QUANTIFYING THE EFFECTS OF RADIATION ON TUMOUR VASCULATURE WITH HIGH-FREQUENCY THREE-DIMENSIONAL POWER DOPPLER ULTRASOUND

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
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Abstract

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Recent evidence suggests that radiation may have a significant effect on tumour vasculature in addition to damaging tumour cell DNA. It is well established that endothelial cells are among the first cells to respond after administration of ionizing radiation in both normal and tumour tissues. It has also been suggested that microvascular dysfunction may regulate tumour response to radiotherapy at high doses. However, due to limitations in imaging the microcirculation this response is not well characterized.

Advances in high-frequency ultrasound and computation methods now make it possible to acquire and analyze 3-D ultrasound data of tumour blood flow in tumour microcirculation.

This thesis outlines the work done to test the hypothesis that single dose 8 $Gy$ radiotherapy produces changes in tumour blood vessels which can be quantified using high-frequency power Doppler ultrasound. In addition, the issue of reproducibility of power Doppler measurements and the relationship between histopathology and power Doppler measurements have been examined.
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Sincerely,

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Chapter 1

Introduction

1.1 Motivation

The mechanism of tumour cell kill in response to ionizing radiation is still not completely clear despite the long proven usefulness of radiotherapy. Classically, the emphasis in radio-biology has been on the negative impact of inadequate vasculature leading to radio-resistance in hypoxic tumour cells, while overlooking the potential therapeutic benefit to microvascular collapse and hypoxic tumour cell death [1]. More recent claims that microvascular damage may regulate tumour cell response to radiation presents a paradigm shift from the classic view of tumour cell kill in which radiation targets tumour clonogens [2].

Although a relationship between tumour cell death and endothelial cell death in response to radiation is clear, there has not been sufficient work done to assess tumour vascular responses in vivo. This may be due to a lack of non-invasive assays of vascular morphology and physiology [3]. There are several imaging modalities which are capable of detecting blood flow in vivo but all suffer from limitations such as inadequate resolution, high radiation doses or the use of toxic contrast agents.
Recent advances in high-frequency ultrasound and the commercial availability of a small-animal high-frequency ultrasound scanner make it possible to acquire and analyze three-dimensional measurements of tumour blood flow in tumour micro-circulation non-invasively.

The motivation for this thesis is to develop and test high-frequency power Doppler ultrasound for monitoring tumour vessel responses to radiotherapy within the context of radio-biology.

In the next part of this chapter, a brief history of radiotherapy in the context of the tumour endothelium is described in order to outline the important concepts developed and contributions made in understanding the role of the tumour micro-circulation in tumour responses to ionizing radiation.
1.1.1 A Brief History

One of the earliest observations of tumours having a blood supply was made by Rudolf Virchow in the 1860’s when he described that tumours were highly vascularized but significantly morphologically different from that of normal tissue [4]. Thirty-five years later, a major breakthrough in physics would change medicine drastically and give rise to the field of radiation oncology. It is interesting that the discovery of X-rays in 1895 by Wilhelm Röntgen would lead to the understanding that ionizing radiation could cause cancer, detect cancer and could also be employed to treat cancer as well.

Within only a few months after the first x-ray image was produced (Figure 1.1) a graduate student, Emil Grubbe, was the first to test the use of X-rays for the therapeutic application to treat breast carcinoma. Emil Grubbe’s hypothesis that X-rays could be used to cause tumours to shrink was motivated by changes in his skin in the form of red lesions which he attributed to the X-rays [5].

In the late 1890’s, other important advancements in medicine were due to the discovery of radioactive decay by Henri Becquerel and the discovery of the radioactive element radium by Marie and Pierre Curie. These achievements led to the Nobel prize in physics in 1903 and a change in the understanding of energy and elements. In radiation oncology, this provided physicians with the ability to target tissues within the body by the implantation of radioactive elements and expanded the use of radiotherapy.

In 1906, the first quantitative radio-biological experiments were performed by J. Bergonie and L. Tribondeau [6]. They carried out experiments to observe the effects of radiation on different cell and tissue types. Their conclusion was that cells that divided rapidly (such as endothelial cells) tended to be more sensitive to the effects of radiation. A year later in 1907, the relationship between a tumour and its vascular supply was suggested, and the invasion of veins by tumour cells was shown by selective staining [7]. However, the idea that tumours were dependent on the formation of new vessels or ‘neovasculature’ had yet to be shown.
Figure 1.1: One of the first x-ray images produced only a short time after Wilhelm Röntgen’s discovery of x-ray fluorescence. The image shows Mrs. Anna Berthe Röntgen’s wedding ring and the bones of her hand. Reproduced from Wikipedia.
In 1927, Muller showed that damage to cells in response to radiation occurred by mutagenesis or by genetic mutation [8]. A dose-response curve was determined using Drosophila following irradiation with x-rays. This mechanism of damage would serve to be the focus of radio-biology in the years to come.

In 1939, it was subsequently firmly established that tumours were dependent on the growth of new vessels, which was proven by Gordon Ide [9]. Ide et al. used the transplant chamber described by Sandison in 1928 [10] to monitor the growth of vessels in tumours transplanted in rabbits. It was discovered by Ide et al. that the implantation of tumours in the ears of rabbits would coincide with the formation of new blood vessels to the tumour [11]. Such blood vessels grow to supply tumours with nutrients and oxygenated blood as a response to hypoxia caused by rapid tumour growth beyond its blood supply.

Tumour cell hypoxia is an important concept in the study of radio-biology as it can influence the resistance of tumours to treatment and is indicative of tumour progression. Chronic hypoxia has been shown to be an important factor in the promotion of neovascular growth and tumour progression [12]. The importance of oxygen concentration in tissue during irradiation was first realized by Thomlinson and Gray [13] in the early 1950's. That discovery is what led to the conclusion that tumour cells which were hypoxic, without an adequate supply of oxygen, were radio-resistant. By histological analysis, it was confirmed that only tumour cells within a radius of 150 \( \mu m \) - 200 \( \mu m \) of a blood vessel were viable confirming tumour cell hypoxia [14]. Tumour cells beyond this distance demonstrated decreased DNA synthesis with increasing distance. This can be related to ‘the viable rim’ concept in which tumour cells at the peripheral edge of the tumour appear to recover quickly after radiotherapy (Figure 1.2). The radius of tissue containing viable tumour cells corresponds to the diffusion distance of oxygen. Since viable tumour cells typically appear in close proximity to capillaries this may be suggestive of a different state of the tumour vasculature in the viable rim [15].
Figure 1.2: Image showing the viable outer rim of a tumour labelled with the white letter 'V'. The black letter 'N' shows the necrotic core of the tumour.
After its discovery, chronic hypoxia was quickly realized as a challenge in the design of appropriate cancer therapies. As a result, much of the research into the tumour endothelium has focused on preserving the tumour vasculature.

More recently, the tumour vasculature has become the focus of targeted treatments of cancer. This may be attributed to the following theoretical advantages of targeting tumour vasculature and endothelium:

1. Endothelial cells are easily accessible to therapies since the endothelial cells make up the lining of the vessels and capillaries. Therefore it is easy to deliver cytotoxic agents to these cells.

2. Endothelial cells are early responders to radiation. Since radiation sensitivity is related to the proliferation rate of the tissue, and endothelial cells have high proliferation rates, they are quick responders to radiation.

3. Endothelial cells are genetically stable meaning they are unlikely to become resistant to new therapies.

These concepts can be attributed to several different lines of studies which emerged in the 1970’s and early 80’s [16, 17]. Of those studies, the discovery that the tumour neovasculature differed greatly in comparison with the structure of normal vasculature has proven to be of significance in identifying the tumour vasculature as a potential target for therapy. These differences can be exploited therapeutically so that the tumour neovasculature is targeted leaving normal vasculature intact.

It was the suggestion to target angiogenesis by Folkman that led to the role of the tumour vasculature being appreciated and considered as a target for therapies. Folkman’s work revealed an important requirement for the development of tumours. He demonstrated that in order for a solid tumour to develop beyond 2-3 mm in diameter, the process of angiogenesis must occur [18, 19]. Tumour angiogenesis is the process by which endothelial cells from the tumours host are recruited to the tumour where they
form the tumour neovasculature. Once the dependence of tumour growth on angiogenesi-
sis was realized, Folkman suggested a therapeutic approach against cancer by targeting
angiogenesis. He suggested the blockade of tumour angiogenesis factor (TAF) would lead
to the inhibition of tumour growth beyond a few millimetres [20, 21].

Over the next 20-30 years the quest to control angiogenesis was the focus of cancer research . In 2002, Ferrara et al. made an important discovery about the control of
angiogenesis by a molecular factor called vascular endothelial growth factor (VEGF)
leading to the development of related therapeutic agents [11].

After angiogenesis became a potential target for therapy, an important question be-
came, and which still remains today, is whether or not a therapeutic strategy should be
developed on the basis of inhibiting angiogenesis to starve the tumour of its blood supply
or by modifying angiogenesis in order to make the entire tumour more radio-sensitive. It
is now recognized that many cancer therapies cause effects on endothelial cells and the
vasculature. These include chemotherapy and radiation therapies and newer vascular
targeting therapies.

A vascular targeting therapy is any therapy which targets a solid tumour via the vascu-
lature. Presently there are several therapies which are targeted to the tumour vasculature
including but not limited to anti-angiogenic therapies such as therapeutic antibodies and
small molecules. While anti-angiogenic therapies serve to arrest or prevent the process
of angiogenesis, other types of vascular targeting therapies include vascular disrupting
agents such as tubulin-binding combretastatins [15]. Vascular disrupting agents rely on
the fact that within a solid tumour a large number of tumour cells may depend on only
a single vessel to provide them with necessary nutrients. This means selectively target-
ing certain tumour vessels can lead to a disruption in the amount of blood flow causing
potential necrosis of tumour cells due to starvation from the essential nutrients required
for tumour cell survival. Such drugs, however, in certain combinations with other drugs,
and at certain doses have been found to produce an undesired effect and instead serve
to normalize the tumour vasculature and aid tumour progression [22]. Therefore it is critical that there be a method to quantitatively monitor the tumour vascular response \textit{in vivo} regardless of the chosen therapeutic approach.

In this thesis the problem of measuring the tumour vascular response \textit{in vivo} is explained and a potential solution to this challenge is shown. In the first half of Chapter 1, an overview of the tumour vascular response to x-ray radiation therapy is presented starting with the tumour endothelium and working up in scale to the tumour microvasculature. In the second part of Chapter 1, a review of blood flow imaging is presented which focuses on high-frequency ultrasound imaging to detect tumour micro-circulation. In Chapter 2, after a brief review, the experimental portion of the M.Sc. thesis is presented. Finally, in Chapter 3, a summary of the previous chapters and a discussion of future work is presented. A modified version of Chapter 2 will be submitted to the International Journal of Radiation Oncology, Biology and Physics for Publication.
1.2 Background

1.2.1 Classic Mechanism of Radiotherapy

Radiotherapy has proven to be critical in the management and control of cancer, with over 50% of cancer patients receiving some form radiotherapy. In radiotherapy, high-energy electromagnetic radiation is administered into tumour tissue so that damage will occur. Ionizing radiation really refers to subatomic particles travelling at the speed of light with very high-frequencies ($> 10^{15} \text{Hz}$). As a result, collisions of beams of ionizing radiation with molecules or atoms cause the ejection of electrons producing damage within the molecules from which they were ejected. The mechanism by which tissue damage occurs as a result of its interaction with X-rays can be summarized in 3 steps:

1. Energy is transferred from incident photons and is absorbed by the target material. The amount of radiation energy absorbed in the absorption of an x-ray photon depends on the photon energy of the incident X-ray as well as the chemical composition of the absorbing medium. The amount of absorbed radiation or the dose of radiation is typically given in SI units of gray ($\text{Gy}$), where a single $\text{Gy}$ is equivalent to a joule of energy absorbed by a kilogram of tissue. There are two mechanisms of absorption namely the Compton process and the photoelectric process. For high energy X-rays such as those used in radiotherapy, the Compton process is the dominant of the two. In the Compton process, energy is transferred from incident photons to nearly free electrons or the electrons in the outermost shell of an atom. The transfer of energy comes in the form of kinetic energy from the electron causing the ejection of the electron and the production of free radicals. This type of energy transfer is called direct ionization. An example of this process is given in Equation 1.1 showing the ionization of water.

$$H_2O \rightarrow H_2O^+ + e^- \quad (1.1)$$

The damage caused by direct interactions is responsible for only a small portion of the overall DNA damage and occurs within a fraction of a second ($10^{-15} \text{s}$) after irradiation.
(2) As the result of many such ionizations, a second interaction is responsible for the majority of damage to DNA within cells. In the case of water which makes up more than 75% of living tissue, direct interactions produces $H_2O^+$ (Equation 1.1) which is both a free radical and an ion. In the presence of other water molecules the following reaction will occur:

$$H_2O + H_2O^+ \rightarrow H_3O + OH^•$$

with highly reactive hydroxyl free radicals as products. Secondary or indirect interactions are the result of damage caused by hydroxyl free-radicals.

(3) Biological effects are observed in the final step which may occur within seconds, days, weeks or even months after irradiation. These biological effects include delayed cell growth, mitotic cell death and apoptotic cell death [23].

Classically in radiotherapy, tumour cure has been thought to require the depletion of clonogenic tumour stem cells. This mechanism of tumour cell death involves mutagensis and the subsequent propagation of mutations to stem cell progeny during stem cell division with the end result being the accumulation of lethal mutations and eventual tumour cell death. Other forms of tumour cell kill such as apoptosis, however have been largely neglected as a method of tumour cure.

The apparent focus of radio-biology which targets only tumour clonogens has been greatly modified in recent years.

Recently, the importance of the cross-talk between tumour cells and host-derived microvasculature has been highlighted [24]. Furthermore, Garcia-Barros et al. have demonstrated in a specialized genetic system that tumours resistant only to endothelial cell apoptosis were completely radio-resistant (15-20 Gy) [2].

In our lab we have previously demonstrated that with single large dose x-ray radiation therapy (XRT) there can be large macroscopic areas of apoptotic death in regions of dense vasculature (Figure 1.3). Also clinically, single dose 8 Gy treatments have been used to cause rapid cessation of bleeding of tumours.
Figure 1.3: Immunofluorescent stained human prostate cancer sections from a SCID mouse 24h after 8 Gy XRT. Green TUNEL staining for apoptosis (left) and red CD-31 staining for endothelial cells (middle) shows corresponding and coincident increased staining. The blue stain DAPI PC-3 (right) indicates cell nuclei of both human PC3 cells and mouse endothelial cells. The yellow line was added to separate the region dense of vasculature (right of line) from that with relatively little vasculature.
1.2.2 Tumour Vascular Response to Radiotherapy

Over the years there have been a wide range of studies which have investigated the complex molecular and cellular responses which occur within vascular endothelium a short time after irradiation [25]. It has been well established that endothelial cells are among the first to respond after ionizing radiation. The vascular effects of radiation are actually seen in two main stages [26]. The first is the ‘early’ response in which endothelial cells undergo apoptosis. Histologically, vessels start to undergo morphological changes with capillaries exhibiting more changes than larger vessels. These changes include; detachment of endothelial cells from the basal lamina, thrombosis, and cell pyknosis with possible microvascular consequences [25]. The early response may begin to occur within 24h after irradiation. In the second ‘late’ stage, which occurs within months after irradiation, permanent capillary collapse, basement membrane thickening, loss of clonognenic capacity, and scarring of surrounding tissue is observed [25]. In the remainder of this thesis, only the initial response to ionizing radiation will be discussed and evaluated.

Various enzymes and growth factors have been implicated in the process of endothelial cell apoptosis, or programmed cell death, within the vascular endothelium [24, 27–29]. One particular pathway leading to endothelial cell apoptosis which has had special attention, is the radiation-induced activation of acid sphingomyelinase and ceramide production [30].

1.2.3 Endothelial Cell Apoptosis

Apoptosis or programmed cell death refers to the biochemical processes by which cells undergo characteristic changes causing cell death. In most multicellular organisms each cell contains a set of instructions, which once activated will lead to characteristic changes in that cells phenotype. In healthy tissue, this process is essential for homeostasis. The mor-
phological changes induced during apoptosis can be summarized in the following steps; (1) blebbing; (2) cell shrinkage; (3) nuclear fragmentation (4) chromatin condensation (5) chromosomal DNA fragmentation.

Activation of an apoptotic pathway varies for different cell types and sub-cellular components, as does the biochemical signalling which causes cell death. Typically, an apoptotic pathway is initiated by physiologic and stress stimuli. One example occurs during tissue repair after an incisional wound where reactive oxygen species such as hydrogen peroxide are generated by phagocytes as part of a tissues inflammatory response. Hydrogen peroxide which causes an oxidative stress at inflammatory sites may then trigger apoptosis.

Endothelial cell apoptosis may occur via several different pathways, however, since the focus of this thesis is on the effects of radiation on PC-3 tumours, the remainder of the thesis will focus on the acid-sphingomyelinase pathway (ASMase). In the ASMase pathway elevated levels of ceramide instruct endothelial cells to undergo apoptosis. It is important to note that endothelial cell death via ceramide signalling has been shown to be independent of DNA damage. Specifically, Bonnaud *et al.* [26] have demonstrated the independence of pre-mitotic apoptosis dependent on ceramide generation from DNA damage-induced mitotic cell death. These studies involved the use of sphingosine-1-phosphate, a ceramide antagonist.

Rather, the mechanism of activation of this pathway is dependent on radiation damage at the level of the cell membrane and in the limit, is independent of double stranded DNA breaks and posits [2, 31, 32].

Therefore in order to understand the radio-biology of tumour cells, one must go beyond the classical views of radio-biology which hold damage to the clonogenic compartment as the most significant response to radiation. Revised views include these compartments which are independent of DNA damage.

Although claims that microvasculature dysfunction via activation of the ceramide
pathway have been suggested, the effect of radiation on the tumour micro-circulation has not been sufficiently studied *in vivo*. This is most likely due to the limited ability to readily and non-invasively image the tumour micro-circulation, as current assays of vascular morphology and physiology such as chronic transparent chambers are extremely invasive [3].

### 1.2.4 Monitoring Tumour Response to Radiotherapy

Current assays of vascular morphology and physiology tend to be invasive [3]. There are non-invasive medical imaging modalities such as magnetic resonance imaging (MRI), multi-detector x-ray computed tomography (CT), positron emission tomography (PET), single-photon emission computed tomography (SPECT) and ultrasound that may be used to image the tumour microvasculature however, each modality suffers from limitations.

Computed tomography allows for non-invasive qualitative and quantitative evaluation of vascular treatment response using iodinated contrast agents [33]. Clinically, contrast-enhanced CT is a useful modality particularly for the detection and characterization of metastatic tumour lesions [34]. However, CT’s contrast agents are toxic and do not provide the appropriate sensitivity to detect small vessels [35].

Steady-state MRI also provides the ability to image the micro-circulation at relatively high-resolution using low toxicity contrast agents to enhance sensitivity to blood flow. However, the high cost, susceptibility to motion artifacts, low temporal resolution and relatively low sensitivity limit the use of MRI for monitoring tumour microvascular responses [36].

MR and CT systems have been scaled down in an attempt to improve the resolution and sensitivity for microvascular imaging in preclinical animal models. Micro-CT and Micro-MR have been implemented with greater resolution than their clinical counterparts [37]. Micro-CT can be used to visualize tumour microvessels in preclinical animal models, but at high radiation doses, making longitudinal studies difficult [38]. Although some
of these imaging techniques described provide the ability to image the micro-circulation, the diffusible nature of the contrast agents make it difficult to study tumour vessel architecture. This is due to the inability to differentiate between contrast which has diffused into the extracellular matrix from that contained within the microvasculature. Therefore quantification requires complex modes which take into consideration the transport of these small molecules. In addition, neither SPECT nor PET clinical systems provide the resolution necessary (3-7mm) to distinguish individual tumour microvessels. Although PET may not be able to resolve individual vessels, it is one of the most sensitive modalities for detecting blood flow [35]. However, micro-PET and micro-SPECT systems have also been developed and offer improved resolution (1.5mm).
1.3 Ultrasound for Imaging the Micro-circulation

One of the most promising imaging modalities for the functional and anatomical imaging of tumour micro-circulation is Doppler ultrasound imaging. Typical clinical ultrasound systems (3-10 MHz) have the ability to detect vessels on the order of 100 µm, one of the best known achievable resolutions. For high-frequency Doppler ultrasound (10-80 MHz), used in preclinical animal imaging, the ability to detect and resolve microvessels is greatly improved (15-20 µm) in comparison with clinical systems [39].

An advantage of Doppler ultrasound imaging is that it does not require exogenous contrast agents, however, contrast agents such as micro-bubbles are available to offer increased sensitivity to detect slow flow\(^1\). In addition, there are multiple operation modes for blood flow detection and measurement (for example: mean-frequency colour-flow imaging mode) which can be selected depending on a particular application.

Power Doppler (PD) ultrasonography is a useful tool for the depiction and quantification of blood flow. Invented for cardiac applications in the early 1990’s and subsequently popularized by Rubin et al. [16, 41], power Doppler has become a particularly useful tool for the detection of blood flow. Power Doppler is currently used clinically for diagnostic applications such as the diagnosis of cancer, ovarian cysts, endomytriosis and many other conditions with uses primarily in gynaecology [42–46].

Although initially invented for use in cardiac imaging of stenosis, the use of power Doppler has been expanded for many applications. It has been shown to be a useful tool for the evaluation of vascular response to therapy in human and animal cancer models. This is due to increased flow sensitivity over conventional mean-frequency Doppler, making power Doppler particularly useful for the detection of blood flow in regions of slow flow, as is the case in tumours [3, 44, 46–52].

\(^1\)Quantifying vascular changes in tumours using micro-bubbles has yet to be established. There are currently techniques which are being developed with promising results [40].
1.3.1 Early Work with Doppler Ultrasound

Although power Doppler is a relatively new ultrasound mode, the use of ultrasound to non-invasively detect blood flow is not a recent invention. The first ultrasound system to study blood flow was described by Satomura in 1957 [53]. Satomura studied the cardiac cycle by sending continuous ultrasound waves toward the heart from the surface of the chest wall. Frequency changes could then be detected in the reflected wave as a result of the motion of the heart. The shift in frequency is termed the Doppler effect. The Doppler effect explains that the observed frequency of a periodic wave will be shifted if there is relative motion between the observer and source of the wave. A familiar example is the increase in pitch heard as a sounding fire truck approaches and then decrease in pitch as it passes and moves away. This can be understood by examining the following equation

\[ \frac{f_{Doppler}}{f_0} = \frac{c + v_o}{c + v_s} \]  

(1.3)

which relates the received Doppler shifted frequency \( f_{Doppler} \) to the velocity of a moving source and/or observer, where \( f_0 \) is the original frequency, \( v_o \) is the speed of the observer, \( v_s \) is the speed of the source and \( c \) is the speed of sound.

The use of Doppler ultrasound to study blood flow in tumours was established over 25 years ago [54]. In the late 70’s and early 80’s, Burns et al. were among the first to study tumour blood supplies in vivo. They used a continuous wave Doppler system to detect and characterize blood flow in breast tumours\(^2\). In a continuous wave system, a sinusoidal ultrasound wave is continuously emitted by one transducer element and the reflected wave detected by another. In the case of ultrasound blood flow detection, two Doppler shifts occur. The first Doppler shift occurs when the transmitted ultrasound is received by moving scatterers namely the red blood cells (RBCs) [55]. Erythrocytes or red blood cells account for approximately 45% of the blood by volume and are approximately 3-10

\(^2\)Interestingly, this was the first in vivo evidence supporting a multiple feed arteries model of a tumour’s blood supply.
\( \mu \text{m} \) in size [56]. The moving RBCs then re-radiate the sound causing a second Doppler shift which is received by the transducer. The reflected signal and transmitted signal are then multiplied and low pass filtered to extract a Doppler shift. Equation 1.4 relates the Doppler shift frequency, \( f_D \), the frequency of the incident ultrasound wave, \( f_o \), the speed of sound, \( c \), and the velocity of the moving RBC’s, \( v \).\(^3\)

\[
f_D = -\frac{2vf_0}{c}\cos\theta
\]  

(1.4)

If quadrature detection is used then it is also possible to determine the polarity of the Doppler shift and therefore the direction of motion away from or towards the transducer. One difficulty with continuous wave Doppler is the inability to distinguish depths. This may be overcome by use of pulsed ultrasound which allows for depth location.

1.3.2 Doppler Ultrasound Today

Today’s scanners typically use pulsed Doppler and do not directly measure the Doppler effect. Instead pulses of ultrasound with a frequency, \( f_o \), are transmitted into tissue at a rate called the pulse repetition frequency (\( PRF \)). Over time, multiple pulse-echoes\(^4\) are sampled at times corresponding to a particular depth within the tissue. If a pulse has been scattered by moving blood, then successive echoes returning from a particular location will be shifted in time (or phase) by an amount which is proportional to the velocity of flow.

The signal produced by the interaction of ultrasound with blood is dominated by scattering with erythrocytes. In addition to the signal received from blood the Doppler signal contains a ‘clutter’ signal produced by tissue motion (See Figure 1.4). In order to accurately depict blood flow, the Doppler signal must be processed to remove unwanted signal and to extract Doppler estimates. The general approach to colour-flow processing

\(^3\)Equation 1.4 is a first order approximation of the two combined Doppler shifts

\(^4\)Pulses returning to the transducer after interacting with tissue
of pulsed Doppler data can be outlined by these 3 key processes which include: clutter filtering, flow parameter estimation and discrimination between flow and noise [39]. In the first step, a high-pass filter, called a wall filter is employed to remove the 'clutter' signal. From Figure 1.4 (a), one can see how this process may be difficult in the case where slow flow is being detected, as there will be overlap of the signal from tissue and blood.

The second processing step involves estimating flow parameters. These parameters are extracted from the Doppler signal and typically reflect the velocity or amount of flowing blood. For mean-frequency estimation, estimates of scatter velocities and power can be calculated using autocorrelation. Since the Doppler signal contains a spectrum of frequencies corresponding to various scatterer velocities it is important that the PRF is set in order to avoid aliasing (PRF must be greater than twice the maximum Doppler shift frequency). Additionally, to obtain accurate estimates of blood velocities, the angle between the ultrasound beam axis and direction of flow must be taken into consideration.

In mean-frequency Doppler, the location of the peak of the blood portion of the spectrum along the frequency axis is estimated. Alternatively, the total integrated power of the Doppler signal may be estimated rather than the mean-frequency. This corresponds to taking the area under the blood portion of the spectrum. It can be shown that the power of the Doppler signal is related to the number of red blood cells being interrogated. There are some key advantages to calculating the power of the Doppler signal rather than the mean-frequency shift. These include the near independence of the scan angle, absence of aliasing, and increased ability to detect slow flow. When the scan angle is perpendicular to flow then no Doppler shift will be observed and therefore no power estimate can be made.

A major limitation in mean frequency colour Doppler is the way in which noise is represented in the Doppler signal. Since noise may contribute across the entire spectrum of frequency shifts, there will be noise contained within the mean-frequency estimate.
This may contribute to artificial estimates of velocity. Since the majority of noise is low power, as shown in Figure 1.4 [57], in the third processing step, separation of noise and flow involves selecting a minimum power level cut-off. This elucidates the primary advantage of power Doppler which is increased flow sensitivity. The increased sensitivity in power Doppler is due to the way in which the noise is represented in power Doppler images. This allows for increased gain with an improved signal-to-noise ratio compared with mean-frequency Doppler.

1.3.3 High-Frequency Doppler

There are a number of modifications that have been made in an effort to improve the detection of tumour micro-circulation in non-clinical research environments. These include the advent of high-frequency animal imaging ultrasound systems as well as three-dimensional imaging techniques. However, there are downsides to ultrasound, of which the most significant appear to be its operator dependence and limited depth penetration. When choosing an ultrasound flow imaging mode to monitor changes in vascular morphology, high-frequency power Doppler is potentially the best choice due to its increased sensitivity to slow flow.

In general, for scattering of sound by blood, the intensity of the signal increases with frequency to the fourth power [3, 48]. Figure 1.4 demonstrates how this corresponds to a larger amplitude of the blood signal with respect to the noise floor and therefore an increased signal to noise ratio. Additionally, if one considers Equation 1.4 it is obvious that the Doppler shift frequency is linearly proportional to the transmit frequency. This means that increasing transmit frequency corresponds to a larger spectrum of Doppler shift frequencies. Additionally, increasing the transmit frequency allows for imaging at a finer spatial resolutions.

Only a brief review of the principles and settings relevant to power Doppler ultrasound has been presented here. For a comprehensive review of Doppler ultrasound the reader
should refer to Burns [55] and Cobbold [58]. For high-frequency 3-D Doppler the reader should refer to Goertz et al. [47, 59].
Chapter 1. Introduction

Figure 1.4: Hypothetical Doppler spectra showing the signal from blood and the 'clutter' signal from tissue. The left figure (a), shows a spectrum obtained at clinical frequencies while on the right (b) shows a spectrum obtained with higher frequencies.

Figure 1.5: A demonstration of the fundamental difference between the Doppler signal and corresponding display for colour-flow imaging and power Doppler image formation. The left side shows that a constant amplitude with varying frequency will be reflected by a change in colour value for the colour-flow image with power Doppler colour values staying constant. On the right side, varying amplitude but constant frequency leaves colour-flow imaging unaffected but changes the colour for power Doppler images.
Figure 1.3.3 (a) shows that a constant amplitude with varying frequency will cause a change in the colour-flow image leaving the hue on the power Doppler image unaffected [60]. In figure 1.3.3 (b), varying amplitude but constant frequency leaves the colour-flow image unaffected but produces a change in hue for power Doppler colour.

Quantified power Doppler is a technique which calculates indicies based on power Doppler signals in an attempt to make quantitative inferences about tissue and in the case of this thesis, tumour vascularity. Typically, quantified power Doppler has been used to estimate relative differences in vascularity among two or more populations. In this thesis, the goal is to detect changes in tumour vascularity over time and with therapy in an attempt to monitor the therapeutic changes induced. The indicies below are typically used in quantified power Doppler for estimation of measurements similar to physiological parameters. It is important to realize that the validity of these indicies relies on the assumption that the integrated Doppler power is related to the ‘clump’ density of red blood cells within the volume. It has been shown by Cobbold et al. that the Doppler signal is a Gaussian random process which arises from fluctuations in scattering from red blood cells [61]. They also theorize that the power of the Doppler signal is proportional to the variance of fluctuating red blood cell concentrations. With this in mind, power Doppler indicies have been created to reflect blood flow measurements [49, 62–64].

**Power Doppler Indicies**

The first index is the vascularization index (VI) which measures the relative number of colour-flow voxels and represents the amount of vasculature. Typically, VI is expressed as a percentage.

\[
VI = \frac{\text{Number of colour voxels}}{\text{Number of voxels in VOI}} \cdot 100\%
\]  

(1.5)
The second index is the flow index (FI) is the average colour-flow value of all coloured voxels, representing the average flow intensity of moving red blood cells.

\[ FI = \frac{\sum \text{Colour voxel values}}{\text{Number of colour voxels in VOI}} \] (1.6)

Also, the vascular flow index (VFI) is the average colour-flow value of all voxels, with voxels not containing flow assigned a colour value of zero. This is the product of FI and VI and might be regarded as ‘perfusion’.

\[ VFI = \frac{\sum \text{Colour voxel values}}{\text{Number of voxels in VOI}} \] (1.7)

Finally, the number of distinctly separate power Doppler signals or objects (NO) can be calculated [65, 66].

The relationship between power Doppler indicies and true blood flow characteristics has recently been examined by Raine-Fenning et al. [62]. They examined the effects of flow rate and erythrocyte density on the VI, FI and VFI. They determined using a controlled blood flow phantom that VI also increases linearly with flow rate. FI increased with flow rate to the third power. VI increased significantly with increased erythrocyte density. FI also increased but plateaued.

Although FI has been shown to change with varying flow rate and erythrocyte density, it has not been established as a good indicy for comparing tumour vascularity. It has been shown that FI does not correlate with expected true blood flow characteristics, particularly when many vessels are included in the calculation. Intuitively, there is a problem with FI in that one would expect the average power to be indicative of blood scatter content, or hematocrit, rather than the amount of flow. One could imagine better representative indicies for flow and perfusion which contains velocity information. Additionally, since VFI is dependent on FI, it may be a non-reliable indicy. However, VFI has been shown to correlate with flow rate and erythrocyte density [67].
1.3.4 Microvessel Density

A gold standard for understanding tumour vasculature is based on histochemical methods. The most common method for measuring tumour vascularity is called *intratumoural microvessel density*. Validation of new techniques to quantify tumour vascularization should thus be compared with standard microvessel density measurement methods. In a microvessel density technique, vascular hot spots are found by scanning stained slides at a low objective power and once the most densely staining regions are identified they are magnified using a 20 X objective and counted. The number of distinct staining regions are then counted [68]. This implies that a clear difference between the stained region and the background staining must be observed. Tumours with no distinct high-quality stain are not included in analyses. This is due to the fact that staining is highly dependent on proper processing and even the smallest error can lead to artifact. It is generally recommended that the staining procedure be performed by someone experienced or only used for quantitative analysis once the experimenter has mastered the technique. Microvessel density has been shown in some cases to correlate with tumour prognosis [35, 68]. Another technique to quantify the amount of vasculature is by calculating the stain positive area fractions, or the ratio of the area stained to the sample area.

Histological staining to measure vascularity is typically done with CD31, CD34 or factor VIII (von Willibrand’s Factor) [69]. Although many studies have used factor VIII, it is generally not recommended as it cross-stains heavily with blood plasma. This however makes factor VIII a potentially better stain for visualizing vessels and their leakiness.
1.4 Hypothesis and Specific Aims

The hypothesis of this thesis is that using high-frequency three-dimensional power Doppler ultrasound imaging, the tumour vascular response to high-dose (8 Gy) single-fraction XRT can be detected and measured immediately (24h) following treatment. This vascular response to therapy is thought to result in significant effects on tumour blood flow which can be detected and quantified.

In Chapter 2 this thesis will address the quantification of the vascular response to x-ray radiation therapy using high-frequency three-dimensional power Doppler. The results of these power Doppler measurements are then compared to immunohistopathology. In addition, the study attempts to address the changes in three-dimensional power Doppler measurements in relation to the size of intra-tumoural blood vessels.
Chapter 2

Monitoring Tumour Vascular
Response to Radiotherapy

2.1 Introduction

The mechanism of tumour cell kill in response to ionizing radiation is still unclear despite its long proven usefulness in radiotherapy. Recent claims that microvascular damage may regulate tumour cell response to radiation presents a shift from the classic view of tumour cell kill in which radiation targets tumour clonogens [2]. Classically, the emphasis in radio-biology has been on the negative impact of inadequate vasculature leading to radio-resistance in hypoxic tumour cells, while overlooking the potential therapeutic benefit of microvascular collapse and hypoxic tumour cell death [1].

Over the years there have been a wide range of studies which have investigated the complex molecular and cellular responses which occur within a short time after irradiation with ionizing radiation [25]. Various enzymes and growth factors have been implicated in the process of endothelial cell apoptosis within the vascular endothelium in response to radiation. One particular pathway leading to apoptosis which has received special attention is the radiation-induced activation of acid sphingomyelinase (ASMase) and
ceramide production [25].

More recently, the importance of the cross-talk between tumour cells and host-derived microvasculature has been highlighted [24]. Furthermore, Garcia-Barros et al. have demonstrated in a specialized genetic system that tumours resistant to endothelial cell apoptosis render them strikingly radio-resistant (15-20 Gy) [2]. We have previously demonstrated that with single large dose X-ray radiation therapy (XRT) there can be large macroscopic areas of apoptotic cell death linked to areas of hypervascularity. Although claims that microvasculature disruption via activation of the ceramide pathway have been suggested, the effect of radiation on the tumour micro-circulation has not been sufficiently studied in vivo. This is most likely due to the limited ability to readily and non-invasively image the tumour micro-circulation. Today, assays of vascular morphology and physiology such as chronic transparent chambers remain extremely invasive [3].

Colour-flow ultrasound imaging has been a popular choice for studying circulation. However mean-frequency colour-flow imaging, which displays the spatial distribution of blood velocities does not provide optimal sensitivity to detect the slow flow present in the tumour micro-circulation. Power Doppler ultrasound has the advantage of being more sensitive to slow flow as it detects the relative power or energy of ultrasound reflected from moving blood. This is desirable when no directional flow information is necessary as it eliminates the dependence on the frequency content of the ultrasound signal and therefore is not susceptible to aliasing. The colour signal shown in power Doppler images is instead representative of the area under the power frequency spectrum and can be related to the number of moving red blood cells or the moving blood volume being detected. Due to its increased sensitivity to slow flow power Doppler is also sensitive to subject or transducer motion potentially resulting in flash artifacts which may simulate blood flow [64].
Power Doppler ultrasonography has previously been used to study the effects of therapy on circulation, however typical ultrasound frequencies used in the clinical setting (< 10 MHz) do not provide the detection limits necessary to image the micro-environment. Recent advances in high-frequency ultrasound technology now make it feasible to study the tumour micro-environment in vivo using small animal tumour models. When extended to three dimensions, power Doppler ultrasound makes it possible to study vascular morphology and flow. High-frequency 3-D power Doppler can thus be employed to study the micro-circulation and monitor the effects of therapy on the micro-circulation [48, 59].

It has already been established that indices calculated from power Doppler ultrasound data correlate with immunofluorescent staining in a treated tumour mouse model using quantified 2D power Doppler ultrasound at clinical frequencies [49]. However, clinical ultrasound scanners do not achieve the appropriate resolution necessary to detect blood flow in the target size range for the tumour micro-circulation. In order to resolve blood flow in the micro-circulation the system resolution must be on the order of tumour microvessel sizes which are approximately 10 µm in diameter with flow velocities in the order of 1 mm/sec [3].

Studies have been done to examine the limits of detection for high-frequency ultrasound systems with small animal models. Since, resolution is proportional to the ultrasound frequency being used, it may be possible to achieve resolution from 15 to 100 µm with ultrasound frequencies in the 20-100 MHz range [3].

In vivo experiments using mouse ears have been conducted by Geortz et al. to determine the size limit to resolve vessel branching patterns. They showed the ability to distinguish closely spaced vessels of 30-100 µm in diameter [59]. They have also verified the ability to identify the morphology of the tumour micro-circulation. Geortz et al. have also shown the ability to use high-frequency Doppler ultrasound to detect changes in blood flow after treatment with a vascular targeting agent. Their results were verified
by measurements of tumour perfusion using Hoechst 33342 staining [48].

In this thesis chapter it is shown that single fraction high dose radiotherapy can lead to microvascular changes in tumours which can be monitored using high-frequency 3-D power Doppler. We demonstrate this in a prostate tumour model with results indicating microvascular effects with large radiation doses. Results indicate a predominant effect of radiation on the smallest detectable Doppler signals with up to 54% reduction in blood flow. This thesis chapter will be submitted for publication to the International Journal of Radiation Oncology, Biology and Physics and represents the experimental work undertaken for this thesis.
Chapter 2. Monitoring Tumour Vascular Response to Radiotherapy

2.2 Materials and Methods

2.2.1 Mouse model

Sixteen solid human prostate cancer tumours (PC-3, American Type Culture Collection) were grown by xenograft transplantation in SCID mice. Approximately $10^6$ PC3 cells in 50 $\mu$L phosphate buffer solution volume were implanted subdermally in the hind left leg of each of the 16 mice [70]. Tumours were allowed to develop to 7 to 10 mm in diameter, after approximately 30-45 days. Eight of the 16 tumours were treated with 8 Gy XRT and 8 were sham irradiated (0 Gy controls).

2.2.2 Treatment

Treatment consisted of irradiation using an X-ray irradiator (Faxitron, IL, USA) calibrated to penetrate the mouses cage. Anesthetized mice were placed in their cage and lead shielding was used to cover the mouse leaving only the tumour exposed. Animals were then placed within the irradiator with the tumour at the center of the X-ray beam. X-rays with an energy of 160 kVp at a dose rate of 200 cGy/minute in one fraction were administered to the tumour volume. An 8 Gy dose was selected as it has previously been suggested as the lowest clinically relevant dose which potentially initiates substantial microvascular destruction [2, 24, 27–32].

2.2.3 Data Collection

In order to assess tumour blood flow, power Doppler data was collected using a Vevo 770 system (VisualSonics, Toronto, ON) with a RMV-710B (25 MHz) probe using the Vevo integrated rail system to facilitate 3-D scanning.

All tumours were scanned before (0 h) and again (24 h) after treatment. Mice were anesthetized with a mixture of ketamine (100 mg/kg), xylazine (5 mg/kg) and acepromazine (1 mg/kg) to reduce motion and subsequent image artifacts. Hair was removed
from tumours prior to scanning using a depilatory agent as performed in previous studies. Mice were mounted on the mouse handling table with their front and hind legs taped to the electrical sensors to reduce motion and facilitate ECG monitoring of mouse vital signs. Clear ultrasound coupling gel was placed over the tumour and the handling table was mounted on the scan stage of the integrated rail system.

Volumes were acquired using the Vevo 770’s 3-D motorized scan stage (VisualSonics) with a step size of 0.1-0.2 mm. A 2D scan at the center of the tumour was employed as a reference image used to determine optimal settings and placement of the power Doppler window. Power Doppler settings were configured for maximum sensitivity to slow flow while reducing noise (wall filter 2 mm/s, scan speed 1-2 mm/s, pulse repetition frequency 5 KHz, low and high power cutoff 14,40 dB, speed medium, Gain 20 dB).

Ultrasound data consisting of B-mode and power Doppler volumes were acquired and then stored for analysis. Power Doppler acquisition over the entire volume lasted approximately 3 min per tumour depending on step size and tumour volume. Volumes were then exported and processed in Matlab (MathWorks, Natick, MA) using custom software. A brief description of the software is provided in the Appendix.

2.2.4 Image Processing

From the B-mode images, tumour voxels were segmented manually by drawing a region of interest (ROI) over the tumour in each slice of the volume constituting a volume of interest (VOI). VOIs were selected to exclude the signal from skin overlaying the tumour. The tumour centroid was calculated using the centre of mass formula. Colour voxels within the VOI were analyzed to evaluate blood flow. Four indices were calculated for each volume. Vascularity Index (VI) was calculated as the ratio of the number of colour voxels to VOI voxels. Vascular flow index (VFI) was calculated as the sum of all colour values over the number of VOI voxels as a measure of perfusion. In addition, the number (NO) and size of distinct clumped colour regions was also calculated as an estimate of
the number and size of tumour vessels and their centroid computed\(^1\).

As power Doppler ultrasound is highly sensitive to motion, artefact’s were common in some mice which exhibited extensive motion. Volumes which contained more than five slices with artifacts were excluded from the study.

### 2.2.5 Histological Analysis

After scanning was complete at 24\(h\) mice were sacrificed and tumours were extracted and processed for histological analysis. Processing included slicing the tumour in half in a plane parallel to the ultrasound scan plane and immediate placement in 10\% buffered formalin. Excised formalin-fixed tumours were paraffin embedded, sectioned and mounted on slides for staining. Tumour sections were sent away for immunohistochemical processing (Toronto General Hospital, Pathology Lab). Tumours were stained with haematoxylin & eosin for tumour cell morphology. For qualitative assessment of apoptototic cell death TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assay was performed. CD-31 staining was employed as a marker for endothelial cells. Additionally, Factor VIII (von Willebrand’s factor) was used to stain endothelial cells as well as blood plasma for qualitative analysis.

Digital images of stained tumour sections were captured using a microscope and digital CCD (Leica Microsystems, DM LB and DC200, Bannockburn, IL) using a 20x microscope objective lens. For sections stained with CD-31, 12 images from 3 sections (4 images/section) were captured in the most densely staining regions within the tumours. The percentage of staining and stained region sizes were calculated using manual segmentation in custom written software in Matlab.

\(^1\)FI was also calculated but not included in the thesis due to the difficulties associated with FI as mentioned in Chapter 1, Power Doppler Indicies. Since FI is related to hematocrit it would not be expected to change with treatment. This is what we observed.
2.2.6 Statistical Analysis

A one-tailed Wilcoxon matched pairs test was performed to test for significant decreases between power Doppler indicies at 0\( h \) and 24\( h \). Reported average changes (ratio of 24\( h \) to 0\( h \)) in indicies and their standard deviations as well as P-values are presented below. Finally, to validate histological analysis a correlation between measured power Doppler vascularity index and corresponding fractional stained area from histology was performed. A linear regression analysis was performed and an \( r^2 \) value was reported. All statistical analysis was performed using GraphPad InStat (GraphPad Software Inc., La Jolla, CA) with 95\% confidence intervals.
2.3 Results

2.3.1 3-D Power Doppler Images

Representative 3-D power Doppler images were constructed using a maximum intensity projection of power Doppler data overlayed on maximum intensity projections of B-mode scalar data with power Doppler values ranging from 0 to 255. Power Doppler data was colour-mapped in hues ranging from dark red to bright yellow respectively. Rendered images typically contained bright yellow vessel-like structures with dark red edges. These structures appear to have branching patterns which appear chaotic and tortuous. The fraction of the tumour which contains vessel-like structures appeared to increase or remain constant in the untreated tumours (Figure 2.1 (a) and (b)). In contrast, the fraction of vessel-like structures appeared significantly reduced after treatment (Fig. 2.1 c) and d)). Although the majority of vessel-like structures are connected, some separate patches were seen primarily in the outer portion of the tumours.

2.3.2 Power Doppler Analysis

In order to quantify apparent changes in ultrasound power Doppler data, indices were calculated for each tumour before and after treatment and compared by graphing the relative change for each tumour for both untreated and treated tumours. Figure 2.2 presents a plot of the relative change of VI, VFI and NO for each tumour individually. A statistically significant difference was observed in VI between 0 and 24h for treated tumours but not control, with treated tumours exhibiting a significant drop in vascularity index (0.68±0.07, P=0.03) with radiation. Sham treatment demonstrated no change (1.07±0.08, P=0.34). The vascular flow index which is dependent on the previous two indicies was calculated and displayed a drop in potential tumour perfusion (0.68±0.07, P=0.03 vs 1.07±0.08, P=0.34).

The relative change in the number of detected objects (0.92±0.10 vs 1.21±0.32)
Figure 2.1: Representative 3-D power Doppler data reconstructed from 2D image planes of B-Mode and power Doppler-mode data. Images were rendered with a maximum intensity projection through the ultrasound volume. Untreated tumours at 0h (a) and 24h (b) appear either to have a slight increase in vascularity or remain relatively unchanged. There appears to be a significant decrease in the amount of signal present in treated case at 24h (d) with respect to 0h (c). The scale bar represents \(1 \text{mm}\). B-mode data is represented in a gray scale mapped from 0-255. Power Doppler data is represented in hues of red to yellow and are also mapped with values of 0-255.
Chapter 2. Monitoring Tumour Vascular Response to Radiotherapy

Figure 2.2: Graphs demonstrating the relative change in power Doppler indices for each tumour for both untreated and treated tumours. On the ordinate, the ratio of indices for 24\textit{h} and 0\textit{h} is plotted for each treatment condition. In the first graph (top) a relative decrease in vascularity index for the treated case is observed. Similarly, a decrease is observed for vascular flow index (middle) however, no apparent difference is observed for the number of objects (bottom). Horizontal lines show the mean values of the relative change for each treatment.
Chapter 2. Monitoring Tumour Vascular Response to Radiotherapy

appeared to have a strong trend towards a lower number of objects in treated tumours, however a wide range of values in treated tumours was observed when compared to control values and no significant difference between 0 and 24h was observed for control and treated tumours. Based on statistical analysis of each treatment group and indicy, only VI and VFI were significantly different after treatment. No other statistically significant differences were observed.

2.3.3 Histology Analysis

Hematoxylin and eosin staining and TUNEL staining (Figure 2.3) provide qualitative information about the tumours structure and areas of tumour cell death. Representative images after treatment (Figure 2.3 b) and d)) show large areas of apoptotic cell death. At higher objective power factor VIII and CD 31 sections (Figure 2.4) showed features of tumour microvessels. In control sections (Figure 2.4 (a) and (c)) staining appeared localized to tumour vessels in both the factor VIII and CD31 sections. However, in factor VIII treated sections (Figure 2.4 b)), staining appeared diffuse surrounding darker staining vessel like structures. Treated CD31 sections appear to contain localized staining but did not appear to contain as many small staining vessels.

To validate histological analysis, a correlation between measured power Doppler vascularity index and corresponding staining index was performed for control and treated groups. However, since calculating the change in staining indices is not possible at more than one time, power Doppler indicies at 24h after treatment were compared with histology measurements.

The average vessel area was calculated for each set of histology sections. The mean value for each tumour treatment group was calculated. The mean average vessel area for 0 Gy and 8 Gy treatment groups were 650±150 µm² and 780±130 µm² respectively. In comparison with measurements made with power Doppler (780±20 µm² for 0 Gy and 970±170 µm² for 8 Gy), these measurements appear to follow the same trend and are of
Figure 2.3: Hematoxylin and eosin (H&E). Untreated (a) and treated 8 $Gy$ (b) 24h after treatment. Hematoxylin colours nuclei blue; eosin colours the cytoplasm pink. TdT-mediated dUTP-biotin nick end-labeling (TUNEL). untreated (c) and treated 8$Gy$ (d). TUNEL stains DNA strand breaks dark. Bar = 1 $mm$. 
Figure 2.4: Factor VIII staining blood plasma and endothelial cells for untreated (a) showing a distinct localized staining pattern, and treated 8 Gy (b) showing diffuse staining extending from the smaller localized darker stain. This may be indicative of damage to tumour blood vessels leading to increased leakiness. Histology cluster of differentiation 31 (CD31) untreated (c) and treated 8 Gy (d). CD31 stains endothelial cells brown. Bar = 0.1 mm
Figure 2.5: Linear regression plot for power Doppler vascularity index and histology average stained area. The dotted line indicates the 95% confidence band of the regression line. Linear regression was performed by fitting the data to a line of the form $y = A \times x + B$, where $A$ was calculated to be $0.019 \pm 0.007$ and $B$, $-3 \pm 5$. Although it appears that the line may fit the data, there is not a significant fit in terms of the regression coefficient $r^2 = 0.3074$. 
the same order of magnitude. Since both ultrasound and histology measurements yield a higher average ‘tumour vessel size’ for treated tumours, this may suggest a loss of smaller vessels after treatment.

To investigate the hypothesis that smaller vessels were potentially most effected by XRT, the size (longest and shortest dimension, area) of distinct power Doppler signals were calculated and compared with the measurements made of histochemical staining. To further explore this idea, a histogram of vessel sizes was produced for each treatment and time for selected histology and ultrasound measurements. For power Doppler data, histograms of vessel area (Figure 2.6) show changes in the number of vessels of a particular size between 0h and 24h. Although there does not appear to be a significant change in the 0 Gy case (top), there appears to be a large reduction in the number of smaller vessels in 8 Gy tumours.

Although a plot of power Doppler vascularity index and the histology vessel area (Figure 2.5) appeared to have a linear relationship, linear regression analysis did not show a statistically strong correlation ($r^2 = 0.3074$). This may be explained by the fact that the entire tumour volume is not being taken into consideration in the histology measurement. Sampled regions are made in ‘vascular hot-spots’ rather than the entire tumour volume which is sampled with ultrasound. Additionally, power Doppler measurements reflect functional blood vessels whereas CD31 stains all endothelial cells.

For histology measurements, a similar histogram of vessel area (Figure 2.7) was produced for comparison with Figure 2.6. From these histograms, it appears that there are less of the smaller vessels seen in the treated case. This is consistent with power Doppler histogram of object size results. This however does not verify a decrease in the number of smaller vessels with treatment, as differences in vessel area at 0h are unavailable with histology.
Figure 2.6: Histogram of power Doppler signal sizes for untreated and treated tumours before and after radiotherapy. No change is observed between 0h (a) and 24h (b) for the untreated case. In the treated case, a decrease in the number of detected power Doppler objects after radiotherapy ((c) and (d), respectively) is observed and appears to be largest for small vessel sizes (indicated by arrow).
Figure 2.7: Histogram of average vessel sizes from histology for untreated (a) and treated tumours (b) after radiotherapy. A significant difference in the number of vessels is observed between the two conditions. It appears that mostly the smaller signals (indicated by arrows) are not present in the treated case when compared to control tumours. The number of vessels for each animal and each bin was calculated and their average computed for each group. Error bars show standard error for each group.
2.4 Discussion

This study examined the ability of high-frequency 3-D power Doppler to detect changes in tumour vascularity in response to XRT in xenograft mouse tumours. It was shown that power Doppler indices such as VI representing the amount of vasculature detected in the tumour can be used to quantify changes in tumour vascularity. Comparison of indices between times after treatment were used to quantify the vascular effect of the treatment. Results from power Doppler indices were compared with histology staining and appeared to be in agreement.

Power Doppler results suggest a reduction in the amount of vasculature at 24h after high dose XRT in treated tumours in the human PC-3 tumour model. This is further supported by the fact that we did no observe significant decreases in control tumours.

Rendered representative 3-D power Doppler images (Figure 2.1) before and 24h after treatment illustrate the treatment effect. In control tumours there was no significant change in power Doppler indices. In the treated animals a decrease in vascularity was observed. Calculated power Doppler indices suggested an exaggerated change in the overall tumour vascularization.

Results with radiation treatments indicated significant changes between vascularity index at 0h and 24h in treated tumours, suggesting a possible reduction in vessel extent. Variability within treated and untreated groups appeared to be potentially due to differences in vascularity at 0h. This was further explored in the Appendix where the response for vascularized and avascular tumours was examined separately. This observation was not explored further due to the small sample size in some groups and therefore inability to form conclusions on this basis.

It is interesting that the average vessel area for 8 Gy treated tumours appeared to be greater than that for 0 Gy for both the power Doppler and histology measurements. This may indicate that it is primarily the smaller vessels which are being destroyed with radiotherapy with fewer smaller vessels contributing to the average area.
In Figure 2.6, the smallest vessels appeared to be affected by treatment the most. This is consistent with the work by Fuks et al. [2].

In conclusion, 3-D high-frequency power Doppler ultrasound is a useful tool for both the visualization and quantification of changes in tumour vascularity in response to therapy. In this study it was shown that there are significant differences between vascularity after treatment in control and treated tumours and that these changes can be measured with 3-D high-frequency power Doppler ultrasound with reasonable agreement to the current standard method of assessing vasculature using histopathology. Although the correlation may be weak, this is expected due to differences in the sampling methods. In power Doppler, only functional blood vessels are detected whereas with histology, endothelial cells for non-function vessels are also stained and counted. Power Doppler provides the advantage of an easy, immediate, non-invasive and quantitative method for assessing a tumour vascular response to treatment. Additionally, 3-D high-frequency power Doppler ultrasound a larger sampling volume. However, since there is a trade-off between frequency (resolution, sensitivity) and depth of penetration, it may be difficult to employ this method for use in non-superficial tumours. In addition, making power Doppler measurements truly quantitative should be further explored and a standardized method for determining power Doppler settings should be employed. This problem will be further discussed in Chapter 3.
Appendix

2.5 Reproducibility of High-Frequency 3-Dimensional Power Doppler Indicies in a PC3 Xenotransplant Mouse Tumour

The objective of this section was to determine the reproducibility of high-frequency power Doppler ultrasound indicies in PC3 tumours grown in SCID mice. Materials and scanning methods were the same as presented in Chapter 2 however, the scan repetitions times were different. One tumour was scanned 5 times per day over 2 days with no treatment given. Before each ultrasound scan was performed, the entire experimental apparatus was completely disassembled and reassembled in order to provide the variation in scanning which would be the result of human error in the orientation of the tumour and transducer surfaces. Power Doppler indicies; vascularity index (VI); flow index (FI); vascular flow index (VFI) and the number of objects (NO) were calculated.

Reproducibility measurements were carried out by repeating scanning procedures on a control mouse over 5 consecutive scans for each of the 2 days and calculating and comparing indicies. Indicies such as the vascularity index appeared to stay constant except when measurements of heart rate started to fluctuate just prior to the mouse awakening from anesthetic. Excluding the scans associated with fluctuating heart rate, the vascularity index (Figure 2.5) showed little variation between scans (VI = 2.97 ±
Figure 2.8: Vascularity index results obtained from reproducibility scans. Scans 1-4 were performed on Day 1 and scans 5-8 on Day 2 showing little variance between each scan and each day.
It was found that depending on the time after the anesthetic was delivered significant changes in indicies could be observed, so extra care in data collection for Chapter 2 was taken to ensure scanning was performed at the same time after administration of anesthetic for both 0 and 24h.

2.6 Preliminary Data

The purpose of this section is to present a sample of some preliminary data which was not included in the bulk of the thesis.

2.6.1 Initial State of Vascularity

Control and treated tumours were further classified into 2 groups which were defined as vascularized ($VI_{0h} > 4\%$) and avascular ($VI_{0h} < 4\%$). The average change for each of the indicies after treatment was calculated for each of four groups, control avascular ($C_-$), control vascular ($C_+$), treated avascular ($T_-$) and treated vascular ($T_+$).

Results with radiation treatments (Figure 2.6.1 and 2.10) indicated significant changes between vascular imaging parameters at 0h and 24h in C-, T- and T+ tumours. Differences in the response in both control and treated tumour groups were observed which appeared to be due to differences in vascularity at 0h. These preliminary findings may provide evidence that vascular response to radiotherapy is dependent on the state of vascularity prior to treatment. Here two regimes were observed. [1] Hypervascular tumours showed a significant vascular response (decrease) to radiotherapy and no vascular changes without radiotherapy. [2] Avascular tumours continued to develop both with and without radiotherapy. Those with radiotherapy appeared stunted in growth when compared to tumours without.

However, we believe that differences in the initial state of the tumour vasculature
may be of significant importance in the tumour vascular response and should be further explored. The most vascular tumours were the ones that seemed to respond the most.

2.6.2 2D slice-by-slice analysis

Three-dimensional power Doppler volumes extracted from the Vevo 770 consists of two sets of two-dimensional ultrasound slice data (B-Mode and Power Doppler Mode). In addition to the overall volumetric tumour analysis, an analysis of vascular indicies was conducted for each slice individually. Below are some representative graphs which show the change in VI (or % Vasculature) between 0h and 24h.

2.6.3 3D Distance Analysis

The purpose of this analysis was to explore the idea that tumour vessels at the edge of the tumour which form the 'viable' rim would be more susceptible to radiation, due to increased vascularity. By calculating the location of the centre of the tumour. Additionally, the location of distinct clumped colour regions was also calculated using the Matlab centroid calculation. A plot of the number of vessels as a function of distance from the centre of the tumour was produced for a representative treated (Fig. 2.6.3) and control case (Fig. 2.6.3. Although differences can be observed at various distances from the centre of the tumour, it is not clear if there is a dominant effect at the outer edge of the tumour. This may be due to an error in the way in which the data is represented since each bin does not contain equal volumes.
Figure 2.9: Box plot showing the relative change in vascularity index for each of the four groups: control avascular ($C_{-}$), control vascular ($C_{+}$), treated avascular ($T_{-}$) and treated vascular ($T_{+}$). The lower edge of the box shows the 25th percentile while the upper edge shows the 75th and the median is shown by the middle line.
Figure 2.10: Box plot showing the relative change in flow index for each of the four groups: control avascular ($C_-$), control vascular ($C_+$), treated avascular ($T_-$) and treated vascular ($T_+$).
Figure 2.11: A slice-by-slice analyses of tumour vascularity for a tumour before and 24\textit{h} after treatment with radiation. There appears to be a consistent decrease across the tumour.
Figure 2.12: Representative graph showing the frequency of power Doppler objects as a function of distance from the centre of the tumour for a treated tumour before and after treatment. There appears to be decreases in the number of objects across the tumour, however there is no obvious trend suggesting a relationship between the radial distance from the centre of the tumour and treatment response.
Figure 2.13: Representative graph showing the frequency of power Doppler objects as a function of distance from the centre of the tumour for a control tumour before and after treatment. There does not appear to be significant changes across the entire range of distances.
2.7 3D Power Doppler Data Analyzer

The purpose of this section is to describe briefly the development of custom software for 3D power Doppler analysis. In order to address the issue of limited data analysis methods for power Doppler data on commercial scanners, a toolbox was developed in Matlab. The 3D Power Doppler Data Analyzer is a graphical user interface which allows one to analyze 3D power Doppler data acquired using the Vevo 770 (Visualsonics, ON). The software allows users to create and analyze 2D or 3D regions of interest (ROIs) within multiple volumes and provides the ability to compare these regions. ROIs can be selected in colour mode, B-mode or cover overlay mode. Once ROIs are selected the user can create their own analysis based on standard power Doppler indices and extended analysis. The indices which are calculated include: percent vasculature or vascular index, flow index, vascular flow index, as well as the number of power Doppler objects in 2D or 3D. Additionally, a comparison of object size statistics is available. The software is currently being further developed.
Chapter 3

Conclusions and the Future Work

3.1 Summary

The objective of this thesis was to examine the application of quantified 3-D high-frequency power Doppler ultrasound for the monitoring of tumour microvascular response to radiation therapy. The tumour treatment model used xenotransplanted PC-3 tumours in SCID mice to investigate the early effect of radiotherapy on the tumour microvasculature, 24\(h\) after irradiation. This was done by collecting power Doppler volumes of tumours before and after treatment with single dose 8\( Gy\) x-ray radiation therapy. Chapter 1 presents salient background information about radiotherapy, tumour microcirculation and basic ultrasound principles. In Chapter 2, power Doppler data collected for both control and treated tumours was analyzed using custom software. Indices indicative of tumour vascularity were calculated and compared for each animal between each experimental time and for each treatment group. Further analysis was carried out on the power Doppler signals to investigate changes in signal sizes. Results were compared with histology measurements as a method of validation.

Differences in the relative change for the vascularity index between untreated and treated tumours indicated a decrease in vascularity in treated tumours by 10 to 54\%.
Although, the vascularity index is thought to represent the fractional amount of vasculature by volume, it is assumed that increases in tumour vascularity in the control group could be potentially exaggerated as a result of rapid tumour growth. This was not a problem however since the difference in changes in vascularity between the 0 Gy and 8 Gy treatment groups make it easy to distinguish between the two effects. Alternatively, it was hypothesized that the small relative increase in the vascularity index for control tumours was due to decreased response to anaesthetic on day 2 of experiments. To test this hypothesis and to ensure the reproducibility of power Doppler measurements, in the Appendix for Chapter 2, a reproducibility experiment is described and some results were shown.

In addition to calculating the vascularity index in Chapter 2, an analysis of the changes in power Doppler signals was carried out. There was both a change in the amount of tumour vasculature as well as a decrease in the number of power Doppler signals in some animals which led to the hypothesis that there are less functional tumour vessels after treatment. To further explore this, an analysis of the size of the power Doppler signals was carried out. From comparisons of histograms of vessel area for all tumours before versus after, a decrease in the number of power Doppler signals which were $< 0.2 \text{ mm}^2$ was observed and appeared to be exaggerated compared with other sizes.

For validation, results obtained from power Doppler measurements were compared with measurements of histology. The sizes of immunohistochemistry stained vessels were measured and the fractional area of vessel staining in tumours was calculated. To compare measurements made by histology with power Doppler measurements, a histogram of vessel area was created. A comparison between histograms made from histology and power Doppler revealed similar relative differences. Finally, a plot of the fractional area calculated by histology was compared with the vascularity index in attempt to detect a correlation between power Doppler and histology measurements and a somewhat linear relationship was established.
In an attempt to ensure the reproducibility of power Doppler measurements a reproducibility experiment was also described in Appendix 2.5. It was demonstrated from these experiments that power Doppler results are extremely reproducible however this is highly dependent on the state of the animal when it is anaesthetized. It was determined that in order for such results to be reproducible that power Doppler volumes must be acquired approximately the same time after the delivery of anaesthetic. Therefore it was decided that any volumes that were not acquired in this fashion should be removed from the analyses.

3.2 Implications

This thesis presents further evidence to support the theory that early tumour microvascular damage is significant and may play a role in regulating the tumour response to therapy. The study in this thesis also demonstrated that high-frequency power Doppler correlates reasonably well with histology, given the small sample size, and can thus be used as a potential non-invasive surrogate for histological based analysis of vasculature.

In the past, most power Doppler studies have looked at differences in indices between two treatment groups. This study differs in that it investigated the change of indices over time within treatment groups. The former is potentially deficient as all tumours may differ in vascularity at 0 h. Here we are looking for a method to detect changes in vascularity in individual animals, not differences in vascularity that may be seen between groups.
3.3 Future Work

Although it has been shown that power Doppler is a useful tool for the depiction and quantitative analysis of the tumour response to therapy, there is still work to be done in order to make power Doppler a better quantitative functional blood flow monitoring tool. Power Doppler suffers from the fact that the cutoff velocity and post scan threshold settings are somewhat arbitrarily set by the user leading to potential subjectivity. Since vessels typically have a higher power at the centre of the vessel, represented by bright voxels, selecting a low power cut-off value which is too high may result in colour regions which are smaller than the true vessel. This is due to the loss of colour at the edge of the vessel. In contrast, selecting a low power value which is too low will result in an overestimate of vessel size and an underestimate in the number of distinguishable blood vessels. The main advantage of power Doppler is the way in which noise is represented. This allows for an increase in gain without overwhelming the power Doppler signal. In order to take advantage of this, a method should be invented to determine the ideal power Doppler settings for maximum sensitivity while maintaining the quantitative nature of power Doppler.

The phenomenon of an increasing vascularity index in control tumours between scans has been consistently reproduced in our lab and must be explored to determine why there is such a large change. This may be related to rapid tumour growth. In contrast observed decreases in vascularity for treated tumours was assumed to be due to changes in the amount of vasculature while neglecting the potential changes in the indices as a result of changes in tissue properties with treatment. Since the ability to detect moving blood is dependent on how much the received ultrasound signal is attenuated by the tissue it is traversing, effects of treatment on relevant tissue parameters should be investigated further.

In addition, a three-dimensional analysis of vessel architecture should be verified using extensive three-dimensional histology methods. New histological methods now can scan
the entire tumour at small intervals between sections, providing an isotropic volume. In the mean time, power Doppler should continue to be used for quantitative analysis of tumour vascular response to treatment in vivo using different tumour models. One such study has already been carried out in the lab. The experiment involves monitoring the tumour microvascular response to micro-bubble and ultrasound radio-sensitization. Experiments have already shown the ability of power Doppler to confirm vascular changes within each treatment group. These changes have been shown to be consistent with the treatment effect which has been verified by measures of apoptosis and histological analysis. It has also been verified that in these tumours which exhibit power Doppler changes that there is ceramide related endothelial cell death occurring.

Further investigation from a biological prospective may be potentially valuable in developing new therapeutic strategies against cancer. It would be useful to conduct a longitudinal study using high-frequency power Doppler which would follow vascular development prior to therapy through vascular destruction caused by therapy and finally tumour outcome.
Bibliography


