Review

Breast tumour angiogenesis

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Abstract

The central importance of tumour neovascularization has been emphasized by clinical trials using antiangiogenic therapy in breast cancer. This review gives a background to breast tumour neovascularization in *in situ* and invasive breast cancer, outlines the mechanisms by which this is achieved and discusses the influence of the microenvironment, focusing on hypoxia. The regulation of angiogenesis and the antivascular agents that are used in an antiangiogenic dosing schedule, both novel and conventional, are also summarized.

Introduction

It has been 3 years since the last critical review of anti-angiogenic therapy was published in *Breast Cancer Research* [1], and since then the central importance of tumour neovascularization has been emphasized by clinical trials in various tumour types, including breast cancer. Many of these trials have used bevacizumab (Avastin™; Genentech, South San Francisco, CA, USA), which was specifically designed to target vascular endothelial cell growth factor (VEGF). Bevacizumab is a recombinant VEGF antibody derived from a humanized murine monoclonal antibody that can recognize all known isoforms of VEGF-A and prevents receptor binding, thereby inhibiting angiogenesis and tumour growth. The critical contribution of this angiogenic factor in controlling many of the processes involved in angiogenesis and its importance as a paradigm for the rational design of an anticancer agent have been among the successes of antiangiogenic treatment, which was first suggested by Judah Folkman more than 35 years ago. The attractiveness of the antiangiogenic approach has always been the wide therapeutic window, since all tumours (including liquid such as leukaemias) are angiogenesis dependent, that angiogenesis is highly restricted in the adult, that endothelium of the vessels are accessible and that any treatment would be amplified through subsequent tumour infarction. Furthermore, the erstwhile problem in oncology of resistance should not be an issue because endothelial cells are non-neoplastic and should have a stable genome [2].

Nevertheless, although these trials have demonstrated significant improvements in response rates, findings to date have not indicated substantial benefits in terms of survival. This is likely to be due to redundancy in breast tumours with an individual tumour being able to utilise several angiogenic pathways at any one time [3] with changes in this profile during tumour progression coupled with the use of other mechanisms to establish a blood supply. Indeed, the central tenet that tumours are angiogenesis dependent (in that for a tumour to grow, this must be preceded by a wave of angiogenesis to deliver nutrients and meet the metabolic requirements of the growing tumour) has been challenged. Thus, a number of nonangiogenic mechanisms may contribute to establishing tumour blood supply; these include co-option, vasculogenesis, vascular remodelling, intussusception and vascular mimicry.

A further important issue that has not been addressed is stratification of patients for appropriate treatment; specifically, individual patients given antiangiogenic agents have yet to be selected based on the characteristics of their tumour. It is therefore likely, as has been demonstrated for other targeted agents such as herceptin, that benefit will be restricted to those patients whose tumours rely largely on VEGF signalling for their angiogenic response. The administration of agents based on the biology of the individual tumour (so-called personalized medicine) will become increasingly important not only to generate maximum therapeutic benefit to the patient but also to realize the optimal economic advantage from the finite resources available.

FGF = fibroblast growth factor; HER = human epidermal growth factor receptor; HIF = hypoxia-inducible factor; PDGFR = platelet-derived growth factor receptor; TAM = tumour-associated macrophage; TKI = tyrosine kinase inhibitor; VEGF = vascular endothelial cell growth factor; VEGFR = vascular endothelial cell growth factor receptor.

Breast tumour neovascularization

Angiogenesis in the normal human adult is highly restricted, largely to wound healing and reproduction. Sustained angiogenesis is pathological and is characteristic of many common diseases, including diabetes, psoriasis and rheumatoid arthritis [4]. Thus, in order to initiate neovascularization, a tumour must switch to an angiogenic phenotype. Evidence from transgenic models that have reproducible distinct tumour stages suggest that the acquisition of this phenotype occurs early in tumour development and that it is rate limiting with regard to tumour progression [5,6]. These experimental models are supported by findings in human tissues, in which 30% of transplanted human hyperplastic breast tissue samples were found to be angiogenic as compared with only 3% of samples from normal breast tissue [7-9]. Interestingly, normal breast adjacent to malignant breast induced angiogenesis twice as frequently as did tissues from non-neoplastic breast, suggesting that the angiogenic switch occurs before morphological changes are identifiable [10]. Using microvessel density as a surrogate for angiogenesis, benign lesions associated with high vascular density are correlated with increased risk for developing breast cancer. It has also been suggested that quantification of angiogenesis might help to predict the likelihood that *in situ* cancers will progress [11,12] or that a tumour will respond to treatment [13-17], and has been shown to correlate directly with the presence of bone marrow micrometastases [18] and survival [19,20].

Although it is likely that different tumour types use different genetic pathways to establish a blood supply, oncogenes and tumour suppressor genes that are frequently associated with transformation also appear to be important in activating the angiogenic switch. Thus, Ras, myc, raf, c-erbB-2, c-jun and src transformed cells exhibit a strong angiogenic phenotype [21-24]. However, the vessels formed under the influence of these pathways are abnormal, leaky with blind sacs, and have reversed and intermittent flow [25]. The result is that although there is an increase in formation of new vessels, drug and oxygen delivery is much poorer than in normal tissues. This leads to hypoxia and microenvironmental stresses that have been demonstrated to have profound effects on tumour biology and resistance to treatment [26].

The microenvironmental influence of hypoxia

Hypoxia is the pathophysiological consequence of a structurally and functionally disturbed microcirculation [26], and it is therefore a common feature in solid tumours. Tumours respond to low oxygen tension by enhancing the hypoxia-inducible factor (HIF) response [27]. The HIF response is mediated through the transcription factor dimer inducible HIF-1α and constitutively expressed HIF-1β (also known as aryl hydrocarbon nuclear translocator).

In normoxia, three prolyl hydroxylases (prolyl hydroxylase-1, -2 and -3) hydroxylate HIF-1α at two proline residues in its oxygen-dependent degradation domain, with oxoglutarate from the Krebs cycle, ascorbate and Fe^{2+} leading to recognition and binding of the α domain by the von Hippel-Lindau protein. This interaction, and through binding of elongin C via von Hippel-Lindau β domain in turn, leads to ubiquitination and targeting for degradation through the proteasome. In conditions of hypoxia, however, molecular oxygen is not available for hydroxylation which results in HIF-1α stabilization and translocation to the nucleus, where it binds to HIF-1β and consensus hypoxia response elements on gene promoters. Co-activators and polymerases are recruited and transcriptional activation of several gene pathways that are involved in angiogenesis, glycolysis, erythropoiesis and apoptosis occurs. The asparagine hydroxylase, factor inhibitor of HIF-1, and CITED4 (CBP p300-interacting transactivator 4), which interfere with co-activator binding, provide a further level of control [28,29].

Over-expression of HIF-1α protein has been identified in various tumour types, with high levels influencing the growth rate and metastatic potential of these cancers. In breast cancers, the frequency of HIF-1α positive cells increases in parallel with increasing clinical stage and is associated with poor prognosis [30-33]. In addition to HIF-1α, other isoforms have been identified, namely HIF-2α and HIF-3α [34,35]. The roles played by these isoforms are complex, but there is evidence that the latter antagonizes hypoxia-dependent gene expression, whereas the former can enhance the hypoxic response element. Interestingly, there is evidence that HIF-1α and HIF-2α activate different sets of hypoxia-inducible genes [36], including those involved in glycolysis, cell survival and proliferation [37]. However, the clinical relevance to breast cancer is not known because there are only limited data on these HIF isoforms. Thus, in breast cancer HIF-2α has been reported to be expressed in both tumour cells [38] and in tumour-associated macrophages (TAMs) [39]. TAM HIF-2α expression was found to be related to tumour vascularity, suggesting that hypoxia induces TAM clustering and over-expression of TAM HIF-2α, thereby inducing an angiogenic phenotype, leading to induction of localized angiogenic hot spots [39]. Thus, hypoxic stress response through HIF is likely to be an important mechanism by which continued remodelling of vessels occurs.

Recognition that HIF plays a significant role in tumour behaviour, conferring an aggressive phenotype and contributing to resistance to both radiotherapy and chemotherapy [40], has led to efforts to target the HIF pathway. Several trials of agents that decrease and/or block HIF-1α expression, including rapamycin/CCI779, quinocarmycin, topoisomerase inhibitors, anti-microtubular agents, YC-1, 17-AAG, thioredoxin inhibitors and 2ME2, have been conducted or are planned [41]. Other potential targets in HIF signalling include the molecules that are involved in oxygen sensing or transcription. For instance, obstructing the interaction between HIF-1α and the co-activator CBP/p300 led to attenuation of

HIF-induced gene expression and inhibition of tumour growth in a xenograft model [42]. An alternative strategy is to use the HIF pathway to activate bioreductive drugs such as tirapazamine, which inhibits DNA repair under hypoxic condition [43] and has been shown to have an antiangiogenic effect as well as direct antitumour activity [44]. Correction of the hypoxic environment by reducing anaemia [45] using human recombinant erythropoietin is also a potentially effective approach.

Mechanisms of neovascularization

Although sprouting-type angiogenesis is an important mechanism in tumour neovascularization, several other mechanisms by which tumours establish a blood supply have been identified; these include vascular remodelling, vasculogenesis, vascular mimicry and glomeruloid angiogenesis. Each may have significance in a particular tumour type or at a particular stage of tumour evolution, but the relative importance of each in human tumours is unknown. However, angiogenesis and vascular remodelling appear to be the major mechanisms in breast cancer with evidence that vascular mimicry may additionally play a role in inflammatory breast cancer. The acquisition of this type of biological information is likely to become more important as patients are treated in a more individuallized manner. Although microvessel density has been used as a surrogate for angiogenesis, many other parameters of tumour neovascularization have also been explored, including angiogenic factor expression, cell adhesion molecules, vessel maturation and endothelial cell proliferation [19]. These measures, including microvessel density, have associated problems [46] and none provides a reliable measure of blood flow, which is extremely variable because of shunting, stasis and even reverse flow occurring through the abnormal tumour vasculature [47].

Angiogenesis

Angiogenesis is the generation of new blood vessels from the existing vasculature. It consists of multiple coordinated, sequential and interdependent steps. The angiogenic programme requires the degradation of the basement membrane, endothelial cell migration and invasion of the extracellular matrix, with endothelial cell proliferation and capillary lumen formation before maturation and stabilization of the new vasculature. The latter requires inhibition of further endothelial proliferation, reconstitution of the basement membrane, and junctional complex formation and organization of endothelial cells into a new luminal space.

Vascular remodelling

In contrast to animal models in which endothelial cells proliferate 30-fold to 40-fold faster in tumour blood vessels than in the vasculature of normal tissue, irrespective of tumour type, growth rate, or size, endothelial cell proliferation in human breast tumours is relatively rare. The corollary of this finding is that vascular remodelling must be the dominant mechanism in establishing the neovasculature in breast cancers [48-50]. This can occur through a variety of processes including co-option, in which tumours hijack the existing vasculature. This has been reported early in brain tumour development, in which existing blood vessels are used in the absence of an angiogenic response [51,52], although continued tumour growth results in angiogenesis. The tie2-angiopoietin and VEGF growth factor pathways may regulate these respective mechanisms of tumour vascularization. Thus, in some circumstances tumours are able to 'parasitize' the normal stroma and sinusoidal vasculature for its metabolic needs.

Intussusception of tumour columns has also been hypothesized to contribute to the establishment of a tumour blood supply. This process is independent of endothelial cell proliferation and is rapid, depending on insertion of tissue pillars into vessels, partitioning the vessel lumen into two or more channels [53,54]. This may be part of vascular remodelling, which may be the dominant mechanism in the establishment of the tumour vascular bed.

Vasculogenesis

Vasculogenesis is the *de novo* generation of blood vessels from endothelial cell progenitors, as occurs in the embryo. In animal models it has been demonstrated that circulating endothelial cell precursors derived from the bone marrow lodge in the cancer vasculature, differentiate into endothelial cells and enhance tumour neovascularization through a combination of vasculogenesis and conventional angiogenesis [55-58]. There appear to be differences in the proportion of tumour neovascularization that can be apportioned to vasculogenesis, depending on the model (up to 90%). In orthoptic models this appears to account for <5% and in human tumours had an average of 4.9% (range 1-12%) when examining tumours from transplant recipients [59,60].

There is evidence to suggest that this process of tumour vascularization may be more frequent and/or significant in early tumour development because inhibition of stem cells or endothelial cell precursor mobilization prevents xenografts from inducing the initial angiogenic response [61]. Nevertheless, there is some debate as to whether such bone marrow derived endothelial cells are actually incorporated into the vasculature and whether they may be acting in a paracrine/support function [62]. The discrepancies between reported findings may be due to replacement of bone marrow derived cells with surrounding endothelial cells with tumour progression [63]. The two models may not be mutually exclusive because several different populations of cells have been reported that may be involved in a support and integral role (for instance, macrophages/monocytes, myeloid progenitors, platelet/megakaryocyte lineages, pericyte progenitors, neutrophils and so-called vascular leucocytes, which express mixed endothelial and white cell lineages). Regulation of these processes may be through angiogenic factors such as

VEGF, which mobilizes precursor cells from the bone marrow, but there are some data to suggest that the subsequent retention of these progenitor cells may require additional factors such as stromal-derived factor 1 [64]. Interestingly, stromal-derived factor 1 is hypoxia inducible via HIF [65]. There are few data in breast, but it has been suggested that vasculogenesis occurs in inflammatory subtype breast tumours [66].

Glomeruloid angiogenesis

Glomeruloid bodies that are characteristic of glioblastomas are also observed in invasive breast cancers [67]. These are highly complex vascular aggregates that resemble glomeruli of the kidney, composed of a network of capillaries that are variably lined by basement membrane and pericytes. Their presence is associated with a significantly shorter survival in breast (and other) cancers [68]. Their formation is related to VEGF, because this angiogenic factor is not only essential for their induction but also for maintenance of these bodies [69]. We have also observed these in breast cancer xenografts transfected with VEGF [70]. This type of tumour neovascularization may also represent vascular remodelling rather than classical sprouting-type angiogenesis [71].

Vascular mimicry

Vascular mimicry is a neovascularization strategy that may largely be restricted to aggressive occular malignant melanomas and ovarian tumours [72], but it has also been reported in breast cancers [66]. Partial lining of the capillary surface by tumour cells has been known for many years [73] and was more recently reported in animal models using advanced techniques [74], but vascular mimicry is defined as a complete capillary network composed of tumour cells themselves rather than vascular endothelial cells that conducts blood [75,76]. The tumour cells not only take on the morphology of endothelial cells but they also acquire phenotypic characteristics of endothelium, expressing a number of vascular markers. It is important to recognize this type of neovascularization because the therapeutic implications of having mimicry as a dominant mechanism are that these tumours may not respond to conventional anti-angiogenic agents.

Angiogenic factors in breast cancer

Whatever the mechanism(s) that a tumour use(s) to establish a blood supply, similar regulatory factors are utilized (although some may preferentially be used in particular processes). The presence of a humoral mediator of tumour angiogenesis was suggested more than 60 years ago, but it was not until 1968 that it was demonstrated that a diffusible tumour-derived factor could induce capillary growth [77,78]. Folkman and coworkers [79] reported the first angiogenic factor, namely tumour angiogenesis factor; this discovery was followed by identification of numerous other angiogenic promoters and inhibitors [80,81]. Most have pleiotropic effects, and the role played by many in human tumours is unknown. However, several important angiogenic pathways have been implicated in human tumour neovascularization.

The angiogenic promoters and inhibitors that underlie establishment of a tumour blood supply through the above-mentioned mechanisms of neovasculrization can originate from the neoplastic cell and/or from other tumour elements. Thus, neoplastic cells can recruit inflammatory cells such as macrophages and mast cells, both of which are rich sources of angiogenic factors and cytokines, or they can induce release of sequestered growth factors or their receptors from the extracellular matrix through protease degradation. Platelets, which also are a rich source of angiogenic factors and are often elevated in malignancy, can be activated by tumour endothelium or epithelium.

Invasive and *in situ* breast cancers express many angiogenic factors, including the VEGF family (see below), fibroblast growth factor (FGF)-1, FGF-2, placenta growth factor, transforming growth factor-β_1, thymidine phosphorylase, pleiotrophin and adrenomedullin [3,82,83]. However, these are expressed preferentially at different stages of tumour development. Hence, thymidine phosphorylase is expressed in *in situ* [84] and T1 breast tumours [85], whereas VEGF expression occurs throughout the tumour stages. Furthermore, breast cancers are likely to express different angiogenic profiles, which will necessitate the use of a different spectrum of antiangiogenic agents.

VEGF and anti-VEGF therapies

VEGF

Studies have shown that the VEGF family plays a central role in many human tumour types (for review [81]). Comprising VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor, these polypeptides exist in a number of isoforms and can form homodimers and heterodimers. They bind variably to three high-affinity endothelial cell tyrosine kinase receptors, namely Flt-1 (VEGF receptor [VEGFR]1), KDR (VEGFR2) and Flt-4 (VEGFR3), which are responsible for initiating intracellular signalling. Receptor activation results in slightly different effects, with VEGFR1 promoting differentiation and vascular maintenance, VEGFR2 inducing endothelial cell proliferation and vascular permeability, and VEGFR3 stimulating lymphangiogenesis. Additional regulation is achieved through the isoform-specific receptors neuropilin-1 and neuropilin-2. The neuropilins bind not only class 3 semaphorins, which are involved in axonal growth, but also some isoforms of VEGF, where they function as co-receptors, increasing VEGF binding to VEGFR2 [86]. Further modulation is achieved by proteolytic processing and/or heparin, which is not only required for binding of VEGF (and basic FGF) but can also compete for receptor sites. This complex pathway enables the VEGFs to have numerous effects, including increasing vascular permeability (thereby augmenting tumour stroma formation), endothelial cell proliferation, endothelial cell survival and tube formation. Although VEGFR expression is

largely endothelial (vascular and/or lymphatic), VEGFRs have also been reported on inflammatory cells such as macrophages and tumour cells themselves.

VEGF-A is highly expressed in many tumours of lung, brain, and gastrointestinal and urogenital tracts, as well as *in situ* and invasive breast cancers [81]. Expression in some studies is associated with microvessel density and prognosis, supporting the importance of VEGF-A in human malignancies. VEGF has a hypoxic response element in its promoter and is one of several genes that are upregulated in a low oxygen microenvironment to elicit a vascular phenotype. However, the role played by the other family members in human disease is still being elucidated and if tumours are unable to express VEGF-A, other VEGF homologues may be induced to augment neo-vessel formation. VEGF-B, VEGF-C and VEGF-D are also expressed in breast cancers, with some pathological correlates with nodal metastases, prognosis and lymphatic density [87-94]. This may be important for two reasons. First, there is large-scale redundancy in blood supply to any tumour, allowing them to switch angiogenic pathways; this suggests that there is a need for several or multifunction agents. Second, many of these angiogenic factors may synergize with each other (for instance, VEGF-A and FGF-2), at least *in vitro*.

Anti-VEGF therapy

Although there are many 'antiangiogenic' targets for anticancer therapies, many therapies have directly targeted the VEGF pathway because of its critical role in pathological angiogenesis and its profound influence of this growth factor on endothelial biology. Many points on the pathway can be targeted, including direct targeting of ligand and receptor (extracellular and intracellular tyrosine kinase domains) at the protein and mRNA levels, interfering with downstream intermediates, and indirect inhibition of upstream regulators of VEGF.

Bevacizumab

The most investigated agent to date is bevacizumab (Avastin™; Genentech). Bevacizumab is a recombinant VEGF antibody derived from a humanized mouse monoclonal antibody (93% human) that is composed of the mouse VEGF-binding site joined to a human IgG framework. Bevacizumab recognizes all isoforms of VEGF-A and thereby prevents receptor binding, which leads to inhibition of angiogenesis and tumour growth. *In vitro* bevacizumab inhibits VEGF-induced endothelial cell proliferation and migration, and in xenograft models of a range of tumour types (including breast cancer) tumour growth is significantly decreased by bevacizumab [95,96]. In some human breast carcinoma models, treatment with bevacizumab is associated with a reduction in microvessel density [97].

In phase I/II clinical trial of 75 patients with metastatic breast cancer treated with bevacizumab [98] there was an overall response rate of 9.3% (confirmed response rate 6.7%) with a median duration of 5.5 months (range 2.3 to 13.7 months); 16% had stable disease or an ongoing response at the end of the trial (after 22 weeks). These data supported the initiation of a phase III clinical trial that combined bevacizumab with capecitabine in patients previously treated with an anthracyclin and a taxane. Although the combination therapy resulted in a statistically significantly increased response rate (19.8% versus 9.1%), neither progression-free nor overall survival differed between arms. Although there are many explanations for the lack of success in terms of the primary end-point of the study, the absence of patient selection (specifically, of those patients whose tumours rely on VEGF) is probably of great importance, in that advanced tumours have redundancy in their ability to establish a blood supply and utilize many pathways. There is ongoing analysis of primary tumour samples for pathological factors that might predict response to bevacizumab in this study.

Nevertheless, a phase III first-line trial comparing bevacizumab plus paclitaxel versus paclitaxel alone in patients with locally recurrent or metastatic breast cancer [99] revealed that this combination increased significantly response rates in all patients (28.2% versus 14.2%; $P < 0.0001$). It also resulted in an increase in median progression-free survival by 4.9 months (6.1 months versus 11 months), which was associated with improved overall survival (hazard ratio 0.674) in the combination arm relative to paclitaxel monotherapy, although this difference did not reach statistical significance. Furthermore, a pilot study conducted in patients with inflammatory breast cancer demonstrated a decrease in tumour cell VEGFR2 (KDR) phosphorylation and an increase in apoptosis after a single cycle of bevacizumab therapy. This response was maintained with the addition of chemotherapy. Results from other trials of bevacizumab are awaited, including studies evaluating neoadjuvant and adjuvant use of this agent, and promising results are emerging for patients with renal cell, colorectal, brain and lung cancers.

In a neoadjuvant study 39 patients with locally advanced breast cancer were treated with docetaxel with or without bevacizumab. There were five complete clinical responses and 24 partial responses, and the therapy was generally well tolerated. Recently, new results for the combination of bevacizumab with doxorubicin and docetaxel in the treatment of inflammatory breast cancer were reported [98]. After treatment, eight out of 13 patients experienced a confirmed partial response, with evidence of a decrease in vascular permeability on dynamic contrast-enhanced magnetic resonance imaging. An ongoing trial plans to evaluate the efficacy of bevacizumab in the adjuvant setting with low dose of methotrexate and cyclophosphamide for cases with residual cancer after neoadjuvant chemotherapy. There is also a planned Eastern Cooperative Oncology Group adjuvant feasibility trial, which will evaluate bevacizumab in combination with dose-dense doxorubicin and cyclophos-

phamide followed by paclitaxel in women with node-positive breast cancer.

Bevacizumab has a relatively long half-life, which allows intervals between intravenous administrations of up to 3 weeks. The tolerability profile of bevacizumab is generally acceptable in clinical trials, and the drug can readily be delivered with other chemotherapeutic agents that, in some circumstances, may be synergistic. Although phase I trials suggested no dose-limiting toxicity, common adverse events of any severity in patients include asthenia, adnominal pain, headache, hypertension, diarrhoea, nausea, vomiting, anorexia, stomatitis, constipation, upper respiratory infection, epistaxis and proteinuria. Most adverse events were mild to moderate in severity, and events such as hypertension, haemorrhage, or proteinuria were clinically manageable. Thus, bevacizumab provides a highly effective addition to standard chemotherapeutic regimens in several common solid tumours.

Tyrosine kinase inhibitors of VEGF

Several receptor tyrosine kinase inhibitors (TKIs) that target the tyrosine kinase portion of VEGFR1 and VEGFR2 have been developed that are being investigated [100,101]. The orally administered VEGFR2 inhibitor ZD6474 was generally well tolerated but exhibited little activity in patients with refractory metastatic breast cancer [102]. A variety of other small molecule TKIs targeting the VEGFRs are being evaluated, as are ribozyme (catalytic RNA molecules that specifically cleave VEGFR mRNAs) and antisense strategies.

VEGF is regulated by other transmembrane receptor tyrosine kinases, the most relevant of which in breast cancer is the human epidermal growth factor receptor (HER)1 and HER2 (c-erbB-2, *neu*) [103-105]. Thus, attenuation of VEGF signalling and therapeutic synergy might be achieved through interference in these other receptor pathways with agents such as trastuzumab in combination with anti-VEGF therapy. The validity and potential of this strategy is supported by the positive correlation between HER2 and VEGF expression in a large cohort of breast cancers [106] and results from a phase I trial of trastuzumab and bevacizumab [107] that indicated a clinical response in five out of nine patients.

Inhibition of the VEGFR mRNA has been attempted both with ribozyme (catalytic RNA molecules), which specifically cleave the mRNAs for the primary VEGFRs [108], and antisense VEGF [109]. Angiozyme is a synthetic ribosome that cleaves the mRNA for the receptor VEGFR1/Flt-1. Preclinical studies confirmed inhibition of both primary tumour growth and metastasis [109]. In patients with refractory solid tumours, a phase I trial of angiozyme demonstrated [110] good tolerability without significant side effects, and phase II trials are ongoing. However, a phase II trial in breast cancer provided no evidence of clinical activity [111], although there was evidence of biological activity, with a decrease in serum VEGFR1 levels.

Recently, various small molecule TKIs targeting the VEGFRs and other critical signalling pathways (for instance, platelet-derived growth factor receptor [PDGFR] and epidermal growth factor receptor) in angiogenesis have been developed. Depending on tumour entity, oral multitargeted TKIs can exert both antiangiogenic and antitumour activities at the same time. As a consequence, they may improve the outcome of cancer patients as single-agent treatment.

A multireceptor targeting agent is PTK787/ZK 222584. It is a pan-VEGF, PDGFR, c-kit and c-Fos receptor TKI. It inhibited the growth of a broad panel of carcinomas in rodent models, with histological examination revealing inhibition of microvessel formation [112]. Patients with a variety of advanced cancers have received this agent and it has been well tolerated. A recent phase I/II study of PTK787/vatalanib in combination with trastuzumab in patients with newly diagnosed HER2-positive metastatic breast cancer has been initiated.

Many extracellular proteolytic enzymes and their inhibitors are active during angiogenesis. Expression of various matrix metalloproteinases has been found to be upregulated in virtually every type of human cancer, and this upregulation correlates with advanced stage, invasive and metastatic properties, and poor prognosis in general [113]. Marimastat, an orally bioavailable hydroxamate, was the most widely studied. E2196 was a phase III trial of 190 patients with metastatic breast cancer who had responding or stable disease after six to eight cycles of first-line chemotherapy for metastatic disease [114]. Patients were randomly assigned to receive marimastat or placebo after chemotherapy. There were no significant differences in median progression-free survival or overall survival, but important musculoskeletal toxicities were noted.

Other agents being evaluated in breast cancer include sunitinib and sorafenib. Future studies are being directed at evaluating these agents in combination with other targeted therapies as well as in the first-line metastatic and/or adjuvant setting. SU11248 (sunitinib malate) is an inhibitor of receptor tyrosine kinases for VEGFR1, VEGFR2, PDGFR, c-kit, and Flt-3. In January 2006, this drug was granted approval by the US Food and Drug Administration for treatment of gastrointestinal stromal tumour after disease progression on, or intolerance to, imatinib mesylate, as well as for the treatment of metastatic renal cell cancer. Sorafenib (BAY 43-9006) belongs chemically to a class described as bis-aryl ureas. It was selected for further pharmacological characterization based on potent inhibition of Raf-1 and its favourable kinase selectivity profile. Sorafenib exhibited significant activity against several receptor tyrosine kinases, including VEGFR2, VEGFR3, PDGFR-α, Flt-3, and c-kit. This molecule is currently being evaluated in phase III clinical trials for renal cell and hepatocellular carcinomas.

Based on these promising findings, these small molecular inhibitors of VEGFR tyrosine kinase activity are being tested in the breast cancer setting. Also, the combination of anti-angiogenic drugs with one another and with other biological agents is also being explored in an attempt to improve efficacy and to overcome the drug resistance observed in the initial studies of antiangiogenic agents. In addition, selecting patients for treatment on the basis of their clinical features and tumour characteristics may be essential in optimizing outcomes with these agents.

Novel use of conventional chemotherapy as antiangiogenic agents

It is likely that over the next few years the designer agents discussed above will be supplemented by conventional chemotherapeutic agents, including cyclophosphamide, paclitaxel, doxorubicin and vincristine, which appear to have antitumour effects through interfering with new vessel formation when used at 'metronomic' doses. This is where chemotherapy is administered frequently at low doses, which avoids myelosuppression and other dose-limiting side effects and which would otherwise require rest periods, but this approach inhibits tumour growth indirectly by damaging endothelial cells. This delivery strategy has several advantages over the conventional maximum tolerated dose approach, apart from reduced toxicity, in that a treatment response should occur irrespective of the resistance profile of the tumour cell population. Thus far, only a few clinical trials have tested this antiangiogenic schedule of chemotherapy [115-118], and the findings of these studies suggest that tumour associated endothelial cells may be sensitive to protracted low-dose chemotherapy. Other common chemotherapeutic agents, for example the camptothecin analogues, have also been shown to modulate angiogenesis as a secondary mechanism of action [119]. Placlitaxel, a microtubule inhibitor that is an active agent in the treatment of many different cancers, was shown to possess antiangiogenic properties that are independent of its antiproliferative action in *in vivo* models [120]. The level of expression of thymidine phosphorylase, a migration but nonmitogenic angiogenic enzyme that converts thymidine to thymine and 2-deoxyribose, may enhance survival in breast cancer through at least two mechanisms [121-123]. The first of these is by activation of intravenous 5-fluoruracil or oral capecitabine through conversion to active metabolites; the second mechanism is by abrogating thymidine rescue in methotrexate regimens and therefore salvaging the methotrexate block on *de novo* DNA synthesis.

In addition to conventional chemotherapeutic drugs that have antivascular effects, hormonal therapies such as tamoxifen may also have antiangiogenetic properties. Oestrogen is known to enhance VEGF expression (which may be partly HIF mediated [124]), and tamoxifen inhibits VEGF and FGF stimulated angiogenesis, resulting in a decrease in microvessel density and an increase in necrosis in MCF-7 xenografts [125-130]. Tamoxifen may also downregulate the angiogenic inhibitor thrombospondin [131]. Other drugs that may be of interest in this setting are the cyclo-oxygenase-2 inhibitors and biphosphonates, which also appear to have antiangiogenic potency [132-134].

Other targets

At the time of writing there are 30 agents on the National Cancer Institute website included in antiangiogenesis trials that interfere with the neovascularization process at many levels. Many promising novel targets have yet to reach this stage of development, the discussion of which is beyond the scope of this review. However, two of these that are of great interest are agents targeting the TAMs (which act as 'conductors' of angiogenesis) and Notch signalling (which is involved in cell differentiation, proliferation and apoptosis).

TAMs in breast cancers are markers of poor prognosis [135]. Evidence suggests that tumours recruit and use macrophage functions to promote tumour growth and metastasis. They achieve this through modulation of immune function, matrix degradation, growth factor production and angiogenesis. TAMs are recruited to avascular areas in breast tumours probably through hypoxic stimulation, in which they can release a variety of potent angiogenic factors, including VEGF (itself a chemoattractant for macrophages), thymidine phosphorylase, cyclo-oxygenase-2 and tumour necrosis factor-α. Matrix metalloproteinases such as urokinase plasminogen activator (also a prognostic factor in breast cancer) from TAMs can further increase local VEGF levels through cleavage from the matrix. Thus, the multifunctional and pivotal TAMs are a target for therapy. Indeed, macrophages are regulated by oestrogen, and crosstalk between the oestrogen receptor and cytokine-mediated pathways provide a potential role for selective oestrogen receptor modulators in prevention and/or treatment of breast cancer [136].

Notch signalling plays an important oncogenic role in breast tumour development in animal models. It is also significant in human breast cancers [137], with upregulation of several of the Notch pathway components occurring in human breast cancer cells; also, pathway members are expressed by endothelial cells in breast cancers. The Notch pathway is critical for angiogenesis, with mutations being associated with abnormal vascular development. Interestingly, like VEGF, haploinsufficiency for the endothelial-specific Notch ligand Delta-like 4 in mice is embryonic lethal. In view of the significance of Notch signalling in oncogenesis and tumour neovascularization, the pathway is a promising target for treatment. The pathway is complex but several points in the pathway may be target (reviewed by Shi and Harris [137]). Central to Notch activation is γ-secretase, which cleaves Notch, allowing its translocation to the nucleus where it activates target genes. Thus, inhibiting γ-secretase function would prevent Notch signal transduction; γ-secretase inhibitors have been developed that perform this function.

Conclusion

It has been more than 35 years since Judah Folkman suggested that the tumour vasculature would be a target for anticancer therapy, and in the interim there has been a huge increase in our understanding of the biology underlying tumour angiogenesis. Unfortunately, early enthusiasm for this approach based on strong preclinical data has not transferred simply to the clinic. Nevertheless, the latest generation of agents provides reason for optimism, such that antiangiogenic therapies are being integrated into routine oncology practice. There is still much to learn, with the full complexity of the mechanisms of tumour neovascularization and their regulators still to be defined, not only in individual tumour types but also in individual patients. Thus, more information in terms of biomarkers that are predictive of response is required so that tailored treatment can be offered. A huge amount of data should become available over the next few years that should help us to use these agents in the most effective manner.

Competing interests

The authors declare that they have no competing interests.

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