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PUSHING THE FRONTIERS OF RADIOBIOLOGY: A SPECIAL FEATURE IN MEMORY OF SIR OLIVER SCOTT AND PROFESSOR JACK FOWLER: REVIEW ARTICLE

Targeting the vasculature of tumours: combining VEGF pathway inhibitors with radiotherapy

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ABSTRACT

The development of blood vessels by the process of angiogenesis underpins the growth and metastasis of many tumour types. Various angiogenesis inhibitors targeted against vascular endothelial growth factor A (VEGF-A) and its receptors have entered the clinic more than a decade ago. However, despite substantial clinical improvements, their overall efficacy proved to be significantly lower than many of the pre-clinical studies had predicted. Antiangiogenic agents have been combined with chemotherapy, radiotherapy and more recently immunotherapy in many pre-clinical and clinical studies in an effort to improve their efficacy. To date, only their use alongside chemotherapy is approved as part of standard treatment protocols. Most pre-clinical studies have reported improved tumour control from the addition of antiangiogenic therapies to radiotherapy and progress has been made in unravelling the complex mechanisms through which VEGF inhibition potentiates radiotherapy responses. However, the efficacy of this combination is variable, and many questions still remain as to how best to administer the two modalities to achieve optimal response and minimal toxicity. One important limiting factor is that, unlike some other targeted therapies, antiangiogenic agents are not administered to selected patient populations, since biomarkers for identifying responders have not yet been established. Here, we outline VEGF biology and review current approaches that aim to identify biomarkers for stratifying patients for treatment with angiogenesis inhibitors. We also discuss current progress in elucidating mechanisms of interaction between radiotherapy and VEGF inhibitors. Ongoing clinical trials will determine whether these combinations will ultimately improve treatment outcomes for cancer patients.

INTRODUCTION

Judah Folkman was first to propose that factors that induce and sustain angiogenesis could be targeted in order to halt tumour growth.[1] Juliana Denekamp also proposed that targeting established tumour blood flow could indirectly and selectively kill tumour cells.[2] Vascular endothelial growth factor A (VEGF-A or simply VEGF) was subsequently identified as a vascular permeability factor and potent endothelial mitogen, capable of stimulating blood vessel growth.[3,4] Ferrara and colleagues provided proof of Folkman's concept and confirmed the importance of VEGF-A as a pro-angiogenic and tumour-promoting factor, by demonstrating that antibodies that neutralised VEGF-A could suppress angiogenesis and tumour growth in a mouse model.[5] Several VEGF-independent pathways have since been identified as capable of initiating and sustaining tumour angiogenesis.[6] Nevertheless, the majority of effort has centred on the development of antiangiogenic s trategies specifically directed against the VEGF pathway, since most cancers overexpress VEGF-A and overexpression is associated with poor prognosis.[7]

Antiangiogenic agents have been combined with other modalities as an attempt to maximise their therapeutic benefits. While addition of VEGF pathway inhibitors to chemotherapy is part of standard care in several instances, to date there are no US Food and Drug Administration (FDA) or European Medical Association approved treatments that incorporate these agents with radiotherapy. Nevertheless, antiangiogenic agents combined with radiotherapy have

been tested extensively in pre-clinical models and in clinical trials. The potential for this combination was recognised in the early 1990s by Teicher et al,[8] who noted that the antiangiogenic agent, TNP-470 combined with minocycline, could increase tumour oxygenation and hence radiosensitivity. These findings were firmly based on the landmark studies of Oliver Scott and colleagues, who first investigated the use of oxygen breathing to increase the radiosensitivity of mouse tumours.[9] Later studies, including the work of Jack Fowler and colleagues,[10] recognised that the favourable interaction of VEGF pathway inhibitors with radiotherapy was not solely due to modifications of tumour oxygenation.

In this article, we review the role of VEGF-A in the tumour microenvironment and report on progress in establishing predictive biomarkers to VEGF pathway inhibitors. We also give an overview of the mechanisms of interaction between radiotherapy and anti-VEGF agents and summarise the progress of clinical trials testing this combination.

VEGF BIOLOGY

The VEGF family consists of several structurally related factors in addition to VEGF-A, namely VEGF-B, VEGF-C, VEGF-D and placental growth factor, all of which contribute to angiogenesis and/or lymphangiogenesis. VEGF-A, the most well-characterised member of the family and the most potent inducer of tumour angiogenesis, is the target of the anti-VEGF-A antibody bevacizumab (Avastin®; Genentech), which was the first antiangiogenic agent to gain approval by the FDA for the treatment of metastatic colorectal cancer (Table 1). Approval was based on successful results of a Phase III clinical trial (AVF2107), which demonstrated that addition of bevacizumab to 5-fluorouracil, leucovorin and irinotecan (FOLFIRI) chemotherapy led to substantial improvements in overall survival (OS) and progression free survival (PFS) in patients with metastatic colorectal cancer.[11] Bevacizumab is now licensed for use in combination with chemotherapy in six different types of cancer that are deemed to be responsive to antiangiogenic therapy (Table 1).

VEGF-A binds to tyrosine kinase receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1) and VEGFR-3 (Flt-4) and activates signalling pathways that promote angiogenesis by stimulating endothelial cells to migrate and proliferate and by enhancing vascular permeability.[12] Upon ligand binding, receptor dimerization occurs and leads to the phosphorylation of several tyrosine residues that reside within the intracellular domain of the receptor, triggering the activation of angiogenesis-inducing signalling cascades. Small molecule inhibitors have been developed to target the tyrosine kinase domain of VEGFR-2, although these drugs also exhibit varying levels of activity against other receptor tyrosine kinases (RTKs), such as VEGFR-1, VEGFR-3, PDGFRs, FGFRs and thus have a wider spectrum of activities. Sorafenib was the first of several RTK inhibitors (RTKi's) to be licensed, initially for newly diagnosed metastatic renal cancer (Table 1). Renal cell carcinoma is relatively responsive to VEGF inhibitors, due to a high dependency on VEGF, which is upregulated by VHL mutations and consequent hypoxia-inducible factor 1α (HIF1α) stabilisation.[13] Aflibercept (Zaltrap®; Regeneron Pharmaceuticals) is a fusion protein of the extracellular domains of VEGFR-1 and VEGFR-2 and the Fc portion of human IgG1 that acts as a "VEGF trap" and sequesters and neutralises VEGF-A, VEGF-B and placental growth factor.[14] Aflibercept gained FDA approval in 2012 for use in colorectal cancer as a second-line treatment in combination with FOLFIRI chemotherapy (Table 1). One of the latest antiangiogenic agents to reach the clinic is ramucirumab (Cyramza®; Eli Lilly (Table 1), which is a monoclonal antibody against VEGFR-2, that acts by preventing binding of ligand to the receptor and is approved for the treatment of metastatic colorectal cancer, gastro-oesophageal cancer and non-small cell lung cancer.[15]

There are now 10 different VEGF pathway inhibitors licensed to treat cancer (Table 1) and although their use has benefited many patients and is transforming the management of patients with renal cell carcinoma[16] their overall efficacy has proved to be less exciting than many of the pre-clinical studies originally predicted. Some tumour types, such as pancreatic and prostate, are inherently resistant to anti-VEGF therapies while treatment-induced resistance develops in initially responsive cancers. Various mechanisms of resistance have been proposed and these include utilisation of VEGF-independent pathways that allow tumours to escape from VEGF-dependent vascularisation.[17]

Benefit from VEGF pathway inhibitors is often restricted to improvements in PFS that do not translate to improved OS. Failure to achieve an OS benefit resulted in the FDA withdrawing the original approval for bevacizumab for the treatment of metastatic HER2 negative breast cancer. Further progress in this field is therefore reliant on identifying mechanisms of resistance to anti-VEGF therapies that can be specifically targeted to improve outcomes. Unlike some other targeted therapies, antiangiogenic agents are not administered to selected patients since there are no validated biomarkers to identify responders. Approaches to identify biomarkers to enable selection of patients responsive to anti-VEGF agents have so far not yielded any definitive answers.

Diverse functions of VEGF within the tumour microenvironment

Tumour blood vessels are chaotic and disorganised, thin-walled and leaky and generally lack sufficient pericyte coverage.[18] Vessel leakiness causes a build-up of proteins and fluid within the interstitial space and a rise in interstitial fluid pressure resulting in a poorly perfused, hypoxic tumour microenvironment. Poor vascular perfusion combined with hypoxia and anaerobic glycolysis also result in acidosis.[18] Aberrant VEGF signalling contributes to the chaotic and immature characteristics of tumour blood vessels within the hostile tumour microenvironment. Enhanced transcription of the VEGF-A gene is promoted through stabilisation of HIF1α while acidosis is also an inducer of cycles of VEGF-A gene expression through HIF1α-dependent and independent mechanisms.[19] VEGF-A is produced by tumour cells, as well as most stroma cells within the tumour microenvironment, as distinct alternatively spliced isoforms that differ in terms of their matrix binding and diffusion properties.[20] VEGF isoforms of 120/121, 164/165 and 188/189 amino acids represent the most predominant mouse and human splice variants respectively, that

Table 1. FDA approved antiangiogenic agents targeting the VEGF axis and their specific indications

Agent	Targets	Disease	Comments	Year of FDA approval
Bevacizumab Monoclonal antibody	VEGF-A	Metastatic colorectal cancer	With chemotherapy, first and second line	2004, 2006
		Non-small cell lung cancer (locally advanced, recurrent and metastatic)	With chemotherapy, first line	2006
		Metastatic renal cell carcinoma	With interferon	2006
		Metastatic renal cell carcinoma	Single agent	2009
		Glioblastoma (progressive disease)	With chemotherapy	2009
		Ovarian, fallopian or peritoneal cancer (platinum resistant and recurrent)	Single agent	2014
		Cervical cancer (recurrent or metastatic)	Single agent	2014
		Ovarian cancer (advanced, newly diagnosed)	With chemotherapy	2018
Ramucirumab Monoclonal antibody	VEGFR-2	Gastric or gastroesophageal junction adenocarcinoma	Single agent or combined with paclitaxel in advanced disease	2014
		Metastatic non-small cell lung cancer (platinum resistant)	With docedaxel	2014
		Metastatic colorectal cancer (previously treated with bevacizumab)	With FOLFIRI chemotherapy	2015
Ziv-Aflibercept Recombinant fusion protein–VEGF trap	VEGF-A VEGF-B, PLGF	Metastatic colorectal cancer (resistant to or progressed on oxiplatin)	With FOLFIRI chemotherapy	2012
Sorafenib RTKi	VEGFR-2, 3 PDGFRs, RAF KIT	Renal cell carcinoma (advanced disease)	Single agent	2006
		Hepatocellular carcinoma (unresectable)	Single agent, first line	2007
		Thyroid carcinoma (dedifferentiated and refractory to radioactive iodine)	Single agent	2013
Sunitinib RTKi	VEGFR-1-3 PDGFRα PDGFRβ, KIT, RET, FLT3	Renal cell carcinoma (metastatic)	Single agent	2006
		GIST (intolerant to or progressed on imatinib)	Single agent	2006
		Pancreatic neuroendocrine tumours, locally advanced or metastatic	Single agent	2011
Pazopanib RTKi	VEGFR-1-3 PDGFRs, KIT	Renal cell carcinoma (advanced)	Single agent	2009
		Soft tissue sarcoma (non-adipocytic or GIST)	Single agent	2012
Vandetanib RTKi	VEGFR-1-3 EGFR, RET	Medullary thyroid carcinoma (unresectable, locally advanced or metastatic)	Single agent	2011
Axitinib RTKi	VEGFR-1-3 PDGFRβ KIT	Renal cell carcinoma	Single agent	2012
Regorafenib RTKi	VEGFR-1-3 PDGFRβ, KIT, RET, Raf-1	Colorectal cancer, metastatic and refractory to other treatments including chemotherapy and other VEGF inhibitor(s)	Single agent	2012
		GIST (no longer responding to sunitibib or imatinib)	Single agent	2012
		Hepatocellular carcinoma (tumours previously treated with sorafenib)	Single agent	2017
Lenvatinib RTKi	VEGFR-1-3 FGFR-1, PDGFRs, KIT	Thyroid carcinoma, differentiated and refractory to radioactive iodine	Single agent	2015

GIST, gastrointestinal stromal tumours; RTKi, receptor tyrosine kinase inhibitor; VEGFR, vascular endothelial growth factor; FOLFIRI, 5-fluorouracil, leucovorin and irinotecan;

display binding affinities to matrix that increase with increasing molecular weight. The differential interactions of VEGF isoforms with matrix generate gradients that are important for regulating angiogenic sprouting, vessel branching and expansion and vascular permeability.[21,22] Although the mechanisms responsible for the actions of each isoform are not clearly understood, studies on endothelial cells have shown that individual VEGF-A isoforms elicit distinct signalling responses and outcomes that are dependent on whether they are matrix bound or not and on their association with specific VEGF receptors and co-receptor neuropilin 1 (NRP1).[20,23,24] Matrix metalloproteinases can further process VEGF-A and release it from matrix,[22] thus adding to the complexities of its signalling. Individual VEGF-A isoforms perform distinct roles during embryonic vascular development,[25] and this is also the case during tumour vascularisation.[26] Our team showed that tumours developed from mouse fibrosarcoma cells that exclusively express the short diffusible $VEGF_{120}$ isoform develop large distended vessels that are highly unstable and leaky and poorly covered in pericytes, in contrast to tumours that produce only the long matrix-bound $VEGF_{188}$ isoform which develop narrow vessels that are more stable and less permeable.[26] The relative abundance of each isoform differs greatly between different tumours,[27–29] and together with differences in matrix composition and protease production may, therefore, contribute to different outcomes of VEGF-A signalling and tumour vascularisation. To date, there are no antibodies specific for individual isoforms and therefore tumour associated VEGF-A isoforms can only be distinguished at the molecular gene expression level. This limits our understanding of the complex role-played by VEGF-A isoforms in tumour growth.

The effects of VEGF-A are not restricted to activating endothelial cells, since many cells within the tumour microenvironment including immune cells, myofibroblasts, macrophages as well as tumour cells themselves express functional receptors and respond to VEGF-A.[30] VEGF-A recruits myeloid cells that differentiate into macrophages and endothelial precursor cells from the bone marrow that are capable of sustaining further angiogenesis and tumour growth.[31,32] Tumour cells express VEGF receptors including NRP-1 and VEGF-A can act in an autocrine and paracrine manner to support growth, survival, initiation and maintenance of cancer stem cell characteristics.[33,34] Furthermore, VEGF-A also promotes tumour invasive properties through induction of epithelial-mesenchymal transition.[35] VEGF-A isoforms appear to play distinct roles in at least some of these angiogenesis-independent processes, although the exact mechanisms have not been clearly defined. We showed that $VEGF_{120}$ and $VEGF_{164}$ expressing sarcoma cells proliferated faster and were less apoptotic *in vitro* than $VEGF_{188}$ expressing cells.[36] *In vivo*, sarcoma cells expressing exclusively the shorter isoform $VEGF_{120}$ metastasised more readily to lung from primary tumours and also homed to lung and survived better following intravenous injection compared to VEGF188 expressing cells suggesting that isoform expression confers distinct survival and invasive characteristics to cancer cells.[37] A similar role for $VEGF_{189}$ was seen in breast cancer where intravenously injected cells overexpressing $VEGF_{189}$ produced fewer lung metastases in mice than $VEGF_{165}$ overexpressing or control breast cancer cells.[38]

In recent years, it has become apparent that VEGF plays an important immunomodulatory role within the tumour microenvironment. VEGF-mediated adhesion molecule expression on tumour blood vessels limits cytotoxic CD8+ T cell infiltration into tumours. VEGF also induces checkpoint molecule expression on CD8+ T cells and prevents dendritic cell maturation.[39] In melanoma, tumours that are resistant to T cell checkpoint immunotherapy often have a highly angiogenic profile and, in pre-clinical studies, antiangiogenic therapy can complement immunotherapy by enhancing dendritic cell maturation and T cell infiltration into tumours.[39] Understanding these complex mechanisms through which VEGF promotes tumour growth and angiogenesis is essential in order to develop optimal antiangiogenic strategies for clinical development.

Identification of biomarkers for predicting response to VEGF pathway inhibitors

The identification of biomarkers for predicting clinical benefit from VEGF pathway inhibitors has met with substantial challenges. Multiple pre-clinical and clinical studies have explored circulating, tumour and imaging biomarker candidates but with somewhat limited success. Anti-VEGF-A therapy could interfere with multiple processes within the tumour and it is, therefore, difficult to identify the exact targets and assess response, especially since in the majority of instances other therapies are co-administered, such as chemotherapy, radiotherapy and immunotherapy.

A well-established association exists between high baseline circulating VEGF-A levels and poor prognosis across different types of cancer.[7,40] Evidence for baseline VEGF-A being of predictive value is, however, inconsistent. Data from four large clinical trials of metastatic colorectal, lung and renal cancer showed that baseline VEGF-A was not predictive of bevacizumab response.[7] Similarly, the results of the more recent prospective MERiDiAN study, a double-blind randomised control trial that assessed the use of baseline plasma VEGF-A for predicting response to bevacizumab as first-line therapy in HER-2 negative metastatic breast cancer, also showed no correlation between baseline VEGF-A and treatment benefit.[41] Plasma VEGF-A levels were also not predictive in patients with metastatic colorectal cancer treated with cediranib.[40] On the other hand, Zhao et al performed a meta-analysis of 11 clinical studies of metastatic colorectal cancer and concluded that high baseline VEGF-A was associated with poor response to bevacizumab.[42] In contrast, retrospective analysis of data collected from large Phase III clinical trials of advanced gastric, breast, pancreatic, and colorectal cancer showed that high levels of circulating VEGF-A were associated with *a better* response to bevacizumab.[43,44] VEGF-A analysis in these latter studies was based on an ELISA with reported selectivity for shorter soluble VEGF-A isoforms and it was therefore proposed that specific isoform expression patterns could be important in predicting response to VEGF pathway inhibition. A small clinical study on metastatic renal carcinoma also points to a predictive role for VEGF-A isoforms, since patients with high tumour levels of $VEGF_{121}$ transcripts benefited significantly from sunitinib.[45] Our own pre-clinical data are also in agreement with the results of the clinical studies, since we found that

sarcoma cells expressing $VEGF_{120}$ were more metastatic to lung and therefore more aggressive but were also more responsive to VEGF-A inhibition with B20-4.1.1 antibody, which targets murine as well as human VEGF-A.[37] Given the complexities associated with tumour vascularisation, it is unlikely that a single factor or protein can predict response to VEGF pathway inhibition. Tumour-associated VEGF-A levels do not necessarily correlate with circulating levels,[7] and therefore, tumour VEGF-A must also be considered as a potential biomarker. However, VEGF-A analysed from tumour biopsies of metastatic colon cancer patients failed to show any association with treatment response with bevacizumab.[46]

Various receptors of VEGF have also been examined for their predictive value. Tumour-associated NRP-1 was retrospectively analysed and low levels were found to correlate with good bevacizumab response in gastric, breast and colorectal cancer.[47] Further clinical studies showed that NRP-1 was predictive for the RTKi tivozanib and bevacizumab in a Phase II trial of metastatic colorectal cancer.[48] Tumour microvessel density has also been explored as a potential predictive biomarker for bevacizumab response in a recent randomised control trial of ovarian cancer patients.[49] Patients who displayed higher pre-treatment tumour microvessel density gained a significant PFS benefit from the addition of bevacizumab to standard chemotherapy, in comparison with patients with low microvessel density.[49] In metastatic colorectal cancer, microvascular density was however not associated with improved response to bevacizumab.[46] Despite some promising leads, the field will only develop if these biomarkers can be validated in large prospective clinical trials and, ideally, across different tumour types.

In addition to predictive biomarkers, response biomarkers have also been explored to monitor disease progression. Ovarian cancer patients treated with carboplatin and paclitaxel and bevacizumab were monitored for circulating levels of the angiopoietin receptor tie2.[50] Disease progression and therefore, loss of any benefit from bevacizumab was seen in patients where tie2 levels rose 50% above the nadir point. This allowed selection of patients for whom bevacizumab treatment continuation would be of benefit.

Combining radiotherapy with VEGF pathway inhibitors: mechanisms of interaction

The abnormal characteristics of the tumour vasculature commonly create tumour regions that are poorly perfused and hypoxic, resulting in a radioresistant microenvironment. The concept of targeting the tumour vasculature with VEGF pathway inhibitors to improve radiation response might appear somewhat untenable, since the primary purpose of VEGF inhibition is to reduce vascularisation, which consequently reduces perfusion and causes hypoxia. Nevertheless, many pre-clinical studies have shown that radiotherapy when combined with VEGF pathway inhibitors generally results in improved tumour responses. Several mechanisms through which VEGF pathway inhibitors and radiotherapy interact to enhance tumour response have been proposed, and these are summarised in Table 2.

More stable, mature "normalised" tumour blood vessels with increased pericyte coverage often emerge within 1 or 2 days after VEGF blockade while the more immature vessels are pruned away.[51] "Normalised" vessels are characteristically less chaotic, better perfused and can therefore help sustain a better oxygenated more radiosensitive microenvironment. However, "normalisation" is considered to be transient, especially if the dosing of a VEGF pathway inhibitor is excessive and/or continuous, in which case vessels subsequently regress and become poorly perfused.[18] In several pre-clinical models, radiotherapy was found to be at its most effective when administered *during* the "normalisation window",[52,53] thereby suggesting that optimal benefit is likely to be highly dependent on the precise scheduling of the two modalities. MRI techniques have enabled the visualisation of vessel normalising effects of anti VEGF therapies in human tumours.[54–59] For example, vessel normalization was evident within 24 h of administration of AZD2171 (cediranib) in

Table 2. Proposed mechanisms through whichVEGF pathway inhibitors and radiotherapy interact to enhance tumour response.

Depending on dosing and timings, VEGF inhibition can "normalise" tumour blood vessels creating a better perfused and oxygenated, radiosensitive microenvironment. Radiotherapy when administered during this period is more effective.
VEGF is a pro-survival factor and can protect endothelial cells from radiation damage. Therefore, blocking VEGF signaling enhances radiation damage to endothelial cells. This can occur in the absence of vessel "normalisation" especially if the dosing of the anti-VEGF therapy is excessive and/or continuous, in which case vessels may regress and become poorly perfused, contributing to tumour cell death.
Fractionated radiotherapy can improve tumour blood flow (normalising effect) via various mechanisms such as reduction in oxygen consumption and tissue pressure due to tumour cell kill, radiation-induced production of vasoactive agents such as VEGF and nitric oxide, pruning of the most radiation-sensitive vessels. These changes are associated with waves of oxygenation that generate reactive oxygen species that in turn stabilise HIF1∝ in tumour cells and increase VEGF production. VEGF then drives more angiogenesis and tumour growth and confers resistance to radiotherapy. Therefore, anti-VEGF therapy can be effective in enhancing response to radiotherapy even when administered after radiotherapy.
High doses of radiotherapy can cause profound damage to tumour blood vessels and cause endothelial cell apoptosis through ASMase-mediated generation of ceramide. Profound apoptosis can contribute to indirect tumour cell kill. VEGF reduces ceramide production following a single high dose of radiation and therefore protects tumour vessels from radiation damage while VEGF inhibition promotes ceramide-mediated apoptosis and enhances the effect of radiotherapy.
VEGF inhibition reduces the acute mobilisation of circulating endothelial cells and endothelial progenitor cells, which could otherwise repopulate the tumour and contribute to radiotherapy resistance.
VEGF inhibition may in some instances radiosensitise tumour cells and reduce their survival and ability to repair DNA damage.
VEGF inhibition and radiotherapy may interact to modify the immune response to cancer cells.

recurrent glioma and remained sustained for at least 28 days,[57] thus providing a reasonably long window that could potentially be exploited for optimal targeting with radiotherapy. Further clinical studies are discussed in the next section of this review.

Several studies have shown that vessel normalisation is not a necessary pre-requisite for improving radiotherapy response by VEGF inhibition. In a HT-29 xenograft model, ZD6474 (vandetanib) enhanced tumour response to radiotherapy independent of scheduling, since a similar enhancement of growth delay was seen if the VEGF inhibitor was delivered just before, during or after radiotherapy.[60] Even in circumstances when VEGF blockade caused a reduction in tumour perfusion and induced hypoxia, addition of radiotherapy still resulted in improved tumour responses.[61,62] A significant increase in tumour growth delay was observed following concomitant administration of radiotherapy and AZD2171 (cediranib) in lung (Calu-6) and colon (LoVo) human tumour xenografts, compared to the growth delay observed with radiation or AZD2171 alone, even though the combination caused a substantial reduction in microvascular density and an increase in hypoxia.[61] Similarly, delivery of an anti-VEGF-A antibody via an oncolytic vaccinia virus caused a substantially greater tumour growth inhibition and reduction in microvessel density, compared to the growth inhibition achieved with the non-antibody expressing virus, when combined with a concomitant course of fractionated radiotherapy.[62] Earlier studies by Gorski et al showed that blockade of VEGF activity in a Lewis lung carcinoma and a range of human tumour xenografts enhanced the tumour radiation response and this was attributed to radiosensitising effects on endothelial cells.[63,64] VEGF is an established pro-survival factor and protects endothelial cells from radiation damage as shown by many studies where VEGF inhibitors radiosensitised endothelial cells in culture.[63,65,66] Therefore, improved response when both modalities are administered concomitantly could be explained by an enhancement of the radiosensitivity of endothelial cells through blocking of VEGF signalling.

VEGF expression is induced in tumour cells that survive radiotherapy through HIF1α-upregulation.[64,67] Improved vascular perfusion and oxygenation are often seen at early times following fractionated radiotherapy probably because oxygen consumption and tissue pressure are reduced following tumour cell death.[68] Fractionated radiotherapy may also cause vessel maturation and pruning of immature vessels in the tumour.[69] Waves of re-oxygenation associated with these effects generate reactive oxygen species that contribute to the stabilisation and upregulation of HIF1α.[64] Tumour cells that survived radiotherapy upregulated HIF1α and were tracked moving towards surviving blood vessels in an elegant study by Harada et al in a colon adenocarcinoma xenograft model.[70] Surviving tumour cells may, therefore, protect the tumour vasculature from subsequent radiation damage and induce further rounds of angiogenesis via their VEGF activity, which could explain the efficacy of VEGF inhibition even when inhibitors are administered post-radiotherapy. Zips et al showed that tumours vascularised by radiation-damaged vessels were significantly more responsive to PTK787/ZK222584 (vatalanib).[71] Similarly, Williams et al showed that ZD6474 was substantially more effective at reducing growth in a non-small cell lung tumour model (calu-6) when administered after the end of a course of radiotherapy rather than by a concomitant protocol.[72] Kleibeucker et al recently showed that fractionated radiotherapy combined with low-dose sunitinib, which alone was ineffective at reducing tumour growth in an HT-29 xenograft, decreased the rise in perfusion seen by radiotherapy and caused substantial reduction in tumour growth compared to radiotherapy alone.[73] Therefore, careful dosing of an anti-VEGF agent may suppress the waves of re-oxygenation and hence reduce resistance without causing too much toxicity and excessive hypoxia. VEGF inhibition may also reduce the acute mobilisation of circulating endothelial cells and endothelial progenitor cells which also contribute to radiotherapy resistance.[74]

The consequences of radiotherapy on the vascular compartment of tumours are complex and dose-dependent.[68] An initial improvement in vascular function following fractionated radiotherapy tends to gradually decline as treatment continues. On the other hand, when radiotherapy is delivered as a high single fraction, vascular damage to tumours can be rapid and profound. Garcia-Barros et al proposed that vascular damage caused by endothelial apoptosis mediated by an acute generation of ceramide through activated acid sphingomyelinase (ASMase) was the major driver of tumour cell death following radiotherapy.[75,76] This suggestion was met with a wave of controversy with many providing experimental evidence disputing that endothelial damage was a major contributor to tumour radiation response.[77,78] Subsequently, it was proposed that while endothelial damage could play a dominant role in tumour response after a single high dose of radiation (>10 Gy), lower fractionated doses (≤3 Gy/fraction) are less damaging to endothelial cells and in this case, the contribution of a vascular response to tumour cell kill by radiotherapy becomes less significant.[79] Truman et al,[80] showed that VEGF abrogated radiation-induced ASMase activation and ceramide production in endothelial cells and protected cells against apoptosis, while VEGF signalling inhibition further activated ASMase and ceramide production and sensitised endothelial cells to radiation damage. *In vivo*, DC101 (a VEGFR-2 antibody) improved the response to radiotherapy in a MCA/129 fibrosarcoma model, achieving tumour eradication, but only if administered approximately 1 h before radiotherapy.[80] This study provided evidence that VEGF protected against radiation-induced apoptosis by repressing the rapid activation of ASMase thus reducing pro-apoptotic ceramide production. Similar results were subsequently shown for axitinib and pazopanib in various tumour models.[81,82]

Cancer patients can now be treated more effectively with techniques such as stereotactic body radiation therapy (SBRT) and stereotactic radiosurgery that typically deliver fewer but high dose fractions of radiation than conventional fractionation techniques, and aim to be curative. Higher dose fractions would be expected to damage the tumour vasculature sufficiently to contribute to tumour cell death. Although very effective, these approaches can be limited by toxicity to surrounding normal tissues. Nevertheless, improvement of therapeutic efficacy by engaging the vascular component in this way would need to be

balanced by considering treatment-induced toxicities, which can be more pronounced when stereotactic body radiation therapy is combined with antiangiogenic agents.[83]

The pro-survival effects of VEGF are not limited to endothelial cells and many tumour cells express functional VEGF receptors and respond to VEGF.[30] Some studies have shown that VEGF inhibition can radiosensitise tumour cells *in vitro*,[84–86] although others have shown that VEGF plays no role in direct tumour cell radiosensitisation.[65] Despite *in vitro* evidence of radiosensitisation, it is nevertheless unclear whether VEGF inhibition contributes to direct radiosensitisation of tumour cells *in vivo*. Brooks et al showed that sunitinib radiosensitised P3 cell *in vitro* but failed to directly radiosensitise cells *in vivo*.[85] However, DNA repair genes were downregulated in human colorectal cancer after treatment with bevacizumab and could therefore, offer a potential mechanism of radiosensitisation.[87]

Clinical trials of VEGF pathway inhibitors combined with radiotherapy

Although to date VEGF pathway inhibitors are not administered together with radiotherapy as part of any standard treatment protocol, numerous clinical trials have tested this combination in a variety of settings, as reviewed recently.[88,89] Two Phase III trials have been carried out in newly diagnosed glioblastoma comparing chemoradiotherapy (with temozolomide) against the same regime plus bevacizumab administered either from Week 1 (AVAglio trial, 921 patients randomised),[90] or Week 4 (637 patients randomised) of chemoradiation.[91] Bevacizumab had a beneficial effect on PFS, albeit only reaching statistical significance when it was started in Week 1 and with some increase in toxicity in both trials. There was no effect on OS in either study, which is difficult to achieve in this disease due to rapid clinical progression and which may have been confounded by substantial cross-over of the placebo group to bevacizumab treatment.[90] Earlier phase clinical trials with a range of VEGF pathway inhibitors and lower patient numbers are ongoing or have been already published in a range of tumour types, including head and neck cancer, non-small cell lung cancer, pancreatic cancer, gynaecological cancer, rectal cancer, renal cancer and soft tissue sarcoma.[89] The majority of the published trials have tested the combination of bevacizumab with chemoradiotherapy, although small molecule receptor kinase inhibitors such as sorafenib, sunitinib, cediranib and pazopanib are also being tested.[89] Although some of these combinations show promise, they remain to be tested in large randomised clinical trials and toxicity remains a concerning issue.

Pre-clinical studies have shown the importance of optimising treatment schedules in terms of dosing and timing of radiotherapy and drugs. However, testing different schedules at the clinical level is a complex issue,[92] especially because standard treatments commonly involve chemoradiation, rather than radiotherapy as a single modality. Information on how different treatment schedules influence therapeutic response in the clinic is limited and optimal timing of VEGF pathway inhibitor administration with respect to radiotherapy has not been established. Moreover, it is highly likely that only subgroups of tumours of a particular type respond well to VEGF pathway inhibitors. As described above, the identification of biomarkers for predicting respond to anti-VEGF therapies has been challenging. It is an even bigger challenge to identify biomarkers to predict response to anti-VEGF/radiotherapy combinations. Since the proposed mechanisms of interaction largely involve changes in the tumour microenvironment, these studies largely rely on sophisticated imaging techniques to estimate parameters such as tumour blood flow, blood volume, vascular permeability, oedema and oxygenation. Validation of these techniques is difficult and, in many cases, there is little consensus on methodology to acquire and analyse data for a particular end-point across different centres.[93] For instance, K_{trans} is a commonly used MRI measure describing a composite of tumour vascular permeability—vascular surface area product and blood flow rate following intravenously administered gadolinium-DTPA. One study showed that absolute measures of K_{trans} can vary by an order of magnitude across centres.[94]

Nevertheless, attempts have been made to monitor tumour vascular changes in the clinic following anti-VEGF treatment and exploit the vessel normalisation "window" for scheduling radiotherapy or chemoradiotherapy. Increased perfusion would potentially increase tumour cell exposure to chemotherapeutic drugs and increase tumour oxygenation to sensitise tumour cells to radiotherapy. Addition of cediranib to chemoradiation caused an increase in perfusion and oxygenation in a subset of newly diagnosed glioblastoma patients, as determined from dynamic susceptibility contrast MRI data,[95] which was suggestive of blood vessel normalisation.[59] In these patients, median OS was significantly longer compared to patients where perfusion either remained stable or declined through the course of the combined treatment (17.0 *vs* 26.3 months). Similar results were seen in a study by the same group investigating treatment outcomes in recurrent glioblastoma patients treated with cediranib and chemoradiotherapy.[58] Response biomarkers such as changes in perfusion appeared rather rapidly after anti-VEGF therapy commenced and so also have potential for predicting those patients likely to benefit from continued dosing of an anti-VEGF agent.[59]

As detailed above, the search for circulating predictive biomarkers for response to bevacizumab and other VEGF pathway inhibitors has received a great deal of attention, but with only limited success. In the AVAglio trial cited above for comparison of chemoradiotherapy ± bevacizumab in newly diagnosed glioblastoma, it was observed that a substantial subpopulation of the patient group appeared to benefit from the addition of bevacizumab.[96] A retrospective study was carried out on blood samples from 563 patients in this trial to determine whether plasma levels of YKL-40, a glycoprotein with angiogenic properties that induces VEGF expression and is upregulated in 55–75% of glioblastoma patients, could help to identify this group. It was hypothesised that low YKL-40 enhances the effect of bevacizumab, and overall results showed that PFS but not OS was improved for patients with the lowest plasma concentration of YKL-40 protein. In addition, there was an indication that this marker may be suitable for the proneural but not the proliferative/mesenchymal subtype of glioblastoma. It is highly likely that

achieving a clinical therapeutic benefit for the combination of VEGF pathway inhibitors with radiotherapy will rely on further development of suitable biomarkers to identify the most appropriate patients for this treatment.

Very few of the studies reported to date have directly compared different scheduling protocols in parallel. Avallone et al compared concomitant vs sequential administration of bevacizumab in locally advanced rectal cancer patients treated with radiotherapy. However, toxicity was found to be a major issue with the concurrent schedule, which had to be terminated early in their trial.[97] Other trials have begun to investigate use of non-standard radiotherapy dosing regimens, such as hypofractionation, in combination with bevacizumab for instance.[98,99] One theory for this approach is that inhibition of VEGF activity reduces vascular permeability, enabling use of more aggressive radiotherapy. The Omuro study[98] was a Phase II study of bevacizumab in combination with chemoradiation (temozolomide) in newly diagnosed glioblastoma. The combination treatment was safe and OS was comparable with that obtained from historical controls. Carlson et al[99] compared results from two consecutive trials of hypofractionated radiotherapy with temozolimide in a similar group of glioblastoma patients, the first without bevacizumab and the second with the antibody treatment. In this indirect comparison, PFS was longer with bevacizumab, but not significantly so and OS was unaffected.

CONCLUSIONS AND THE WAY FORWARD

Although antiangiogenic agents have not lived up to the original expectation that they would cause tumours to revert to an avascular dormant state, nevertheless their use still holds great promise for improving therapeutic outcomes when used in combination with radiotherapy and other modalities. The vessel normalising activity of antiangiogenic agents and the consequence of such "normalisation" on improving tumour oxygenation clearly play a major role in enhancing the radiotherapy response. And yet, the normalising effects of VEGF inhibition are at best only transient, while normalisation is not always the outcome of VEGF inhibition. Indeed, antiangiogenic therapy can also cause hypoxia or fail to alter tumour oxygenation altogether. Siemann et al reviewed the evidence for the effects of antiangiogenic therapy on tumour oxygenation in the pre-clinical setting and reported many studies showing variable effects thus illustrating the difficulties of assessing this even in the pre-clinical setting when various key parameters are more easily controlled.[100] Parameters such as dose and duration of treatment, time of assessment of oxygenation, type of antiangiogenic agent used or indeed tumour model all could account for such variability. As stated above, optimal protocols for establishing a predictable and sustained "window or normalisation" are required so that radiotherapy can be administered in an effective way.

Considering the plethora of possibilities for testing, it is unlikely that clinical trials reported to date have fulfilled the potential for combining VEGF pathway inhibitors with radiotherapy. Further development of well-validated imaging and/or blood-based bio-markers for monitoring changes in the tumour microenvironment will be crucial in meeting the challenges associated with optimising this approach, notably identifying those patients most likely to benefit, identifying the best antivascular agents and determining the best scheduling regimens. The focus here should perhaps be on dynamic biomarkers of response that can be used to monitor patients during treatment to allow therapy to be delivered to individual patients in the most effective way. Vascular biomarkers such as circulating tie2 and Ang2 were recently shown to closely correlate with continued bevacizumab benefit in colorectal and ovarian cancer patients and used as a means of deciding on continuation of treatment.[50] Progress in this area has been reviewed recently elsewhere.[101]

Increasing the tumour's own immune clearance mechanisms is also likely to be an important way forward. Radiotherapy itself can trigger the tumour's own immune clearance but can also result in the development of an immune suppressive environment through complex mechanisms. It is now becoming apparent that both radiotherapy and antiangiogenic approaches can benefit from combination with immune checkpoint inhibition. Understanding how the two modalities work together to modulate the immune response is an important requirement for optimising treatment protocols. Further progress in both pre-clinical and clinical research is indeed needed to determine whether combining VEGF pathway inhibitors with radiotherapy will ultimately benefit cancer patients in the future.

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