of findings is exhibited in figure 5.1. Our results can be used as basis for future exploration of the role of tumour blood vessels in treatment response. The implications and limitations of these conclusions are discussed in their respective chapters. Overall, the main challenges encountered in this thesis relate to: i) tumour variations in treatment response, ii) dosing and delivery scheduling of each treatment in strategic combination therapies (especially when translating from mice to men), and iii) limitations associated with the primary imaging modality employed here (i.e. power Doppler ultrasound). The following section highlights these three topics, while shedding light on future investigations based on other separate literature.
Figure 5.1 – Summary of findings. Taken together, our results demonstrate the importance of targeting tumour blood vessels in radiation therapy. Treatments inducing a vascular destruction effect often undergo a vascular rebound; maintenance therapy may be a solution to blocking blood vessel regrowth and causing pertinent tumour damage. On the other hand, treatments that normalize tumour vasculature increase overall clonogenic cell death compared to low-dose (< 8 Gy) radiation doses delivered alone.
5.2 Future Directions

5.2.1 Variations in Treatment Response Associated with Tumour Type

As described in chapter 1, the tumour vasculature is abnormal in comparison to normal vasculature. Furthermore, it tends to be characteristically different from one tumour cell line to another. These differences are regulated by the varying levels of vascular growth signals released by different tumour cell lines, and contribute to variations in tumour aggressiveness. Tumour vascular properties result in distinct tumour microenvironments, and impact tumour response to therapy. A study by Eberhard demonstrated that the features and rate of angiogenesis differed significantly amongst six different tumour cell lines \(^\text{194}\). In chapter 4, results demonstrate that, although USMB combined with radiation therapy has been shown to cause a significant response in tumours derived from PC3 and MDA-MB-231 cell lines, overall tumour response was minimal in the LS174T cell line \(^\text{91,190}\). These results indicate the importance of adopting differing vascular strategies in radiation therapy for different tumour types, sites and grade. In addition, it may be that endothelial cells derived from differing tumour cell lines are also characteristically different from one another \(^\text{195}\). This should be investigated in future work. It is likely that future vascular strategies will require close monitoring of vascular treatment response in order to tailor treatments as needed at different cancer sites.

In addition, a number of combined VTAs and cancer treatment permutations remain to be explored. Future work requires a closer examination of the effects of microbubble radiosensitization on different endothelial cell lines in order to determine the biomechanical and biochemical pathways involved in this process. In this work, we have only examined single doses of radiotherapy. Future work should examine fractionated radiation therapy in vascular targeting strategies, as well as USMB combined with other VTAs. The mechanism of treatment response fundamentally differs when delivering a prescribed dose over a period of time (fractions) than when delivering a single dose, often leading to repair, redistribution, reoxygenation and repopulation of cancer cells \(^\text{196}\). It will hence be important to study how vascular targeting strategies, such as the ones proposed here, affect these. Studying the inter-relationship of vasculature response and tumour physiological characteristics will require advanced imaging tools capable of monitoring respective treatment response.
5.2.2 VTA Timing and Dosing

Questions on the timing, scheduling, dosing and duration of use of VTAs in conjunction with radiation therapy need to be addressed. Anti-angiogenic agents are discussed in chapter 1 in the form of vascular normalizing agents aiming to enhance oxygenation and leading to enhanced radiosensitivity of tumour cells. In chapter 2, our results suggest that vascular normalization agents may be radiosensitizing tumours via a ‘normalization’ mechanism. It is then important to determine the optimal timing for radiation delivery (the ‘normalization’ window). This has so far proved to be a challenge and will require the use of specialized imaging tools sensitive to biomarkers associated with vascular normalization (Figure 5.2). When attempting to normalize vasculature, anti-angiogenics are typically administered over a period of time. Ideally, agent effects should be monitored through the use of imaging modalities seeking an optimal treatment delivery window. Anti-angiogenic agents have been shown to begin inducing ‘normalization’ effects anywhere from 1 hour to days (up to 15 days) after the first administration\textsuperscript{14,61,64,76,197}. The elevated oxygenation time-window is dependent on dosing and tumour stage. Furthermore, low drug doses (compared to those prescribed when anti-angiogenics are used alone) have been used when combined with radiation therapy to induce vascular ‘normalization’\textsuperscript{162,198}.

VDAs seem to work best on late-stage tumours, characterized by necrotic and/or hypoxic cores\textsuperscript{199,200}. These are generally delivered through pulsed dosing schemes, whether alone or in combination with other treatments. As to the timing of such agents, studies have suggested that delivering VDAs after radiation therapy may be most effective, possibly to avoid elevating tumour hypoxia levels before radiation delivery. Results obtained in chapter 4 are in agreement with this and demonstrate the potential effect of VDAs when used as a maintenance therapy. This sort of treatment acts to prevent reperfusion occurring due to tumour revascularization, in turn aiding to maintain tumour control or preventing tumour growth\textsuperscript{201}. In an ideal situation, the vascular targeting strategies described in this thesis, if optimized, should act to destroy most tumour cells in targeted disease sites without the need for a maintenance therapy. However, the physiological complexities of cancer make this near impossible, stressing the potential importance of maintenance therapies. As for strategies that employ angiogenesis-deregulators, which also lead to vascular flow shutdown, much remains to be studied in regards to optimal methods by which these could be used. Our studies have found that these can act by temporarily shutting down tumour vasculature, similar to VDAs, but that their mechanism of action is
Figure 5.2 – Various biomarkers that indicate tumour vascular normalization. Novel imaging modalities should be able to detect these biomarkers in vivo to optimize radiation delivery during the normalization window. Figure is adopted from Jain.\textsuperscript{76}
completely different as they promote endothelial cell proliferation. Theoretically, these can also be used to balance angiogenesis signalling, potentially resulting in a vascular ‘normalization’ process. Finally, there may be an optimal pre-radiation dose and delivery schedule that would result in endothelial cell radiosensitization. Further studies will be required to investigate how to be use such treatments.

5.2.3 Imaging Vascular Response – Focus on Ultrasound

Future studies of the tumour vasculature will depend on novel imaging methods. These will enable researchers and clinicians to assess the tumour specific vascular network before a treatment is delivered, allowing for better-tailored treatments. It is likely that multi-modality imaging protocols will be employed in the future to assess different aspects of tumour vasculature. For instance, photoacoustic imaging systems have recently been made commercially available as part of an ultrasound system, permitting researchers to assess oxygenation parameters co-registered within an ultrasound image (Figure 5.3).

Novel algorithms for processing Doppler ultrasound data, augmented with advances in computing power, are allowing for new real-time parameter extractions that may be capable of instantaneously characterizing tumour vasculature (i.e tumour normalization/remodelling)\(^ {115,202–204}\). Studies have demonstrated the potential of analyzing regional power Doppler signal, as well as volumetric branching and vessel shape parameters\(^ \text{10,99,115}\). Vessel sizes could further be extracted from volumetric data, and used to analyze distribution changes following therapy. In a pilot study, I have demonstrated that using the image processing software Imaris (BITPLANE, South Windsor, CT) can be used to improve three-dimensional power Doppler image visualization, and to generate vessel tortuosity maps in the tumour volume (Figure 5.4). Other key physical parameters can then be extracted from such images and used as biomarkers in vascular response to therapy.

Alternatively, using contrast-enhanced ultrasound (CE-US) in conjunction with power Doppler ultrasound, whether it be to delineate vessel location in order to examine specific perfusion rates, or to enhance power Doppler signal, may be another avenue to explore. Registering power Doppler images to CE-US images could yield simultaneous anatomical and functional vascular information. This could further be augmented with photoacoustic-based
Figure 5.3 - High-frequency (25 MHz) photo-acoustic images showing co-incident (left-hand image) total haemoglobin and (right-hand image) O² saturation images of a PC3 prostate cancer xenograft. Tumours shown have been imaged 24 hours after treatment with ultrasound-stimulated microbubble and radiation therapy. Note the lack of blood flow in the tumour core after treatment. The scale bar indicates 2.5 mm. (courtesy of Dr. Azza Mahrouky and Dr. Gregory Czarnota)
Figure 5.4 – A volumetric rendering (right image) of a power Doppler image stack obtained from an LS174T tumour grown in vivo on the hind leg of an athymic nude mouse after 10 days of growth. Data was collected with the Vevo2100 (VisualSonics, Toronto, ON) and the MS550S transducer. Data was then segmented (left image) with the Imaris (BITPLANE, South Windsor, CT) software using an automatic segmentation algorithm based on flow regions. Colouring is representative of vascular tortuosity.
Figure 5.5 – Poorly perfused tumour before and after microbubble infusion. VI is enhanced 10-fold, increasing the amount of vasculature detected in a 2D plane.
oxygenation measurement and quantitative ultrasound cell death maps in a volumetric therapy-response monitoring toolbox. I have examined the potential of CE-US to improve power Doppler signal. Results (Figure 5.5) suggest an increase of up to 10-fold in power Doppler signal in a single 2D tumour ROI in comparison to power Doppler images without CE-US.

5.3 Conclusion

Several reasons warrant the investigation of vascular targeting strategies combined with radiation therapy. It could be argued that a greater antitumour effect will be achieved when combining agents that have fundamentally different mechanisms of action, different cellular targets and non-overlapping toxicities. These may overcome factors known to adversely affect the efficacy of radiation therapy by altering the tumour microenvironment and its respective blood vessels, further leading to enhanced classic clonogenic cell kill. On the other hand, evidence that radiation targets endothelial cells differently than clonogenic tumour cells at high radiation doses suggests that there may be more potent and strategic methods of delivering radiation therapy to tumours. Here, the emphasis would be on destroying tumour vasculature, which would result in secondary tumour cell death. Furthermore, the use of endothelial radiosensitizers may increase blood vessel destruction both at low and high doses of radiation, and enhance secondary tumour cell death. Observations that radiation-induced lesions in tumour cells were often not lethal, and that conversion to lethal damage is tightly coupled to an endothelial apoptotic response support this. As blood vessels are common to different tumour types and play an important role in the growth and survival of tumour cells, it makes them an ideal target for therapy. In addition, radiation therapy is a common therapeutic modality, employed for treating various cancer sites. As vascular imaging modalities are further developed (i.e. resolution, stability, image processing, multi-modality imaging), their use will be essential in better understanding the changing dynamics of tumour blood vessels (i.e. tortuosity, vascular density, flow rate, permeability) and the tumour microenvironment (i.e. hypoxia, cell death) following cancer therapy. This will be necessary in order to reinforce this new paradigm, which assigns a greater role to tumour blood vessels in how tumours respond to radiotherapy.
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Appendix A

Ultrasound and Power Doppler

The earliest recorded use of ultrasound in imaging dates back to the late 1940s. Ultrasound’s potential for monitoring tissue motion and blood flow using Doppler concepts was realized shortly after. By the early 1970s, continuous wave (CW) ultrasound had become a widely accepted medical imaging modality; duplex devices that use a pulse-echo method soon followed. These allowed imaging anatomy and blood flow with a single instrument. Today, ultrasound imaging is second only to conventional x-ray in medicine. It is recognized as a relatively cheap and widely accessible modality, permitting rapid medical diagnostics. Before the recent development of contrast enhanced ultrasound methods, most ultrasound-based blood flow imaging techniques relied on the Doppler effect. This phenomenon takes advantage of the frequency change occurring in ultrasound echoes returning from moving scatterers in blood (i.e. RBC). The Doppler equation can be written as follows:

\[ f_d = \frac{2v(\cos\theta)f}{c} \]

where \( v \) is the blood velocity, \( f \) is transmitted frequency, \( c \) is the speed of sound in tissue, \( \theta \) is the angle of the ultrasound beam and \( f_d \) is the Doppler frequency. This equation works well when an ultrasound signal is continuously emitted while another transducer samples the returned ultrasound backscatter (termed continuous wave ultrasound – CW). However, it becomes very difficult to detect the Doppler frequency using this equation in ultrasound systems that employ a pulse-echo method (where a single transducer is used to send an ultrasound signal and then goes into ‘listen’ mode to sample the returned backscatter). In pulse-echo ultrasound imaging (the most common systems today), although still termed Doppler, blood flow and motion imaging relies on algorithms (often proprietary to device’s manufacturer) to detect flows specific to a time-gated voxel along a scan line. These act by detecting phase changes in the ultrasound backscatter at a specified time-gated voxel in an image (Figure A.1). For example, in modern ultrasound devices, the rate of speckle pattern change can be measured at the time-gated location using a ‘sample and hold’ method – various algorithms have been implemented to estimate the Doppler frequencies from an ultrasound scan line. Pulse-echo Doppler shift estimates are
Figure A.1 – As RBCs move within a voxel (broken black line box; left-hand image), an ultrasound signal phase change occurs at the specified time-gated voxel in the returned ultrasound backscatter (voxel with the two broken lines; right-hand image). The phase change can then be used to derive the Doppler frequency.
advantageous over CW Doppler ultrasound as they permit to differentiate the origins of the Doppler signal within an ultrasound beam and a 2D plane. A number of algorithms have been devised commercially to detect the Doppler signals across a two-dimensional image\textsuperscript{205}. Generally, these are capable of estimating the Doppler shifts at multiple time-gated voxels along a scan line very rapidly – on the order of 50-20 ms; this allows visualizing blood flow superimposed onto a Bmode image.

Conventionally, two types of Doppler information can be superimposed onto a Bmode image – color Doppler and power Doppler information. The difference between these two is in how the Doppler frequencies are processed and presented to the end user. In color flow, an average of all Doppler frequencies is color coded to display flow velocity (direction) estimates within the 2D image. Power Doppler uses the total integrated power under the Doppler frequency spectrum, and is hence a function of flow velocity and the number of scatterers within each voxel. Color flow ultrasound is susceptible to aliasing and angle effects. However, power Doppler does not contain flow velocity estimates nor direction, but is more sensitive to smaller flow, while being minimally angle dependent and exempt from aliasing effects.

**High-Frequency Power Doppler Ultrasound**

As discussed in chapter 1, at high frequencies, power Doppler ultrasound is more sensitive to slow flow than at low frequencies. In our studies, we have utilized the Visualsonics Vevo 770 device to probe vascular response to therapies. Scan heads associated with this device are based on a single element ‘swinging’ (single transducer which is moved to scan a surface line by line) transducer.

Generally, high-frequency power Doppler ultrasound is sensitive to flow in vessel diameters of 100-50 μm at 25 MHz. Several studies have demonstrated its potential in imaging tumour vasculature and assessing VTAs\textsuperscript{99,114,141,206}. During scanning, parameters (Table A.2) were set accordingly to maximize power Doppler signal and minimize noise for each mouse.
<table>
<thead>
<tr>
<th>Setting Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Scan Speed:</strong></td>
<td>Time it takes the transducer to do a whole swing. This setting controls the dwell time. Can cause clutter and/or false signal if set too high. Optimal at 0.5-0.8 mm/s – causes clutter and false signal - slows down scanning</td>
</tr>
<tr>
<td><strong>Wall-Filter:</strong></td>
<td>Cut off Doppler frequency/ flow velocity. If set too low, may allow clutter signal to be detected. Must always be set higher than scan speed. Optimal setting in mouse imaging between 1 and 2 mm/s.</td>
</tr>
<tr>
<td><strong>Dynamic Range:</strong></td>
<td>Coloring of each voxel based on the provided range of power Doppler intensities.</td>
</tr>
<tr>
<td><strong>Priority:</strong></td>
<td>Eliminates power Doppler signal at areas of high bmode intensity.</td>
</tr>
</tbody>
</table>

**Table A.2** - Machine settings that affect the image are described in. Parameters highlighted in red can be set after data collection is complete, while those in black affect live data collection.
Power Doppler Analysis Tools

We have developed a software package that permits the analysis of the power Doppler flow signals in 3D volumes. The package allows for user selection of min/max dynamic range threshold and regions of interest (ROI). The threshold sets the limits of the intensity of all detected power Doppler signals. This means that a minimum intensity set too low may inherently allow for noise artefacts to appear in the power Doppler image plane, while a maximum intensity set too low may degrade the true flow signal. We have found from preliminary optimization experiments, that this threshold is optimal when set at a minimum of 12-16 dB and maximum of 40 dB. These settings are based on observations that a min threshold lower than 12-16 dB displays random noise artefacts, while a max threshold set lower than 40 dB takes away from the center of the true flow signals. The software also computes a number of volumetric parameters based on the user-selected ROIs. The vascularity index (VI) is computed by obtaining the volume of all coloured objects (coloured pixels) over the total tumour volume formed by the selected ROIs:

\[ VI = \frac{\text{Colored Voxels}}{\text{All Volume Voxels}} \times 100\% \]

ROIs are generally carefully selected by user to exclude the surface skin of the tumour and the lower hind-leg bone, including only tumour tissue. Future versions of the software will also yield information about the size distribution of flow signal areas, the vessel dimensions in 3D as well as vessel density throughout the tumour volume. The VI will be the primary vascular surrogate for the proposed experiments.
Appendix B

Peer Reviewed Publications


10. Robin Castelino, Matthew Rodrigues, Ahmed El Kaffas, Omar Falou and Eduardo Galiano "A Markovian approach to prostate cell survival under fractionated
radiotherapy", 2010 - Journal of Radiation Physics and Chemistry


Full Paper Publications in Peer-Reviewed Conference Proceeding


2. Ronesh Puri, Ahmed El Kaffas, Alagan Anpalagan, Sridhar Krishnan, Bern Grush, “Multipath Mitigation Of GNSS Carrier Phase Signals For An On-Board Unit For Mobility Pricing”, Canadian Conference for Electrical and Computer Engineers (CCECE05), 2005

Conference Presentations


19. Ronesh Puri, Ahmed El Kaffas, Alagan Anpalagan, Sridhar Krishnan, Bern Grush, “Multipath Mitigation Of GNSS Carrier Phase Signals For An On-Board Unit For Mobility Pricing”, Canadian Conference for Electrical and Computer Engineers (CCECE05), 2005

Patents and Intellectual Property Rights

1. Intellectual property disclosure for novel 3D high-frequency power Doppler analysis software.

2. Intellectual property disclosure for novel quantitative ultrasound texture analysis.


4. Intellectual property disclosure for a mobile application for counting colony formations (pending).