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Commentary

The angiogenic process as a therapeutic target in cancer

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ABSTRACT

Angiogenesis has emerged as a critical process for tumour progression. Identifying key pathways involved in the regulation and promotion of angiogenesis has resulted in the development of numerous approaches targeting pro-angiogenic signalling pathways. The most prominent and characterised pro-angiogenic pathway is the vascular endothelial growth factor signalling pathway. This review will describe several inhibitors of angiogenesis currently in clinical trial and their various targets. Targeting pro-angiogenic pathways has improved outcome for many patients, however, the emerging problems with drug resistance with clinically approved angiogenic inhibitors will also be discussed in this review. It is hoped that identifying the causes of tumour re-growth and disease progression following treatment will enable future anti-angiogenic therapies to circumvent resistance.

1. Introduction

Advances in molecular biology are carefully dissecting the mechanisms underlying tumourigenesis (the formation and progression of a tumour). The growth of new blood vessels, termed angiogenesis, and low oxygen levels (hypoxia) are two well established hallmarks of solid tumours [1]. In pathological conditions, such as cancer, a chronic unregulated angiogenic state exists, whereas in physiological conditions, such as pregnancy and wound healing, angiogenesis is tightly regulated. As the tumour mass increases, so does the demand for a sufficient supply of nutrients and oxygen. Tumour cells in areas within the tumour that become nutrient and oxygen depleted release angiogenic promoting signals, which drive the angiogenic switch to expand the tumour vascular network [2].

Briefly, the initial process of angiogenesis involves the stimulation of the endothelial cells lining the luminal surface of the blood vessel, resulting in the release of proteases, including matrix metalloproteinases, which subsequently leads to the extracellular matrix degradation. The second phase of angiogenesis, known as sprouting, is spearheaded by leading endothelial tip cells that enter the underlying tissue and migrate along the chemotaxic gradient towards the source of the angiogenic stimuli [3]. The stalk cells follow the tip cells and proliferate in response to the environmental cues. It is the stalk cells which co-ordinate the formation of the vascular lumen and extracellular matrix [4], as well as establish adherins/tight junctions [5] to maintain the integrity of the new sprout. Mural cells, such as pericytes, become sparse or absent in growing vessels, but reappear and stabilise mature non-growing capillaries [6].

The vascular network within the tumour enables malignant cells to escape from the primary tumour and enter into circulation and establish distant metastases elsewhere. Not surprisingly then, angiogenesis has emerged as a critical process driving tumourigenesis. The concept of attacking tumours by cutting off their blood supply was first described in the early 1970s [7] and subsequently many studies have demonstrated in preclinical models that targeting tumour angiogenesis will compromise tumour growth. Numerous agents that are currently undergoing clinical development aim to interfere with signals promoting angiogenesis. The first part of this review will briefly highlight several pathways targeted by inhibitors of angiogenesis, both clinically approved and those in development. This approach is distinguished from the acute vascular damage and tumour necrosis induced by vascular targeting [8]. The second part will discuss the concerns raised regarding anti-angiogenic therapy following emerging problems with drug resistance, and potential solutions.

2. Targeting signals promoting angiogenesis

2.1. Pathways regulating tumour blood supply

A finely tuned equilibrium between anti- and pro-angiogenic molecules modulate the complex and dynamic events during angiogenesis [2]. In physiological conditions the balance between

Abbreviations: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; FGF2, fibroblast growth factor 2; HGF, hepatocyte growth factor; PDGF, platelet derived endothelial growth factor; PlGF, placental growth factor; Dll4, delta like-4.

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the anti- and pro-angiogenic results in the limited new blood vessel development, whereas in pathological conditions the balance is tipped towards excessive blood vessel development, due to the abundance of pro-angiogenic stimulii. Key mediators of angiogenesis, and therefore potential targets of anti-angiogenic therapy, include the vascular endothelial growth factor (VEGF, also known as VEGF-A), fibroblast growth factor 2 (FGF2, also known as bFGF), angiopoietins 1 and 2, hepatocyte growth factor (HGF) and ephrin signalling (Fig. 1).

Anti-angiogenic strategies have been developed to prevent stimulation of endothelial cells by pro-angiogenic molecules released from the extracellular matrix by proteases or secreted by a variety of cell types (stromal and tumour cells) [13]. Of all the pro-angiogenic molecules the most prominent and best characterised is the VEGF signalling pathway, a key component in both the early and late phases of angiogenesis. VEGF is produced at high levels by tumour cells and the immediate tumour stroma, and its receptors can be found expressed on both stromal and tumour cells. High expression of VEGF is an independent factor predicting poor prognosis in various types of malignant tumours, including colon cancer, breast cancer, gastric carcinoma, prostate cancer, non small cell lung cancer, sarcoma, hepatocellular carcinoma and melanoma [14]. The VEGF receptor (VEGFR) family has different roles in regulating angiogenesis. VEGFR-2 (also known as KDR, Flk1) is prominently expressed in the polarised extension of tip cell filopodia, enabling migration along the chemoattractant gradient [3]. The VEGFR-1 (also known as Flt1) modulates VEGFR-2 signalling, by acting as a decoy receptor and sequestering VEGF from VEGFR-2 [15]. A recent study also demonstrated VEGFR-3 (also known as Flt4) signalling influenced sprouting angiogenesis [16]. Not surprisingly then, many components of the VEGF signalling pathway are the current major targets for anti-angiogenic therapies. Ephrin-B2 reverse signalling can control VEGFR2 and VEGFR3 internalisation, and subsequently regulate endothelial tip cell migration and angiogenic sprouting [17]. Therefore, blocking the ephrin-B2 reverse signalling may be another ideal candidate for anti-angiogenic therapy.

The emerging picture is that the VEGF pathway acts as a potent upstream activating stimulus for angiogenesis, whereas cross talk with other signalling pathways, such as Notch signalling, helps to shape that activation appropriately [18]. Notch signalling is dependent on cell-to-cell contact and can regulate cell fate, proliferation and survival in the signal receiving cells. Delta like-4 (Dll4)/Notch signalling acts as negative feedback of VEGF signalling to restrict tumour angiogenesis by mechanisms including decreasing endothelial cell proliferation, reduce VEGFR2 expression and induce VEGFR1 expression (reviewed in [18]). Hypoxia (low oxygen levels) and growth factor (e.g., VEGF, FGF2) stimulation in endothelial cells results in the up-regulation Dll4 expression, particularly in the tip endothelial cells [19–21]. Interfering with the Notch signalling pathway, as described in more detail later, leads to poorly perfused blood vessels [22–24].

Alternatively, another anti-angiogenic strategy being developed is to hinder the recruitment of various cell types within the tumour. HGF, the c-met activating ligand can be secreted by a variety of cells within the tumour, including fibroblasts, smooth muscle cells, as well as tumour cells. Although HGF/c-met signalling can induce VEGF and VEGF receptor expression in endothelial cells, this pathway can also act independently in promoting angiogenic proliferation and survival signals [25]. Another key angiogenic growth factor is FGF2, a member of a family of heparin-binding proteins; FGF signalling can modify angiogenesis independently to the VEGF stimulus [26]. Tumour cells, as well as fibroblasts, can secrete FGFR ligands. FGF-2 expression levels have been found to correlate with cancer progression and metastasis in many different tumour types [26]. In response to angiogenic stimuli, endothelial cells can secrete platelet derived growth factor (PDGF)-B, in order to recruit

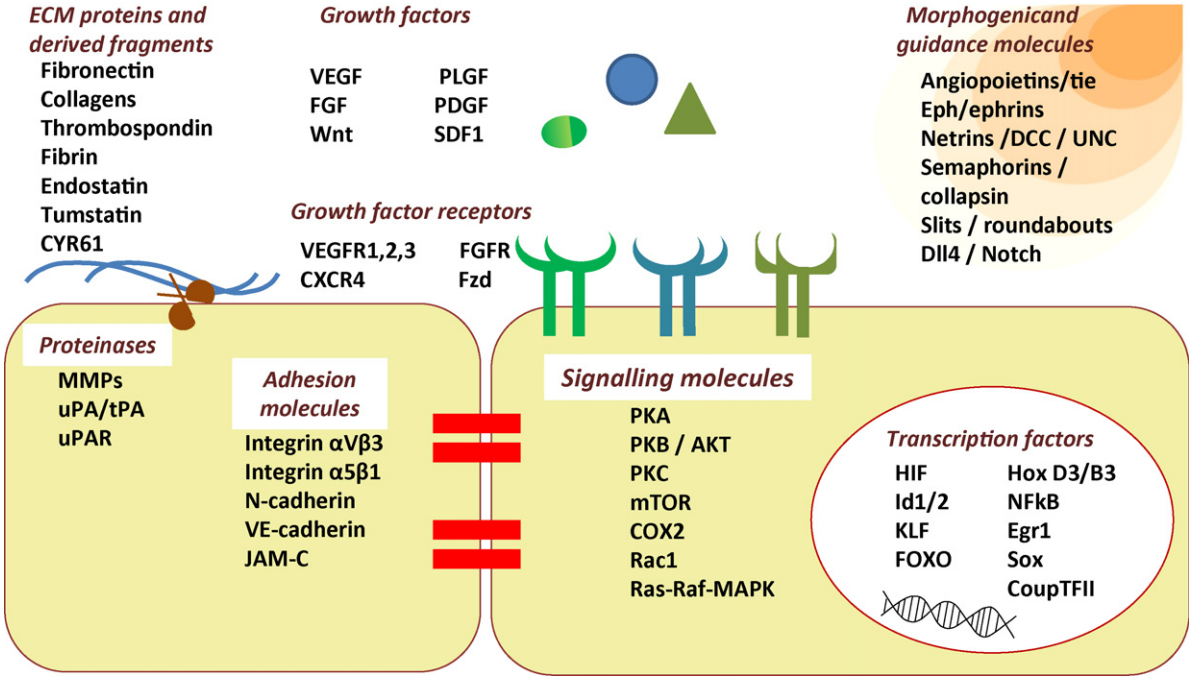

Fig. 1. A summary depicting some of the major molecules that are implicated in mediating angiogenesis. Cysteine-rich angiogenic inducer 61 (CYR61), matrix metalloproteinases (MMPs), urokinase (uPA), uPA receptor (uPAR), tissue-type plasminogen activator (tPA), vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR), fibroblast growth factor (FGF), FGF receptor (FGFR), platelet derived growth factor (PDGF), stromal cell derived factor 1 (SDF1), SDF1 receptor (CXC chemokine receptor; CXCR4), wnt receptor (Frizzled, Fzd), junctional adhesion molecule C (JAM-C), protein kinase A (PKA), protein kinase B (PKB), protein kinase C (PKC), mammalian target of rapamycin (mTOR), cyclooxygenase 2 (COX2), ras related C3 botulinum toxin substrate 1 (Rac1), mitogen activated protein kinase (MAPK), netrin4 receptor (DCC), delta like 4 (Dll4), hypoxia inducible factor (HIF), inhibitor of DNA binding 1/2 (Id1/2), Kruppel-like factor (KLF), forkhead box O (FOXO), nuclear factor kappa B (NFκB), early growth response transcription factor 1 (EGR1).

pericytes cells to the new blood vessel [27]. PDGF-B signals through PDGFR, which is found expressed on pericytes [27]. Angiopoetin1/Tie2 signalling can also aid the recruitment of pericytes [28]. Pericytes play an important role during the later stages of angiogenesis by stabilising the maturing blood vessel, support endothelial cell survival and also release pro-angiogenic molecules [13]. Placental growth factor (PIGF), signalling through VEGFR1, is another growth factor that not only can induce endothelial cell proliferation, migration and survival but also acts as a chemo-attractant for macrophages [29]. Tumour associated macrophages are also essential promoters of tumour angiogenesis, tumour cell migration, invasion and metastasis [30]. Endothelial progenitor and other bone marrow derived cells, such as inflammatory cells, can also be recruited in response to growth factors such as VEGF and FGF2, and may be suitable targets of anti-angiogenic therapy [9,31]. For instance, *de novo* generation of endothelial cells from endothelial progenitor cells contributes to the growth of new blood vessels and tumourigenesis, in a process referred to as vasculogenesis [9,10].

2.2. Current anti-angiogenic strategies

The crucial role of blood vessels in cancer and the assumed absence of angiogenesis in (most) healthy conditions have primed tremendous efforts to develop different approaches to block pro-angiogenic signalling pathways. Angiogenesis research is based on the assumption that preventing the growth of new blood vessels will impair the viability of tumour cells, but tumour cell susceptibility to hypoxia induced death may be modified by mutational events occurring as part of tumourigenesis or as a consequence of the treatment, as discussed later. The aim of anti-angiogenic therapy is to stabilise the disease and keep tumourigenesis in check, rather than eradicating tumour mass, as in the case of the endpoints of chemotherapy and radiotherapy.

Out of the several hundreds of molecules with anti-angiogenic activity in experimental models, about one hundred of these have entered clinical testing in cancer patients, and around 10 of these so far have become approved for clinical use (source of information NCI clinical trial database, www.cancer.gov/clinicaltrials) [32]. Table 1 highlights the main targets of anti-angiogenic therapy being evaluated in clinical trials now encompassing a wide range of targets other than VEGF and its signalling pathway; recent reviews have also indicated the progress of therapy, reporting the patient outcome and response rates of treatment in phase III as well as featuring additional lists of anti-angiogenic agents undergoing clinical evaluation (i.e., [33,34]). However, why some inhibitors work in one cancer type and not another is not understood and highlights the complexity of angiogenesis when applied to an individual tumour and the pathways to target, as discussed later.

Several anti-angiogenic inhibitors have been licensed by the FDA in various tumour types, however, only in patients presenting metastatic disease and without a curative potential [34]. In 2004 the FDA approved the first anti-angiogenic molecule to be utilised in the clinic, which was bevacizumab (Avastin®, ranibizumab). Bevacizumab is a humanised neutralising antibody against VEGF and was first shown to extend survival of patients with advanced colorectal cancer [35] and later extension of progression free survival was also observed in non-small cell lung cancer, breast cancer, glioblastoma, and metastatic renal cell carcinoma [36–39]. In contrast to preclinical studies however, efficacy in patients was only observed following combination therapy with conventional chemotherapy and/or radiotherapy, with the exception of patients with renal cell carcinoma [40]. The small molecule receptor tyrosine kinase inhibitors sunitinib (Sutent®), sorafenib (Nexavar®; BAY 43-9006) and pazopanib (GW786034, Votrient) have also been approved for the treatment of metastatic renal cell carcinoma [41–43]. Additionally, sunitinib has been approved for use treatment of gastrointestinal stromal tumours [44] and sorafenib for treatment of hepatocellular carcinoma [45]. These receptor tyrosine kinase inhibitors affect angiogenesis by targeting multiple pro-angiogenic signalling pathways, which include the VEGF and PDGF receptor families.

Some other therapeutic approaches have been shown to exert anti-angiogenic activity, such as hormonal agents, metronomic chemotherapy, bisphosphonates and others. For example, thalidomide and its analogues lenalidomide and pomalidomide are a class of immunomodulatory drugs that target Tissue Necrosis Factor α and display anti-angiogenic properties in preclinical models [46]. Lenalidomide lacked some of the severe toxic effects observed by thalidomide and has been approved by the FDA for treatment in multiple myeloma, whereas pomalidomide is still in preclinical trial [46]. Peptibody (AMG-386), which prevents the interaction of the angiopoietins with the Tie2 receptor, demonstrated anti-angiogenic activity in preclinical trials and is subsequently being evaluated in phase II trials in a variety of tumour types (Table 1), based on the findings of the combinational phase I studies [47]. Currently small kinase inhibitors have been developed to target the HGF/c-met pathway, which includes TAK-701, SCH900195, humanised antibodys against HGF, as well as MetMAb, a antagonist antibody to the c-Met receptor, all of which are being screened in early clinical trials [25]. Antibodies (Dovitinib, BGJ398) and small molecule FGF receptor kinases inhibitors (Inedanib/BIBF1120, TSU-68) that target multiple pathways, including the FGF signalling pathway, are currently being evaluated in clinical trials [26]. Multiple preclinical and early clinical studies are also providing evidence of bisphosphonates (including clodronate, pamidronate and zoledronic acid) improving tumour response, disease-free survival, and overall survival in patients in various cancer types including myeloma and breast cancer [48,49]. The mechanisms of anti-cancer activity of bisphosphonates include interfering with recruitment of cells into the tumour, including endothelial progenitor cells, and reducing tumour angiogenesis [50]. Recent trials have also investigated whether endothelial cells are sensitive to chemotherapeutic agents administered at low and frequent doses, known as metronomic chemotherapy. Although the tumour response in clinical trials has been variable, metronomic chemotherapy has low toxicity, as well as enhancing the efficacy of some anti-angiogenic and cytotoxic agents [51].

3. Does anti-angiogenic therapy have a dark side?

In many preclinical tumour models blockage of the VEGF pathway was found to reduce tumour vascular density and inhibit xenograft tumour growth. However, although blocking the VEGF pathway can improve progression-free survival, the overall survival is not prolonged in a variety tumour types [40,52]. Recent preclinical [53,54] and clinical [55,56] studies indicated that the therapy might induce a more invasive phenotype as a consequence of the anti-angiogenic therapy utilised. This view is not supported when looking at other clinical trial data and the benefits of therapy to patients, recently covered in [57], i.e., randomised trials have not shown patients treated with the drugs do worse than controls or have more metastases on progression.

Nevertheless, the preclinical and clinical studies clearly demonstrate the emergence of resistance affecting the overall survival rate of patients receiving anti-angiogenic therapy. Resistance to anti-angiogenic therapy and subsequent tumour re-growth following treatment could reflect the intertwined consequence of the tumours displaying adaptive (evasive) resistance and intrinsic (pre-existing) non-responsive resistance; such mechanisms have been more extensively discussed in recent reviews (e.g., [58]). However, the difference between the preclini-

Table 1
List of several anti-angiogenic agents and their targets currently been evaluated in clinical phase trial involving a variety of tumour types.

Compound	Target	Tumour type compound is being investigated in
Antibodies		
AMG-386	Angiopoietin 1 and 2	Colorectal cancer, renal, liver, gastric. Gastroesophageal and distal esophageal adenocarcinoma
Bevacizumab	VEGF	Breast cancer, colorectal cancer, glioblastoma, head and neck cancer Liver cancer, neuroectocrine tumours, non small cell lung cancer, ovarian cancer, pancreatic cancer, renal cancer
IMC-1121b	VEGFR2	Non small cell lung cancer, breast, colorectal, lung, ovary, prostate, renal, liver and melanoma
IMC-18F1	VEGFR1	Solid tumours
VEGF trap	VEGF	Colorectal, lung, pancreas, glioma, prostate, ovary, endometrial, soft tissue sarcoma, renal cancer, ovarian cancer
Receptor tyrosine kinase inhibitors		
AV-299	HGF	Non small cell lung cancer
Apatinib	VEGFR2 Ret c-KIT c-SRC	Colorectal cancer
Axitinib	VEGFR PDGFR	Renal cancer, breast, lung, liver, prostate and colorectal cancer, melanoma
BIBF 1120	VEGFR PDGFR FGFR	Colorectal cancer, liver cancer, non small cell lung cancer
Brivanib alaninate	VEGFR2 FGFR	Advanced cancer
Crizotinib	c-Met/HGFR	Non small cell lung cancer
Erlotinib hydrochloride	EGFR	Breast cancer, colorectal cancer, glioblastoma Head and neck cancer, liver cancer, neuroectocrine tumours Non small cell lung cancer, ovarian cancer, pancreatic cancer Prostate cancer, renal cancer
Enzastaurin hydrochloride	PKC-beta	Brain metastasis
Foretinib	VEGFR c-Met Flt3 c-Kit	Non small cell lung cancer, breast and liver cancer
Linifanib	VEGFR PDGFR FLT1 FLT3 CSF-1R c-KIT	Liver cancer
MetMab	c-Met	Breast
Pazopanib hydrochloride	VEGFR PDGFR c-KIT	Renal cancer, breast, lung, cervical, liver, thyroid, prostate, colorectal cancer, melanoma, glioblastoma, neuroectocrine tumours, ovarian cancer
Sunitinib malate	VEGFR PDGFRb FLT3 c-KIT Ret CSF-1R	Renal cancer, breast cancer, brain metastasis, glioblastoma, colorectal cancer, carcinoma of the oesophagus, head and neck cancer, liver cancer, prostate cancer, non small cell lung cancer, melanoma, ovarian cancer
Sorafenib tosylate	VEGFR PDGFR c-KIT RAF	Acute myeloid leukemia, breast cancer, cervical cancer, colorectal cancer, glioblastoma, head and neck cancer Liver cancer, non small cell lung cancer, melanoma, thyroid, ovarian cancer, prostate cancer
Vandetanib	VEGFR2 EGFR	Esophageal cancer, non small cell lung cancer, ovarian cancer, breast cancer, colorectal cancer, prostate cancer, renal cancer
Vatalanib	VEGFR1,2 PDGFR	Melanoma
XL184	c-MET/HGFR VEGFR RTK FLT3 TIE2	Glioblastoma
Integrin inhibitors		
Cilengitide	αvβ3, αvβ5 antagonist	Glioblastoma, head and neck cancer, lung cancer
mTOR inhibitors		
Temsirolimus	mTOR	Glioblastoma, melanoma, colorectal cancer, renal cancer
Everolimus	mTOR	Leukemia, breast cancer, colorectal cancer, glioblastoma Head and neck cancer, liver cancer, melanoma Non small cell lung cancer, ovarian cancer, prostate cancer
Ridaforolimus	mTOR	Neurofilbromatosis

cal efficacy of anti-angiogenic agents and activity displayed in the clinic likely reflects the preclinical model utilised, the cancer type being clinically treated, as well as differences in the dosages and schedules utilised in the preclinical and clinical setting. Understanding the limitations of pre-clinical models should narrow the discrepancies observed when treatment is translated into the clinic. For example, sunitinib promoted metastasis in animal models of metastasis (e.g., tumour cells injected intravenously),

where resistance to the therapy emerged, as well as revascularisation of the tumour [53,54], compared to outcome of sunitinib therapy in localised orthotopic models [53,59]. Metastatic models, such as injection of tumour cells into the bloodstream mimic the circulating tumour cells from the primary tumour, which then form metastatic tumour deposits. However, only the tumour cells, such as cancer stem cells, with a highly aggressive tumour phenotype will have the ability to survive and invade from blood vessels and form the metastatic tumours. Therefore, it is likely that the selection of such tumour cells also lead to a population of cells with an acquired adaptive/evasive resistance to the anti-angiogenic therapy used, and thereby presented a different response pattern to anti-VEGF to that observed in the primary orthotopic models. Similarly, it is likely that in the clinic patients presenting with macro- or micro-metastatic disease, that the variety in response to the anti-angiogenic therapy also reflects tumour cells having alternative pro-angiogenic signalling pathways promoted which evoke an angiogenic response independently to the loss of the target (e.g., VEGF in anti-VEGF therapy). Selection of highly aggressive resistant tumour cells is also likely to occur as a consequence of the more hostile macro-environment promoted by anti-angiogenic therapy. Hypoxia induces many pro-angiogenic pathways, including matrix metalloproteinases, which in turn can promote more tumour invasion [13]. Such mechanisms of acquired resistance are likely to be missed in pre-clinical mouse models evaluating tumour response in primary tumours over relatively short time periods, compared to the months of therapy that a patient receives. Additionally, it is likely in patients that the recruitment of cell types from the bone marrow (e.g., active immune cells such as macrophages) can contribute to promotion of tumour growth and metastasis [13], a phenomenon not easily capsulated in immuno-compromised mice. The following sections discuss in further detail the various factors that ultimately could lead to the promotion of resistance to anti-angiogenic therapy.

3.1. Factors effecting patient response to anti-angiogenic therapy

1. Requirement for continuous knockdown of anti-angiogenic target and tumour size.

   Anti-angiogenic therapy can induce significant cell death and necrosis within the centre of the tumour. However, tumour and stromal cells often survive in the peripheral rim of the tumour due to the passive diffusion of nutrients and oxygen from the surrounding tissue. When therapy stops endothelial cells can sprout back within the tumour and re-growth of the tumour is again supported. Patients with metastatic breast cancer or metastatic renal cell carcinoma were found more likely to have tumour progression, 'flare', during anti-angiogenic drug free break periods using low molecular weight inhibitors; these breaks are not required with antibodies [60,61]. Therefore future treatment schedules should take into consideration the growing evidence that tumour progression is able to occur in discontinuous anti-angiogenic treatment schedules (i.e., 4 weeks on/2 weeks off). The breaks due to the toxicity of these inhibitors may reflect off-target effects and highlight the need for greater selectivity of treatment appropriate to the patients.

   The timing of starting anti-angiogenic therapy could also be influencing response; currently therapy is started in late stage presenting patients. Anti-angiogenic therapy is generally more efficient in preventing tumour growth, rather than causing regression of established tumours. Therefore anti-angiogenic therapy may perform better in patients with minimal residual disease, or at stage involving growth initiation of dormant tumour cells or micro-metastic tumours. Recent real time imaging preclinical study tracking the fate of individual metastasizing cancer cells demonstrated that VEGF inhibition induced long-term dormancy of lung cancer micrometastases in the brain by preventing angiogenic growth to macrometastases [62]. However, the recent adjuvant trial of bevacizumab in colorectal cancer showed no benefit, raising many questions on mechanisms for early angiogenesis and the need to understand and identify the pro-angiogenic events driving angiogenesis within the tumour during tumourigenesis [63,64].

   Anti-angiogenic therapies can also 'normalise' the tumour vasculature, which has important implications on the efficacy of chemotherapeutic agents and sensitivity to radiation (recently reviewed in [8,65]). Tumour vasculature is structurally and functionally abnormal compared to vasculature within normal tissues. The continuous input of pro-angiogenic stimulus within the tumour results in the development of leaky, tortuous, dilated and saccular blood vessels, and poor blood flow. It has been observed in anti-VEGF therapies that blocking the pro-angiogenic signal resulted in the remodelling and pruning of tumour blood vessels, which lead to transient period of time where blood flow within the tumour increased [65,66]. It was subsequently observed that during this time period that the delivery of chemotherapeutic agents increased, as well as the sensitivity of tumour cells to the effects of radiation. However this is not a uniform finding and needs further study. The feature of blood vessel normalisation may partly explain the clinical trials where tumour response was only observed in combinational studies with bevacizumab, rather than with bevacizumab alone [36–39]. As the time period where vessels are normalised is transient and is likely to vary between tumour types, it will be difficult to schedule anti-angiogenic therapies optimised such to provide the maximum delivery of chemotherapy.

2. Switching to alternative pro-angiogenic pathways.

   Rather than one predominant pro-angiogenic factor being solely present within a tumour, the reality is that a pool of pro- and anti-angiogenic factors is present that create the overall angiogenic potential of a given tumour. Therefore sources of adaptive resistance could be due to production of other compensatory pro-angiogenic proteins within the tumour. For example, a variety of pro-angiogenic proteins were found highly expressed within advanced tumours, compared to expression levels found present in other stages, in neuroblastoma [67] and breast cancer [68], or up-regulated during the course of therapy as observed in preclinical models [69].

   Alternatively additional pro-angiogenic growth factors could be produced as an indication of the mutational events occurring within the tumour during tumourigenesis. These pro-angiogenic proteins can lead to rescue of angiogenesis and revascularisation of tumours, leading to tumour growth and override the effects of the VEGF blockade treatment [69,70].

   New understanding of the VEGF pathway is also emerging that could explain some aspects of resistance to anti-angiogenic therapy. Recently intracrine effects of VEGF have been shown, which would make tumours resistant to anti-VEGF antibodies [71,72]. Also a splice variant of VEGF, which inhibits the VEGFR and is bound by bevacizuamb, could affect the balance of the anti-angiogenic effect [73]. A mutation in the kinase domain of the target receptor tyrosine kinases following therapy could also compromise the outcome of the treatment [57,58]. Patients may therefore benefit from anti-VEGF therapy being administered together with other angiogenic inhibitors over a long-term treatment to disrupt tumour growth, survival and metastasis more effectively, than specific inhibition of each pro-angiogenic pathway alone. As highlighted in the following section, this requires a much better knowledge of the status of the VEGF and other pro-angiogenic pathways within the tumour.

3. Targeting the appropriate cell type and pathway to improve therapy outcome – lack of biomarkers.

As indicated earlier, the process of angiogenesis depends on various cell types, in addition to endothelial cells, in order for the new blood vessels to become stabilised and perfused with blood. Additionally increased pericyte coverage of the tumour vasculature, activation of stromal fibroblasts or the recruitment of macrophages can lead to increased integrity of the new blood vessels and promote survival of endothelial cells, even in the absence of VEGF [6,30]. Targeting pro-angiogenic factors involved in recruiting cells to within the tumour could improve the outcome of the therapy. For example, PDGF expression was found up-regulated in patients treated with anti-VEGF therapy [74]. Targeting PDGF enhanced the efficacy of chemotherapy and anti-VEGF therapy in combinational preclinical studies, including tumours resistant to anti-VEGF therapy [75].

Understanding additional mechanisms that enable tumour cells to adapt and survive may also yield further targets for anti-angiogenic therapy. For instance, intussusceptive angiogenesis, where the vessel splits and remodels itself, is a process that does not primarily require cell proliferation in order to improve the blood supply to the tumour [12]. Tumour cells can also grow in close proximity to established blood vessels, in a process known as vessel co-option, without evoking an angiogenic response. Vessel co-option by glioblastoma cells was observed to increase following anti-VEGF therapy in a preclinical animal model [76]. Vessel co-option provides tumour cells with sufficient nutrients to survive independently from the anti-angiogenic therapies and thus promotes another avenue of resistance and tumour progression. Vasculogenic mimicry has also been observed in several tumour types, where highly aggressive tumour cells form vessel-like structures themselves, containing plasma and red blood cells [11,77]. The occurrence of vasculogenic mimicry has been found to correlate with an increased risk of metastasis and therefore poor clinical outcome, for example in melanoma and colon cancer [77,78].

Other key angiogenesis signalling pathways are being explored as therapeutic targets (Table 1), particularly those that interact with the VEGF pathway. One such pathway is Notch signalling. The Notch ligand, Dll4, was recently identified as a novel target in tumour angiogenesis [79]. Notch signalling, in particular Dll4/Notch signalling, enables the selection and discrimination between the tip cells and the stalk cells, during the early sprouting phase of angiogenesis [18]. In the absence of Dll4/Notch signalling all endothelial cells can respond indiscriminately to the VEGF stimulus and form tip cells with filopodia, resulting in dramatically increased sprouting, branching and fusion of endothelial tubes [22,24]. However, such vessels are poorly functional, which leads to increased hypoxia, poor perfusion and decreased tumour growth [22–24]. Importantly though, blocking Dll4/Notch signalling overcame resistance to bevacizumab treatment [22]. The presence of glioblastoma stem-like cells in the perivascular niche microenvironment has been demonstrated to drive glioblastoma progression. Recently, comprehensive studies revealed that these glioblastoma stem-like cells could differentiate into functional tumour endothelium [80,81]. Ricci-Vitiani and colleagues observed that these stem-like cells promoted angiogenesis through the release of VEGF [80]. An exciting observation was also made whereby inhibiting Notch signalling by γ-secretase or Notch1 silencing blocked the transition of the glioblastoma stem-like cells into endothelial progenitor cells [81]. Future therapeutic strategies preventing the process of stem cell differentiation into endothelial cells in tumours, such as glioblastoma stem-like cells and targeting Notch signalling, represents a novel target to inhibit angiogenesis and tumourigenesis.

Currently there are no validated methods in place in the clinic to define and identify the key pro-angiogenic pathways driving angiogenesis in patient tumours over the course of treatment and few clinical trial studies incorporate imaging modalities to assess vascularisation, vascular permeability, response to drugs and relate this to the active pathways expressed in tumour biopsies.

4. Selecting more aggressive tumour cells, leading to tumour progression.

Anti-angiogenic therapies were initially developed with the notion that by inhibiting tumour angiogenesis the tumour would subsequently become starved of necessary oxygen and nutrients, ultimately reducing tumour growth and progression. Yet there are dangers associated with inducing hypoxia within tumours. Hypoxia is associated with radiation therapy and chemotherapy resistance and not surprisingly therefore, a poor clinical prognosis [82]. Increased hypoxia, as a consequence of anti-angiogenic therapy, is likely to promote tumour selection of more aggressive tumour cells that are better adapted to survive and proliferate under stressful oxygen deficient growing conditions partly through induction of hypoxia inducible factor 1 [83]; as exemplified when examining the change in p53 status and subsequent contribution to resistance during the course of treatment in preclinical studies [84]. Mechanisms include free radical generation and defects in DNA repair [58,85]. In response to hypoxia tumour cells can regulate many angiogenic growth pathways besides VEGF, which contributes to resistance to anti-angiogenic therapy [86]. These include growth factors involved in stimulating proliferation and migration of endothelial cells (e.g., VEGF, PlGF, FGF2, PDGF), pericytes recruitment and stabilisation of maturing blood vessels (e.g., PDGF) and recruitment of macrophages (e.g., PlGF) [86,87]. In addition the metabolic consequences of hypoxia are highly favourable for tumour growth and these pathways provide many new targets [58,83,88]. Such resistance mechanisms have been more extensively reviewed in [58].

3.2. Addressing the issues raised by anti-angiogenic therapy

It is crucial that the complexity of the signalling pathways that promote or regulate angiogenesis is translated into the clinical situation when selecting patients for the appropriate anti-angiogenic therapy. In order to achieve this, specific strategies and assays in biomarker screens are needed to characterise the presence and concentration of not only the therapeutic target, but also the pharmacodyamic efficacy of anti-angiogenic agents.

Therefore research on the genetic basis of the tumour response to anti-angiogenic treatment will have important implications for the development of angiogenesis based biomarkers and the optimisation of anti-angiogenic therapy to limit toxicity effects. Anti-VEGF therapy is unfortunately not without side effects: hypertension and proteinuria are often induced [89]. Interestingly though it has been reported that hypertension induced by bevacizumab or an increase in antihypertensive medication while receiving bevacizumab were associated with a longer progression free survival [90–92].

Biomarkers, present in either the blood or urine, may be identified through proteomic studies [93], cDNA microarrays and serial analysis of changes in gene expression [94]. Although biomarkers present in the blood or urine could provide cost-effective screens, being non-invasive and therefore repeatable, no validated biomarker has been so far identified. Imaging of the

tumour vasculature enables the regional blood flow, blood volume, permeability, pH and oxygen levels to be determined. Techniques such as dynamic contrast enhanced magnetic resonance imaging, dynamic computed tomography and positron emission tomography are being utilised in many clinical trails and provide key information about the tumour [95,96]. The advantage of such imaging is the ability to provide a functional analysis of the whole tumour and visualise tumour angiogenesis by a relatively non-invasive procedure, and thereby provide clear indications of the efficacy of the anti-angiogenic therapy. However, these imaging techniques have been infrequently utilised in prospective trials, where specific angiogenic targets being investigated can be exploited by the use of labelled probes, which will enable a more specific evaluation of the changes in angiogenesis following therapy. The ECOG 2100 study of bevacizumab in metastatic breast cancer was one of the first to describe biomarkers that seem to be associated with efficacy and toxicity for bevacizumab in cancer [97]. In this study two *VEGF* polymorphisms (VEGF-2578AA and VEGF-1154) were found to correlate with overall survival, however these biomarkers did not correlate with progression free survival; VEGF tumour expression also did not correlate to outcome [97]. Interestingly, a polymorphism at position −460 in the *VEGF* gene, leading to increased VEGF levels, has been shown to positively correlate with the likelihood of developing renal cell carcinoma [98]; although, as recently reviewed in [99] the effect of polymorphism in *VEGF* on VEGF serum levels and subsequent risk of developing solid tumours can vary between tumour types (e.g., −936 in breast and colorectal cancer [100,101]). A recent review also discusses the putative clinical, radiological, and molecular biomarkers of bevacizumab efficacy, derived from recent clinical trial data [102]. For example in a trial in advanced breast cancer it was observed that elevated baseline biomarkers vascular cell adhesion molecule 1 was associated with a reduction in tumour response [103] and lower levels of baseline VEGF were associated with longer time to progression [104], whereas examining tumour VEGF baseline levels was found to be higher in the responders to treatment than in the non-responders [105]. The clinical importance of serum or plasma soluble forms of VEGFR-1 has been reported in different cancer types, including leukemia, lung cancer and colorectal cancer, but overall is not a robust marker [106].

In addition to utilising biomarkers, understanding the effects of anti-angiogenic therapy on tumours could enable identifying features that will provide therapeutic advantages. For example, as discussed anti-angiogenic therapy will increase hypoxia. A number of groups are exploiting this feature by treating tumours with hypoxic pro-drugs or hypoxia directed gene therapies (i.e., hypoxic-response promoters for the expression of pro-drug acting enzyme) that enable selective delivery to affected areas [83].

It will be critical to obtain the angiogenic profile of the tumour prior to therapy in order to define the 'resistance potential' of a tumour (reviewed more extensively in [96,107]). Biomarkers could be utilised in clinical trials in the following manner: in phase I the functional analysis of the tumour vasculature, at baseline and early after therapy e.g., 1–2 weeks, together with concentrations/expression of the angiogenic target and other biomarkers will enable the range of the biological active dosages of the drug to be defined, and the impact of the pharmacodynamic and pharmacokinetic parameters on the angiogenic target to be determined. In combinational or single agent studies the data collected will able the maximum tolerated dose of the anti-angiogenic drug to be calculated. In phase II the parameters and biomarkers determined for the anti-angiogenic drug in the previous phase, in a run-in phase of early imaging response can be integrated and correlated with the effect of the agent on tumour response. In phase III the criteria of patient selection will be based on the run-in imaging response parameters and angiogenic biomarkers of the tumour (e.g., expression of the angiogenic target).

4. Conclusions

Angiogenesis plays a critical role in the local growth and metastasis of many different solid tumours. Over the past years great progress has been made in developing molecules with anti-angiogenic activity. Although there have been a number of positive results observed in the clinical trials and treatment of various cancers, concern is being expressed at the increased observance of resistance to anti-angiogenic therapy and subsequent tumour re-growth following treatment. This resistance could reflect tumours displaying adaptive (evasive) resistance and intrinsic (pre-existing) non-responsive resistance. However, resistance develops to all treatments used in advanced disease (radiation, endocrine therapy, chemotherapy and immunotherapy) and is induced by the treatments, so anti-angiogenic therapy is no different in this regard and many strategies to overcome it are being developed.

Agents that target multiple pro-angiogenic molecules need to be selected based on the tumour biology. Angiogenesis markers are needed to correlate with progression or response of tumours. Tumour vascular imaging and determination of short-term response may provide the necessary data to develop and validate such markers. These markers of angiogenesis will serve an important role in predicting a particular patient's clinical course, guiding which patients may benefit most from anti-angiogenic therapies and biomarkers to monitor patient's response to these therapies. In the future, tailored treatments based on dynamic assessment of response should result in individualised patient therapy and improved progression free and survival free outcomes, as well as being more cost effective and importantly reduce unnecessary toxicity in patients and promote more a more cost-effective treatment.

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