Vascular Strategies for Enhancing Tumour Response to Radiation Therapy

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Radiation therapy is prescribed to more than 50% of patients diagnosed with cancer. Although mechanisms of interaction between radiation and tumour cells are well understood on a molecular level, much remains uncertain concerning the interaction of radiation with the tumour as a whole. Recent studies have demonstrated that single large doses of radiation (8-20 Gy) may primarily target tumour endothelial cells, leading to secondary tumour clonogenic cell death. These studies suggest that blood vessels play an important role in radiation response. As a result, various strategies have been proposed to effectively combine radiation with vascular targeting agents. While most proposed schemes focus on methods to disrupt tumour blood vessels, recent evidence supporting that some anti-angiogenic agents may “normalize” tumour blood vessels, in turn enhancing tumour oxygenation and radiosensitivity, indicates that there may be more efficient strategies. Furthermore, vascular targeting agents have recently been demonstrated to enhance radiation therapy by targeting endothelial cells. When combined with radiation, these agents are believed to cause even more localized vascular destruction followed by tumour clonogenic cell death. Taken together, it is now crucial to elucidate the role of tumour blood vessels in radiation therapy response, in order to make use of this knowledge in developing therapeutic strategies that target tumour vasculature above and beyond classic clonogenic tumour cell death. In this report, we review some major developments in understanding the importance of tumour blood vessels during radiation therapy. A discussion of current imaging modalities used for studying vascular response to treatments will also be presented.

Key words: Tumour vascular imaging; Radiation therapy; Anti-angiogenic agents; Vascular normalization; Tumour vasculature; Vascular disrupting agents; Vascular targeting agents.

Introduction

Folkman first hypothesized and subsequently demonstrated that for tumours to survive and continue growing after reaching a volume of 2-3 mm³, angiogenesis has to occur (1). Angiogenesis refers to the process of new vessel formation within tissue. It occurs via endothelial sprouting and intussusceptions, and involves the upregulation of vascular endothelial growth factor (VEGF), interleukin (IL-8), epithelial growth factor (EGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). Tumour blood vessels tend to differ significantly from normal tissue blood vessels (Table I). 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. Tumour functional

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Abbreviations: Ionizing Radiation Therapy (XRT); Vascular Targeting Agent (VTA); Anti-Angiogenic Agent (AA); Vascular Disrupting Agent (VDA); Intravital Microscopy (IVM).
Abnormalities are also caused by tumour blood vessels. These include increased interstitial blood fluid pressure, decreased tumour oxygenation and increased intratumoural metabolites contributing to the hypoxic environment characteristic of many tumour lines (2).

Radiation therapy is prescribed to more than 50% of patients diagnosed with cancer in North America. Treatments are usually administered using small fractionated doses (1.8-4 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 (3). Nonetheless, it has been demonstrated that tumour-derived cell lines respond to radiation differently in vitro than in vivo (3, 4). 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 (5), resulting in hypoxic radioresistant regions. Moreover, low-dose fractions of radiation may further promote radioresistance by causing the secretion of cytokines such as VEGF and bFGF, increasing the complexity of the tumour microenvironment (6). Radioresistance has been associated with poor prognosis and treatment response (7, 8). Taken together, the general understanding of how radiation interacts with the tumour as a whole (including host-derived stroma) is not well understood, and new treatment strategies should be considered. Recent studies indicate that tumour blood vessels may play a crucial role in the response to radiotherapy (7). This has motivated researchers and clinicians to question and begin addressing the role that blood vessels play in tumour response to radiation therapy. Here, we review some recent developments, which attempt to address this question, as well as some proposed vascular strategies aiming to enhance tumour response to radiation therapy. We conclude this review by discussing the potential use of various imaging modalities for quantitatively and qualitatively characterizing tumour vascular response to treatments strategically combining radiation therapy with VTAs.

Table I
Table summary of general characteristics of normal tissue vasculature vs. tumour vasculature (45, 72, 88, 89).

<table>
<thead>
<tr>
<th>Normal vasculature</th>
<th>Tumour vasculature</th>
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<tbody>
<tr>
<td>Well constructed walls with full pericyte and basement membrane coverage.</td>
<td>Absent or detached pericyte covering.</td>
</tr>
<tr>
<td>Responds to innervations. Can increase blood flow in response to demand.</td>
<td>Absent or thickened basement membrane.</td>
</tr>
<tr>
<td>Organized hierarchical vessel diameter and distribution.</td>
<td>Minimal response to innervations.</td>
</tr>
<tr>
<td>Low interstitial flow pressure.</td>
<td>Endothelium relies on cytoskeleton to maintain integrity.</td>
</tr>
<tr>
<td>Resistant to radiation therapy. Can withstand high depletion of endothelial cells</td>
<td>Random vessel diameter variability and heterogeneous vascular densities.</td>
</tr>
<tr>
<td>(in arterioles).</td>
<td>Low pO2 tension and high interstitial flow pressure. Increased permeability/leakiness</td>
</tr>
<tr>
<td>Slow endothelial proliferation.</td>
<td>Generally more sensitive to irradiation. Vessels can easily become dysfunctional.</td>
</tr>
<tr>
<td></td>
<td>Rapid endothelial cell proliferation.</td>
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### Vascular Strategies in Radiation Therapy

#### Targeting Tumour Blood Vessels

The role of blood vessels in tumour responses to ionizing radiation has been a topic of debate in recent literature. Although recognized that radiation therapy targets endothelial cells differently than epithelial and tumour cells (Table II), it remains unknown how the irradiated tumour vasculature contributes to the overall tumour response. Substantive evidence suggests that tumour blood vessels may act to regulate tumour response to radiotherapy (7-13), challenging the canonical notion that tumour response is primarily dependent on an inherent radiosensitivity of clonogenic tumour cancer cells. This paradigm shift originates in a controversial study by Paris et al. (14) 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 clonogen lethality via reproductive cell death. This lethality is linked to early upregulation of acid-sphingomyelinase (ASMase) dependent endothelial apoptosis causing dysfunction of crypt blood vessel networks (14-19). The authors further demonstrated that bFGF administration before whole body irradiation counters

Table II
Common biophysical responses of vasculature and endothelial cells when irradiated.

<table>
<thead>
<tr>
<th>Biophysical effects of radiation on vasculature and endothelium</th>
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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>
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the ASMase-dependent apoptosis signaling pathway, protecting gut endothelial and epithelial cells (14). 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 (16). However, other researchers have challenged this notion, demonstrating that other factors (i.e., p53) control the radiation-induced gastrointestinal syndrome, independent of endothelial apoptosis (20). 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 (21) 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 in vivo and 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 et al. (7) 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. As the ASMase enzyme is present in endothelial cells 20-fold more than in epithelial and tumour cells, it makes this apoptotic pathway primary in blood vessels (Figure 1). On the other hand, standard 2 Gy fractionated radiation was suggested to wave effects due to hypoxia, reperfusion and reactive oxygen species formation (8, 22, 23). The exact mechanism of the endothelial-tumour linkage is still unknown. It was suggested that it might 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 (15).

Consequently, a series of letters were published opposing this potential new paradigm (24, 25). The primary criticism relied on data published in 1993 by Budach et al. (26), which demonstrated that the TCD50 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 stromal 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 (27, 28) 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 García-Barros et al. (10) investigated the above-mentioned concerns in asmase knock-out SCID mice. Results indicated that the asmase−/− 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 how important the ASMase pathway is for endothelial apoptosis. A second important comment suggested that the original Garcia-Barros et al. (7) results might be caused by a host immune response.

Figure 1: 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 sphingomyline into ceramide, which then acts as an apoptosis messenger. Because ASMase is present 20-fold more in endothelial cells than in epithelial and tumour cells, this is a common pathway of apoptosis only in the endothelium. Methods of suppressing this pathway include pre-treatment with bFGF and VEGF as well as asmase knock-out.
response. This speculation was derived from observations that the TCD 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{\textminus/-} than in \textit{asmase} \textsuperscript{\textplus/+} littermates. This comment was however refuted in 2004, where 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{\textminus/-} phenotype is not deficient in antitumour immunity (9).

Taken together, it is likely that the role of blood vessels in radiation response is not a black and white issue, considering the complexity of cancer and the tumour microenvironment. Nonetheless, a paradigm shift from the accepted concepts of classic radiobiology is suggested and is likely to follow through from 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 or biophysical vascular targeting agents to enhance tumour response.

\textbf{Combining Radiation with Vascular Targeting Agents}

Vascular targeting agents (VTA) are currently under investigation as potent tumour radiosensitizers. VTAs are categorized into two types of agents: vascular disrupting agents (VDA) and anti-angiogenic agents (AA). VTAs disrupt tumour vasculature leading to vessel collapse or destruction, while on the other hand AAs inhibit the growth and development of new blood vessels. Up-to-date reviews are available on both types of agents (2, 29, 30). A vast amount of published evidence suggests an additive or synergistic tumour response to radiation when combined with such agents (31-33). Furthermore, studies have shown that VTAs can cause endothelial radiosensitization \textit{in vivo} and \textit{in vitro} (34-36). 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 tumor radiosensitizing agents, or immediately after irradiation as a maintenance therapy (2, 29). 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 single doses of radiation, or in a complementary manner, targeting tumor regions which tend to be radioresistant.

A wide variety of AAs are currently available. These include agents which are in use in the clinic as well as agents which are at various stages of clinical trials or preclinical testing. These target various aspects of molecular pathways involved in tumour angiogenesis. Most clinically approved AA agents target VEGF by binding and neutralizing it (\textit{i.e.} Bevacizumab and DC101), or act as small molecule tyrosine kinase inhibitors, which prevent the activation of a wide-spectrum of receptors including VEGF receptors (\textit{i.e.} SU11248, ZD6474 and PTK787/ZK 222584). These act by inhibiting downstream signaling pathways rather than binding directly to VEGF (37-41). 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 (5, 38, 39, 42, 43). However, the majority of ongoing research focuses on targeting vascular growth factors or their receptors. Teicher \textit{et al.} (44) first advocated the use of AA 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 (44). It was later demonstrated that there may be a more-than-additive effect when combining radiation with AA agents (6, 33). Following this, Jain suggested that there may be a timing and dose window where AA agents act to ‘normalize’ the tumour vasculature by balancing “pro” and “anti” angiogenic factors (45-47). The term vascular ‘normalization’ has been generally associated with the pruning of immature and leaky vessels involving a remodeling 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. Moreover, with exposure to AAs, 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 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 (45). A number of earlier studies have yielded data supporting the ‘normalization’ theory using DC101 and thalidomide, both AA agents (46, 48, 49), while a recent study demonstrated that radiation therapy to glioma (U87) tumours orthotopically implanted in rodents was enhanced when combined with AA therapy (50). These studies linked the enhanced radiosensitivity of tumours to enhanced diseased tissue oxygenation after AA administration and a potential vascular normalization process.

Vascular disruption encompasses a wide variety of treatments, including physical treatments such as hyperthermia and photodynamic therapies, biological response modifiers (\textit{i.e.} tumour necrosis factor and interleukins), certain chemotherapies (\textit{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 (2, 30). There exist two major classes of small
molecule VDAs. Agents derived from flavon acidic acid (*i.e.* 5,6-dimethylxanthenone-4-acetic acid (DMXAA)) which act through a cascade of direct and indirect effects leading to tumour hemorrhages and heavy necrosis. More common however are a class of tubulin-depolymerizers, which have become increasingly popular over the past decade (*i.e.* CA4P, CA1P and OXi4503). Tozer et al. (30) 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, hemorrhage 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 depolymerization 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 tumors. However, the effects of VDAs have been shown to be much less severe in well-oxygenated tissues such as the rim of tumors 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 is 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, where radiation has been shown to have relatively minimal effects.

Imperative questions on the timing, scheduling, dosing and duration of use of these VTAs in conjunction with radiation therapy require answers. Anti-angiogenic agents are discussed above in the form of vascular normalizing agents aiming to enhance oxygenation and radiosensitivity of tumour cells. Determining the optimal time for radiation delivery (during a ‘normalization’ window) has so far proved to be a challenge. Anti-angiogenics are typically administered daily for a specified period, and their effects are often monitored through the use of imaging modalities. These agents have been shown to begin inducing ‘normalization’ effects anywhere from 1 hour to days after the first administration (36, 37, 45, 51, 52). The elevated oxygenation time-window has been thought to be dependent on dosing and tumour stage. Furthermore, low AA doses (compared to those prescribed when AAs are used alone) have been used when combined with radiation therapy to induce vascular ‘normalization’ (53, 54). VDAs on the other hand seem to work best on late-stage tumours, characterized by necrotic and/or hypoxic cores (55, 56). 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. It has also been suggested to deliver AAs as maintenance therapy after radiation treatment or combination therapies involving VDAs and radiotherapy. This sort of therapy would act to prevent repopulation occurring due to tumour revascularization, in turn aiding to maintain tumour control or preventing tumour regrowth (57). The proposed use of maintenance therapy is primarily for the combination of different kinds of VTAs or subsequent to radiation therapy. The vascular targeting strategies described above, if optimized, should act to destroy most, if not all, tumour cells in the targeted site without the need for maintenance therapy.

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 microspheres on the order of 3–4 μm, 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 (58–61). Acoustic microbubble disruption has been noted to also perturb the function of surrounding cells (primarily endothelial cells). Disruptions have been associated with cavitational microstreams and microjets, stress fields and shockwaves capable of destroying cells or permeabilizing their membranes (62–64). More recently, it has been demonstrated that causing microbubbles to lightly oscillate near the surface of endothelial cells can activate angiogenic pathways (65) and alter physical properties of the cellular membrane (66). Moreover, it has been suggested that bubble microstreams could be controlled by altering the ultrasound field parameters (67), 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 (62). Proposed applications for the microbubble-cellular interaction include permeabiliyzing 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 xenograft tumours in mice, pre-treated with microbubbles followed by single doses of radiation yield significant amounts of cell death and a synergistic tumour growth suppression effect. Along the same lines, the use of gold nanoparticles has recently been proposed as endothelial-specific radiosensitizers. Using simulations, their work has shown that the enhancement of radiation dosing is localized to the tumour endothelium, sparing surrounding organs (68-70). This would allow for vascular destruction effects at lower doses than those suggested by Fuks (8). Microbubbles could potentially further be used to permeabilize vascular endothelial cells for gold nanoparticle delivery.

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 (Figure 2). Moreover, with the development of a vast number of chemical and biophysical VTAs, it will be necessary to comprehend the benefits and pitfalls 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 AA drugs have recently been published, which suggests that there is a growing interest in bringing this type of combination into clinical use (62).

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 which can be used non-invasively offer new ways to assess response to vascular targeting strategies.

### Tumour Vascular Imaging

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 common 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 unique properties, including advantages and disadvantages, which emphasizes the need to use multiple imaging techniques to begin gaining an understanding of 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 (71). However, to uncover mechanisms of action and to comprehend the dependence tumour cells have on their vasculature, multi-imaging modality studies must be conducted in pre-clinical studies. In the following sections we briefly discuss the use of imaging to evaluate tumour blood vessel response to radiotherapy and anti-vascular strategies in pre-clinical models.

#### Optical Imaging

Light-based imaging techniques are well 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 (40). These have however been historically known to 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 cater the probe size and fluorescence wavelength for various structural, functional and biological imaging applications (72).

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 (see reference for review). 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 (72). 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 (73).

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 currently only applicable for highly invasive window chambered tumour models.
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. Its superior resolution over common clinical-frequency ultrasound devices (1-10 MHz) comes at the sacrifice 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. 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 artifacts. Power Doppler (an extension of Doppler based methods) is capable of overcoming some of the limitations encountered with color flow ultrasound. 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 (>20 MHz), power Doppler ultrasound detects blood flow down to 1-2 mm/s and vessel sizes of 30-100 μm. 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. Its main limitation is that it is unable to detect the smaller blood vessels of the tumour micro-circulation (which can be as small as 5 μm in diameter).
other hand, changes in smaller capillaries of the tumour often affect the whole microcirculation and might then be detectable in the signal from the detectable vessels. Ultrasound microbubble contrast agents are theoretically ideal for imaging tumour vascular perfusion and perfusion rates (79, 82, 83). Contrast enhanced imaging can be performed in conventional Doppler mode, enhancing the Power Doppler signal from smaller blood vessels with slower flows, or in contrast-mode. Contrast mode imaging exploits the unique non-linear harmonics from resonance of water protons, yielding a unique contrast between tissue scattering (84). Because this technique is still in its infancy, obtaining images of whole 3D tumour volumes is 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 complexities in studies involving a large number of subjects (animals). However, contrast-enhanced ultrasound is useful in assessing tumour perfusion (in contrast mode), representing the smallest of vessels in the tumour. Ultrasound flow techniques are ideal for assessing vascular destruction strategies, but may not be capable of assessing vascular normalization yet. It may however be possible to assess structural characteristics such as vascular distribution and vessel sizes during vascular normalization using ultrasound.

**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 (58, 85). 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 yields information on perfusion, permeability and 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 AA agents (86). Such a vascularization index would be very useful in assessing tumour response when radiation is combined with AA 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.

**Computed Tomography**

Nuclear medicine modalities such as PET, SPECT and CT have dynamic tracer imaging capabilities also very good for imaging various tumour vasculature hemodynamics (87). 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 volume, and mean transit time. The major limitations to CT 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.

**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 therapy targets blood vessels differently than clonogenic tumour cells (especially at high radiation doses) suggests that there may be more potent ways of treating tumours. The use of endothelial radiosensitzers may optimize 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 sufficiently 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. Nonetheless, it may be that different treatment strategies should be employed with different cancer sites, an idea that may be explored in upcoming research. As vascular imaging modalities develop further, their use will be essential in better understanding the changing dynamics of tumour blood vessels and the tumour microenvironment. 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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