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Angiogenesis as a therapeutic target

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Inhibiting angiogenesis is a promising strategy for treatment of cancer and several other disorders, including age-related macular degeneration. Major progress towards a treatment has been achieved over the past few years, and the first antiangiogenic agents have been recently approved for use in several countries. Therapeutic angiogenesis (promoting new vessel growth to treat ischaemic disorders) is an exciting frontier of cardiovascular medicine, but further understanding of the mechanisms of vascular morphogenesis is needed first.

Early pioneers of angiogenic research observed over a century ago that the growth of human tumours is often accompanied by increased vascularity. They suggested that a key aspect of the cancer process is a disease of the vasculature in the whole area affected (reviewed in ref. 1). The existence of tumour-derived factors responsible for promoting new vessel growth was postulated over 65 years ago[2], and a few years later it was proposed that tumour growth is crucially dependent on the development of a neovascular supply[3]. In 1971, it was hypothesized that inhibition of angiogenesis (antiangiogenesis) would be an effective strategy to treat human cancer, and an active search for angiogenesis inducers and inhibitors began[4]. Extensive research has led to the identification and isolation of several regulators of angiogenesis, some of which represent therapeutic targets.

Despite some initial setbacks and negative clinical trial results, major progress has been made over the past few years in targeting angiogenesis for human therapy. In February 2004, the US Food and Drug Administration (FDA) approved bevacizumab, a humanized anti-VEGF (vascular endothelial growth factor)-A monoclonal antibody, for the treatment of metastatic colorectal cancer in combination with 5-fluorouracil (FU)-based chemotherapy regimens. This followed from a phase III study showing a survival benefit[5]. In December 2004, the FDA approved pegaptinib, an aptamer that blocks the 165 amino-acid isoform of VEGF-A, for the treatment of the wet (neovascular) form of age-related macular degeneration (AMD)[6].

These achievements have validated the notion that angiogenesis is an important target for cancer and other diseases. These advances notwithstanding, much progress is needed on a variety of important issues; for example, how do we achieve the most effective combinations of antiangiogenic agents with chemotherapy or other biological agents and how do we select patients that are most likely to respond to the treatment? Another issue is that resistance to antiangiogenic therapy is emerging[7] and thus a better understanding of pathways that may mediate tumour angiogenesis in various circumstances is necessary. Furthermore, the hope that 'therapeutic angiogenesis' will provide a treatment for ischaemic disorders still remains unfulfilled, in spite of considerable preclinical and clinical efforts.

The main purpose of this review is to summarize recent progress and emphasize the issues that need to be resolved before the field of angiogenic therapy can make further significant advances.

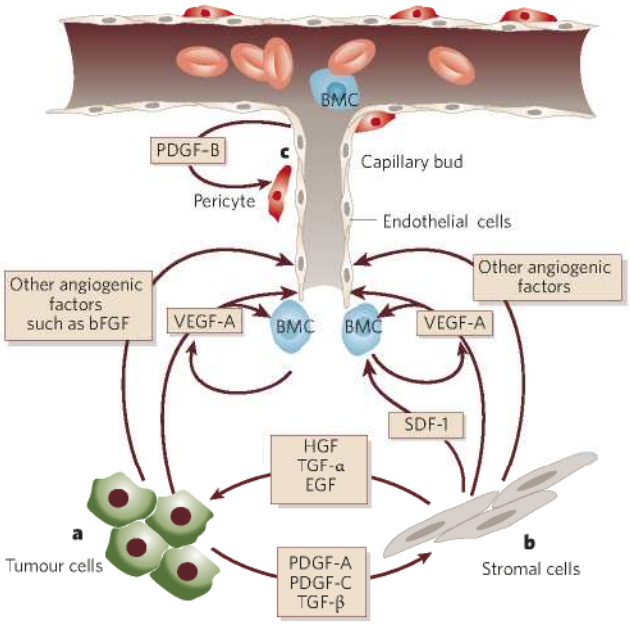

Figure 1 | A few of the molecular and cellular players in the tumour/microvascular microenvironment. a, Tumour cells produce VEGF-A and other angiogenic factors such as bFGF, angiopoietins, interleukin-8, PlGF and VEGF-C. These stimulate resident endothelial cells to proliferate and migrate. **b**, An additional source of angiogenic factors is the stroma. This is a heterogeneous compartment, comprising fibroblastic, inflammatory and immune cells. Recent studies indicate that tumour-associated fibroblasts produce chemokines such as SDF-1, which may recruit bone-marrow-derived angiogenic cells (BMC). The various hypotheses on the nature and role of such cells in angiogenesis and tumour progression are discussed in the text. VEGF-A or PlGF may also recruit BMC. Tumour cells may also release stromal cell-recruitment factors, such as PDGF-A, PDGF-C or transforming growth factor (TGF)-β. A well-established function of tumour-associated fibroblasts is the production of growth/survival factor for tumour cells such as EGFR ligands, hepatocyte growth factor and heregulin. **c**, Endothelial cells produce PDGF-B, which promotes recruitment of pericytes in the microvasculature after activation of PDGFR-β. HGF, hepatocyte growth factor.

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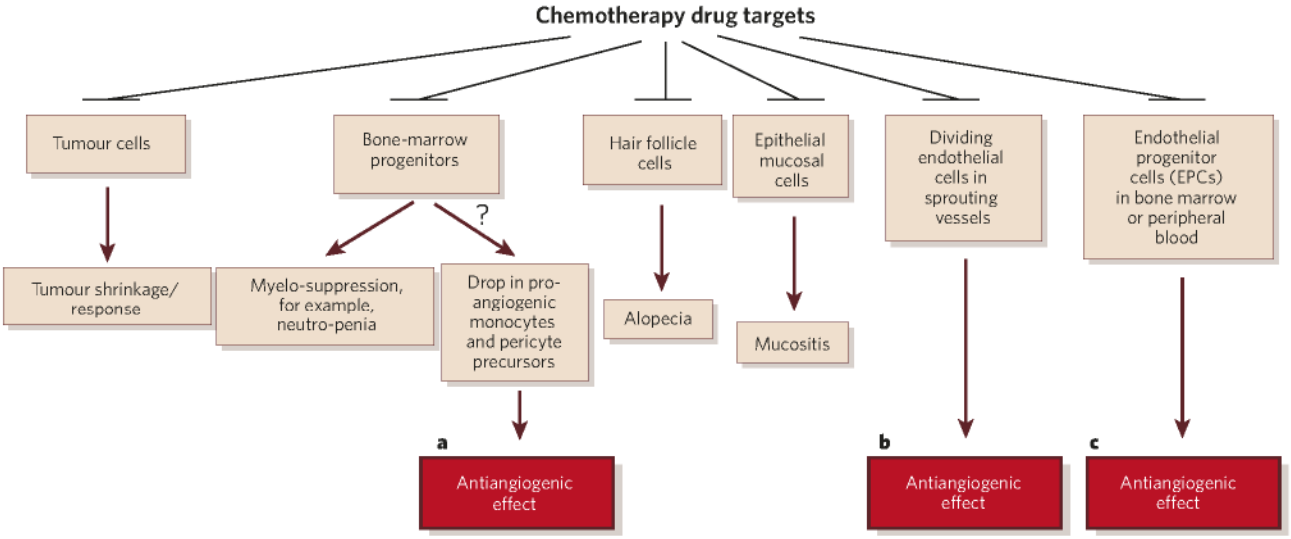

Figure 2 | Chemotherapy targets. In addition to tumour cells, the intended target for chemotherapy in cancer patients, conventional chemotherapy drugs can inhibit the proliferation of, or kill, a number of normal host cell types, including several that, in principle, can contribute to an antiangiogenic effect. Targeting of various normal cell populations is generally associated with harmful or undesirable side effects such as myelosuppression, alopecia or mucositis. A desirable effect could be antiangiogenesis as a result of targeting. **a**, Bone-marrow-derived proangiogenic cells that adhere to the walls of new blood vessels and further stimulate their growth by paracrine mechanisms. Whether these latter cell types, which probably include monocytes and pericyte precursors, are affected directly by chemotherapy or are reduced in numbers by elimination of more primitive bone marrow progenitors which give rise to such cells is not yet clearly established. **b**, Cycling endothelial cells present in sprouting blood vessel capillaries; and **c**, authentic bone-marrow-derived circulating endothelial progenitor cells (EPC) that can incorporate into the lumen of growing vessels and differentiate into endothelial cells. Inhibiting the levels or function of VEGF can augment these various antiangiogenic mechanisms of chemotherapy. For example, VEGF is a potent mobilizer of EPC, a pro-survival (anti-apoptotic) factor for differentiated, activated endothelial cells, and also may be one of the more important paracrine growth factors secreted by proangiogenic vessel adherent bone-marrow-derived monocytes.

The major signalling pathways in tumour angiogenesis

VEGF/VEGF receptors

Angiogenesis is a fundamental developmental and adult physiological process, requiring the coordinated action of a variety of growth factors and cell-adhesion molecules in endothelial and mural cells (reviewed in this issue by Coultas, Chawengsaksophak and Rossant, p. 937). So far, VEGF-A and its receptors are the best-characterized signalling pathway in developmental angiogenesis[1,8,9]. Loss of a single VEGF-A allele results in embryonic lethality[1,8,9]. This pathway also has an essential role in reproductive and bone angiogenesis[8]. Much research has also established the role of VEGF-A in tumour angiogenesis[8,10]. VEGF-A binds to two receptor tyrosine kinases (RTK), VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1) (reviewed in ref. 10). Of the two, it is now generally agreed that VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF-A. The significance of VEGFR-1 in the regulation of angiogenesis is more complex. Under some circumstances, VEGFR-1 may function as a 'decoy' receptor that sequesters VEGF and prevents its interaction with VEGFR-2 (ref. 10). However, there is growing evidence that VEGFR-1 has significant roles in haematopoiesis and in the recruitment of monocytes and other bone-marrow-derived cells that may home in on the tumour vasculature and promote angiogenesis[11–13]. In addition, VEGFR-1 is involved in the induction of matrix metalloproteinases (MMPs)[14] and in the paracrine release of growth factors from endothelial cells[15]. Thus the VEGFR-1-selective ligands VEGF-B and placental-like growth factor (PlGF) may also have a role in these processes. Furthermore, in some cases VEGFR-1 is expressed by tumour cells and may mediate a chemotactic signal, thus potentially extending the role of this receptor in cancer growth[16].

VEGF-A gene expression is upregulated by hypoxia[17]. The transcription factor hypoxia inducible factor (HIF), which operates in concert with the product of the von Hippel–Lindau (VHL) tumour suppressor gene, has a major role in such regulation. Under normoxic conditions, the VHL protein targets HIF for ubiquitination and degradation[17].

In situ hybridization studies demonstrate that VEGF-A messenger RNA is expressed in many human tumours[18]. Renal cell carcinomas have a particularly high level of VEGF-A expression, consistent with the notion that inactivating VHL mutations occur in about 50% of such tumours[19], thus providing a further explanation for the responsiveness of this tumour type to a VEGF-A blockade[20]. However, VEGF-A upregulation in tumours is not only linked to hypoxia or VHL mutations. Indeed, a very broad and diverse spectrum of oncogenes is associated with VEGF-A upregulation, including mutant ras, erbB-2/Her2, activated EGFR and bcr-abl[7,21]. Besides VHL, inactivation/mutation of various other suppressor genes can also result in VEGF upregulation. These genes include those associated with familial syndromes characterized by well-vascularized hamartomas[22].

In 1993, it was reported that a murine anti-human VEGF-A monoclonal antibody inhibited the growth of several tumour cell lines in nude mice, whereas the antibody had no effect on tumour-cell proliferation *in vitro*[23]. Subsequent studies have shown that many additional tumour cell lines, regardless of the tumour's origin, are inhibited *in vivo* by the same anti-VEGF monoclonal antibody (reviewed in ref. 24). Tumour-growth inhibition has also been demonstrated using independent anti-VEGF approaches including a dominant-negative VEGFR-2 mutant[25], anti-VEGFR-2 antibodies[26], small molecule inhibitors of VEGF RTKs[27] and soluble VEGF receptors[28,29]. VEGF-A gene inactivation also suppresses angiogenesis in a transgenic model of multi-stage tumorigenesis[30].

Platelet-derived growth factor (PDGF) and angiopoietins

Other signalling molecules that have an established role in the development and differentiation of the vessel wall such as PDGF-B/PDGFR-β[31] and the angiopoietins (Ang), the ligands of the Tie2 receptor[9], may also be therapeutic targets. PDGF-B is required for recruitment of pericytes and maturation of the microvasculature[31]. Inhibition of PDGFR-β signalling has been reported to result in a tumour microvascular tree that is particularly dependent on VEGF-mediated survival signals. Withdrawal of VEGF-A leads to endothelial apoptosis and vascular regression[32]. In this context, newly formed

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vessels, whether they are tumour-associated or not, are particularly vulnerable to VEGF-A blockade, whereas mature vessels, covered by extracellular matrix and pericytes, may be resistant to VEGF inhibitors and other antiangiogenic agents. Furthermore, recent studies have emphasized the significance of tumour-derived PDGF-A (and potentially PDGF-C) and PDGFR-α signalling in the recruitment of an angiogenic stroma that produces VEGF-A and other angiogenic factors[33] (Fig. 1). Therefore, combining PDGF and VEGF inhibitors is an attractive anti-vascular and anti-tumour strategy.

Ang-1 is required for further remodelling and maturation of the initially immature vasculature. Unlike mouse embryos lacking VEGF-A or VEGFR-2, embryos lacking Ang-1 or its receptor Tie2 develop a rather normal primary vasculature, but this vasculature fails to undergo effective remodelling (reviewed in ref. 9). The generally accepted view is that Ang-1 is the major agonist for Tie2, whereas Ang-2 may act as an antagonist or a partial agonist[34]. However, more recent evidence indicates that, unexpectedly, Ang-2 has a positive role, at least in tumour angiogenesis[35]. Administration of Ang-2 inhibitors to tumour-bearing mice has been reported to result in delayed tumour growth, accompanied by reduced endothelial cell proliferation, consistent with an antiangiogenic mechanism. Therefore, inhibitors of Ang-2 may be candidates for clinical development[35].

Axon-guidance molecules

Recently, the role of axon-guidance receptors and ligands in developmental angiogenesis has received much attention. There are four main families: the neuropilins (NRP)/semaphorins, the ephrins, Robo/Slit and netrin/Unc5. For recent reviews, see refs 36, 37. Although the significance of these pathways in tumour angiogenesis is far from clear, there is emerging evidence that they have a role in some cancer models and therefore may be potential therapeutic targets.

NRP1 and NRP2, previously shown to bind the collapsin/semaphorin family and implicated in axon guidance, are also receptors for the heparin-binding isoforms of VEGF-A and seem to potentiate the activation of VEGFR-2 by VEGF165 (ref. 38). Therefore, NRPs may participate in tumour angiogenesis as positive modulators of VEGF signalling in endothelial cells. Furthermore, NRP1 and NRP2 are expressed on the cell surface of several tumour cell lines that bind VEGF165 and display a chemotactic response to this ligand, suggesting a pro-tumour activity of NRPs, with or without the involvement of VEGF RTK signalling[37].

The ephrins and their tyrosine kinase Eph receptors are a large family, initially implicated in neuronal guidance during development and subsequently found to have activities in other cell types, including vascular cells (for a review see ref. 39). The earliest evidence for a role of this family in angiogenesis was the report by Pandey *et al.* that ephrin A1 mediates TNF-α-induced angiogenesis *in vivo*[40]. Ephrin B2 and its receptor EphB4 are important for distinguishing between developing arterial and venous vessels (see p. 937). Recent studies suggest a role for Eph/ephrin interactions in malignant tumour progression and angiogenesis. Soluble EphB4-expressing human melanoma A375 cells grown subcutaneously in nude mice showed reduced tumour growth compared with control tumours[41]. Interfering with EphA signalling has been also reported to result in some inhibition of angiogenesis in tumour models[42].

Slits are secreted proteins that function as chemorepellents in axon guidance and neuronal migration through the Roundabout (Robo) receptor (reviewed in refs 36, 37). Wang *et al.* reported the expression of Slit2 in several tumour cell types and that Robo1 expression was localized to vascular endothelial cells[43]. Recombinant Slit2 protein attracted endothelial cells and promoted tube formation. Neutralization of Robo1 reduced microvessel density and growth of A375 cells transplanted in nude mice[43].

Negative regulators of angiogenesis

Angiogenesis is a tightly regulated process and seems to be under the control of both positive and negative regulatory factors. Although several potential negative regulators of angiogenesis have been identified, relatively little is known about their role in the physiological regulation of angiogenesis. Thrombospondin, a large multifunctional glycoprotein secreted by most epithelial cells in the extracellular matrix, inhibits angiogenesis associated with tumour growth and metastasis[44]. Several fragments of larger proteins have been described as endogenous inhibitors of angiogenesis including endostatin[45], tumstatin[46] and vasostatin[47]. The most recently described endogenous inhibitor of angiogenesis is vasohibin, which seems to be derived from the endothelium and to operate as a feedback regulator[48]. The precise mechanism of action of these proteins remains to be more clearly defined, although several hypotheses have been proposed, including that they bind to specific integrins in the case of endostatin and tumstatin[49].

Role of bone-marrow-derived cells in angiogenesis

An intensively debated issue in the field is the contribution (as well as the precise nature) of bone-marrow-derived endothelial progenitor

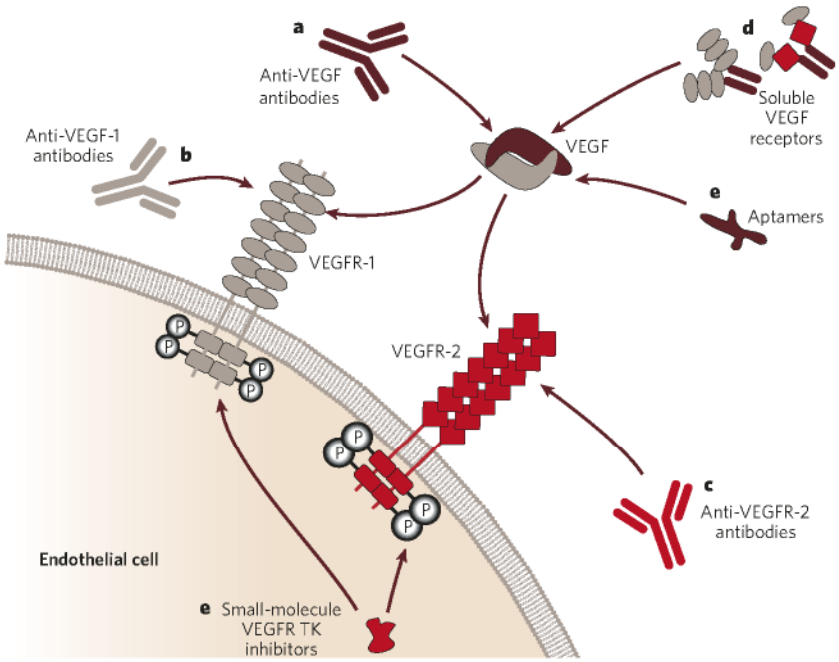

Figure 3 | Various strategies to inhibit VEGF signalling. These include monoclonal antibodies targeting VEGF-A (**a**) or the VEGF receptors (**b**, **c**). **d**, Chimaeric soluble receptors such as the 'VEGF-trap' (domain 2 of VEGFR-1 and domain 3 of VEGFR-2 fused to a Fc fragment of an antibody) are also undergoing clinical development. **e**, Additional extracellular inhibitors are aptamers that bind the heparin-binding domain of VEGF165 (pegaptanib). A variety of small-molecule VEGF RTK inhibitors that inhibit ligand-dependent receptor autophosphorylation of VEGFR-1 and VEGFR-2 are being tested. Additional strategies to inhibit VEGF signalling include antisense and siRNA targeting VEGF-A or its receptors.

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cells (EPC) to angiogenesis. However, there is little doubt that bone-marrow-derived cells participate in angiogenic processes, at least as a source of angiogenic factors. In 1997, Asahara *et al.* reported the isolation of putative EPC from human peripheral blood, on the basis of cell-surface expression of CD34 and other endothelial markers[50]. These cells were reported to differentiate *in vitro* into endothelial cells and seemed to be incorporated at sites of active angiogenesis in various animal models of ischaemia. These findings suggested that incorporation into the lumen of bone-marrow-derived endothelial precursor cells contributes to the growing vessels, complementing resident endothelial cells in sprouting new vessels. Also, ischaemia and various cytokines, including VEGF and granulocyte-macrophage colony-stimulating factor (GM-CSF), were reported to mobilize EPC into sites of neovascularization[51]. However, the precise contribution of these cells in various pathophysiological circumstances was not clearly defined.

Subsequent studies have suggested that the contribution of such cells to angiogenesis is dependent on the experimental system employed. In the angiogenic-defective, tumour-resistant *Id*-mutant mice, EPC accounted for a large proportion of endothelial cells associated with xenografted tumours[52]. Rafii and collaborators proposed that mobilization of EPC from bone marrow requires angiogenic-factor-mediated activation of MMP-9, which leads to the release of the soluble KIT ligand. This ligand would in turn promote proliferation and motility of EPC within the bone-marrow microenvironment, thus creating permissive conditions for their mobilization into the peripheral circulation[53]. However, in spontaneous tumours occurring in *Id*-deficient mice in the tumour-prone PTEN$^{+/-}$ genetic background, the contribution of EPC was less significant[54]. Also, De Palma *et al.* suggested that the percentage of EPC that are truly incorporated into a growing vessel wall is very low and that the majority of bone-marrow-derived cells homing in on the tumour vasculature are adherent perivascular mononuclear cells, which may contain angiogenic factors[55]. Peters *et al.* recently analysed the tumour endothelial cells in six individuals who developed cancers after bone-marrow transplantation with donor cells derived from individuals of the opposite sex and found that an average of 4.9% of cells of the total endothelial cell population were derived from the bone marrow[56].

In summary, bone-marrow-derived cells seem to contribute to tumour angiogenesis, of which a small and variable proportion are probably true EPCs. Bone-marrow-derived circulating pro-angiogenic cells, regardless of their precise nature, may be a common target for antiangiogenic therapies and may be exploitable as surrogate biomarkers for the angiogenic process as well as antiangiogenic therapies[57].

Combination therapies

It is increasingly likely that cancer therapy, with a few exceptions, will need to be combinatorial. It seems logical to target multiple pathways simultaneously. Much preclinical evidence indicates that combining antiangiogenic agents with conventional cytotoxic agents or radiation therapy results in additive or even synergistic anti-tumour effects[58]. So far, it is unclear whether such positive interaction takes place preferentially with specific types of antiangiogenic or cytotoxic agents. An issue that is being debated is the mechanism of such potentiation, as it would seem counterintuitive that 'tumour-starving' antiangiogenic drugs that suppress blood flow in tumours actually increase the efficacy of chemotherapy. Browder *et al.*[59] and Klement *et al.*[60] proposed that chemotherapy, especially when delivered at close regular intervals using relatively low doses with no prolonged drug-free break periods ('metronomic therapy'), preferentially damages endothelial cells in tumour blood vessels. These cells are presumably dividing, and the simultaneous blockade of VEGF-A is thought to blunt a key survival signal for endothelial cells, thus selectively amplifying the endothelial cell targeting effects of chemotherapy, leading to improved subsequent killing of cancer cells.

A similar process, in principle, may take place when combining more conventional maximum-tolerated dose chemotherapy regimens with a drug such as bevacizumab[61]. In addition, bone-marrow-derived pro-angiogenic circulating cells, probably including authentic EPC, seem to be very sensitive to both conventional cytotoxic and low-dose metronomic chemotherapy[62]. However, levels of such cells can rapidly rebound, returning to normal or even increased levels during the drug-free break periods after maximum-tolerated dose cytotoxic chemotherapy[62]. Because VEGF-A acts as a mobilizing and probably a survival agent for such cells, co-administration of a VEGF-targeting agent, especially one with a long half-life in the circulation (for example, anti-VEGF antibodies), would be expected to amplify and sustain the suppressive effects of standard (as well as metronomic) chemotherapy on bone-marrow-derived circulating pro-angiogenic cells[63]. Furthermore, it has been proposed that progressively accelerated proliferation and repopulation of cancer cells during intervals of radiotherapy or chemotherapy is an important cause of treatment failure[64]. It is tempting to speculate that antiangiogenic treatment during these intervals inhibits such repopulation process. Figure 2 illustrates the various cellular targets of chemotherapy.

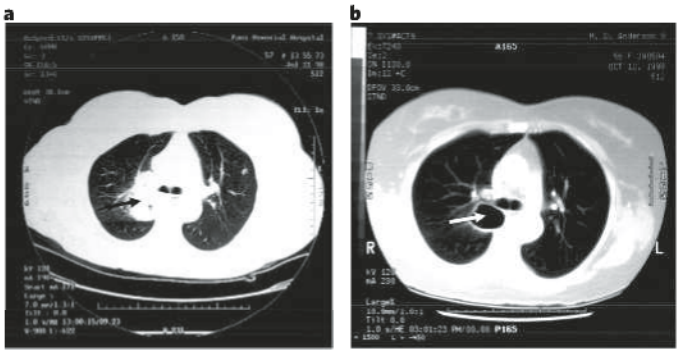

Figure 4 | Computed tomography chest scans. These scans were taken of a NSCLC patient before (**a**) and after (**b**) three cycles (nine weeks) of treatment with bevacizumab plus carboplatin and taxol (reproduced from ref. 71 with permission from American Society of Clinical Oncology). Note that the tumour mass in **a** (arrow) underwent extensive necrosis and cavitation in **b** (arrow). This pattern was seen more frequently in patients treated with bevacizumab plus chemotherapy relative to chemotherapy alone. Cavitation may be associated with serious bleeding, especially when it occurs in proximity to large vessels[71].

An alternative hypothesis has been proposed by Jain[65]. Submaximal doses of an antiangiogenic agent such as an anti-VEGFR-2 antibody would 'normalize' the vasculature that is characteristic of many vessels in tumours. This would result in pruning of excessive endothelial and perivascular cells, in a decrease in the normally high interstitial pressures detected in solid tumours and in temporarily improved oxygenation and delivery of chemotherapy to tumour cells[65]. However, according to recent studies, the tumour vasculature can be 'normalized' transiently, and eliciting synergistic effects through this mechanism requires administration of chemotherapy or radiation therapy over a defined time window after the angiogenesis inhibitor[66]. Considering also that in most clinical protocols no such intentional sequential administration is performed, it remains to be established whether such a mechanism accounts for the long-term beneficial effects of combination treatments observed in some trials. By contrast, acute administration of angiogenic inhibitors induces vascular changes consistent with 'normalization' in humans. In this regard, Willett *et al.* reported that a single infusion of bevacizumab to patients with rectal carcinoma rapidly decreased tumour perfusion, vascular volume, microvascular density and interstitial fluid pressure as well as the number of viable, circulating endothelial cells in six colorectal cancer patients[67].

Combinatorial therapies with antiangiogenic agents are not limited to those including cytotoxic chemotherapy. Several preclinical and clinical trials are exploring the combination of various angiogenesis

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inhibitors with other targeted therapies, such as EGFR or Her2 inhibitors (cetuximab, erlotinib and trastuzumab), PDGFR/ bcr-abl inhibitors (imatinib), proteasome inhibitors (bortezomib) and other antiangiogenic agents such as inhibitors of integrins (for example $\alpha v\beta 3$ and $\alpha 5\beta 1$).

Clinical trials for antiangiogenesis

Many angiogenesis inhibitors are currently in clinical trials. It is noteworthy that, in parallel to angiogenesis inhibitors, another class of vascular-targeting or vascular-modulating drugs is being tested, namely 'vascular-disrupting agents'. These drugs primarily target existing, recently formed vasculature and cause acute vascular occlusion and disruption of tumour blood flow[68]. For an overview of these trials, see http://www.cancer.gov/clinicaltrials/developments/anti-angio-table. The inhibitors tested include a variety of agents with diverse mechanisms of action (several of which are not known). At present, inhibitors of the VEGF pathway are the most clinically advanced, and bevacizumab, a humanized variant of a murine anti-VEGF-A monoclonal antibody that was used in early proof-of-concept studies[23], is the only FDA-approved antiangiogenic treatment for cancer therapy[69]. Figure 3 illustrates several methods for inhibiting the VEGF pathway.

Several important clinical studies testing angiogenesis inhibitors have been presented at recent oncology meetings, such as the American Society of Clinical Oncology meeting. Typically, clinical studies are presented and discussed at such meetings in advance of peer-reviewed publication. Therefore, in the interest of an up-to-date overview of the field, a discussion of some of these studies will be included here, with the caveat that the data are preliminary and require further analysis.

The clinical trial that resulted in FDA approval of bevacizumab was a large, randomized, double-blind, phase III study in which bevacizumab was administered in combination with bolus IFL (irinotecan, 5FU and leucovorin) chemotherapy as first-line therapy for metastatic colorectal cancer[5]. Median survival was increased from 15.6 months in the bolus-IFL plus placebo arm of the trial to 20.3 months in the bolus IFL plus bevacizumab arm. Similar increases were seen in progression-free survival, response rate and duration of response. The clinical benefit of bevacizumab was seen in all subject subgroups, including those defined by performance status, location of primary tumour, number of organs involved and duration of metastatic disease[5]. Although bevacizumab was generally well tolerated, some serious and unusual toxicities have been noted, albeit at low frequencies. Bevacizumab was associated with gastrointestinal perforations and wound healing complications in about 2% of patients. In addition, the incidence of arterial thromboembolic complications were increased about twofold relative to chemotherapy alone, with patients 65 years or older with a history of arterial thromboembolic events being at higher risk. Although the precise mechanism of this effect is unknown, it is conceivable that vascular damage induced by cytotoxic agents can be exacerbated by the blockade of VEGF-A.

Preliminary data of a phase III study indicate that bevacizumab confers a survival advantage on patients with previously treated, relapsed, metastatic colorectal cancer in combination with FOLFOX4 chemotherapy (5-fluorouracil, leucovorin and oxaliplatin), relative to chemotherapy alone (B. Giantonio, P. J. Catalono, N. J. Meropol, E. P. Mitchell, M. A. Schwartz *et al.*, unpublished data).

The role of bevacizumab in other tumour types and settings is currently under investigation, and phase III clinical trials of this drug in non-small-cell-lung cancer (NSCLC), renal cell cancer and metastatic breast cancer are ongoing. An early phase III trial of advanced, heavily pretreated, metastatic breast cancer showed that adding bevacizumab to capecitabine chemotherapy did not improve progression-free survival, despite a doubling of the response rate (that is, tumour shrinkage of 50% or more) in the bevacizumab-treated arm of the trial[70]. Thus, the responses seemed to be very short in duration. However, an interim analysis of a phase III study of women with previously untreated metastatic breast cancer treated with bevacizumab in combination with weekly paclitaxel chemotherapy showed that the study met its primary efficacy endpoint of improving progression-free survival, compared with paclitaxel alone (K. Miller, unpublished data).

Furthermore, administration of bevacizumab in combination with paclitaxel and carboplatin to patients with NSCLC resulted in increased response rate and time to progression relative to chemotherapy alone in a randomized phase II trial[71]. The most significant adverse event was serious haemoptysis. This was primarily associated with centrally located tumours with squamous histology, cavitation and central necrosis and proximity of disease to large vessels[71]. Figure 4 illustrates the extensive tumour necrosis and cavitation that may result from the combination treatment[71]. More recently, preliminary results from a large, randomized phase III clinical trial for patients with previously untreated advanced non-squamous NSCLC show that patients who received bevacizumab in combination with paclitaxel and carboplatin lived longer than patients who received chemotherapy alone (A. B. Sandler, R. Gray, J. Brahmer, A. Dowlati, J. H. Schiller *et al.*, unpublished data). Serious bleeding was infrequent but occurred more commonly in the bevacizumab arm of the trial.

Besides bevacizumab, several other VEGF inhibitors are being clinically pursued. A variety of small-molecule RTK inhibitors targeting the VEGF receptors have been developed. The most advanced are SU11248 and Bay 43-9006. SU11248 inhibits VEGFRs, PDGFR, c-kit and Flt-3 (ref. 72) and has been reported to have considerable efficacy in imatinib-resistant gastrointestinal stromal tumour (R. G. Maki, J. A. Fletcher, M. C. Heinrich, J. A. Morgan, S. George *et al.*, unpublished data). Bay 43-9006 was initially identified as a raf kinase inhibitor and subsequently shown to inhibit several RTKs including VEGFRs. An interim analysis of phase III data indicates that Bay 43-9006 monotherapy results in a significant increase in progression-free survival in patients with advanced renal cell carcinoma (B. Escudier, C. Szczylik, T. Eisen, W. M. Stadler, B. Schwartz *et al.*, unpublished data). Follow-up of such phase III data is ongoing to determine whether an overall survival benefit occurs. An earlier randomized phase II study had shown that bevacizumab as a single agent also results in an increase in time to progression in renal cell carcinoma patients, providing further evidence that this tumour type may be particularly responsive to anti-VEGF treatment[20].

AG-013736, which has a similar spectrum of kinase inhibition to SU11248, has also shown promise in metastatic renal cell carcinoma in a phase II monotherapy study (B. Rini , O. Rixe, R. Bukowski, M. D. Michaelson, G. Wilding *et al.*, unpublished data). Twenty-four patients (46% of those on the trial) experienced partial responses to the treatment. Stable disease was observed in an additional 21 patients (40%). These interesting efficacy data will need to be followed by an appropriately designed and powered phase III trial.

An additional VEGF RTK inhibitor in late-stage clinical trials is PTK787 (ref. 27). This molecule is in phase III trials in colorectal cancer patients, in combination with FOLFOX4 chemotherapy. Recently, interim findings of this trial have been presented (J. R. Hecht, T. Trabech, E. Jaeger, J. Hainsworth, R. Wolff *et al.*, unpublished data). According to investigator-based assessment, there was a statistically significant increase in progression-free survival in PTK787-treated patients. However, a central review failed to document any significant difference. Subgroup analysis suggested that patients with high lactic dehydrogenase have the best response to PTK787 in terms of progression-free survival.

A chimaeric, soluble VEGF receptor ('VEGF-trap')[29] is also undergoing clinical development as an anti-cancer agent and preliminary results of a phase I study have been recently presented (J. Dupont, M. L. Rothenberg, D. R. Spriggs, J. M. Cedarbaum, E. S. Furfine *et al.*, unpublished data).

Recombinant human endostatin has been tested in phase I studies over the past few years to determine its safety and pharmacokinetic characteristics in patients with solid tumours, and these studies have documented a lack of dose-limiting toxicity[73].

Neovascularization and vascular leakage are a major cause of visual

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loss in the wet form of AMD[74]. Currently, several anti-VEGF strategies are being explored in AMD clinical trials. The most clinically advanced inhibitors are pegaptanib, an aptamer that interacts with the heparin-binding domain of VEGF165 (ref. 75) and ranibizumab, a humanized high-affinity anti-VEGF Fab that neutralizes all VEGF-A isoforms and proteolytic fragments[76]. Pegaptanib has been approved by the FDA for the treatment of AMD, after phase III studies showing that intraocular administrations of the drug reduced visual loss relative to the placebo[6]. Very recently, data of a controlled phase III study with ranibizumab indicated that the treatment is efficacious and maintains or improves vision in patients with wet AMD (P. J. Rosenfeld, D. M. Browne, J. S. Heier, D. S. Boyer & P. K. Keiser *et al.*, unpublished data).

Resistance to antiangiogenic therapies

Many patients treated with VEGF inhibitors, especially in combination with chemotherapy, may survive longer, but they eventually succumb to their disease. There is emerging evidence that VEGF-A may be replaced by other angiogenic pathways as the disease progresses. These potentially include VEGF-unrelated pathways and others mediated by other members of the VEGF gene family such as the lymphangiogenic factors VEGF-C and VEGF-D, which may bind to and activate VEGFR-2 after proteolytic cleavage (see the review in this issue by Alitalo, Tammela and Petrova, p. 946). Other possible mechanisms for acquired resistance to antiangiogenic drugs[77,78] include selection and overgrowth of tumour-cell variants that are 'hypoxic resistant' and thus less dependent on angiogenesis[79] and tumour-vessel remodelling resulting in a shift to mature, stabilized vessels that are less responsive to antiangiogenic drugs[80]. The recruitment of bone-marrow-derived angiogenic cells may also provide a potential mechanism of escape to some antiangiogenic strategies.

Orimo *et al.* have recently reported that one of the mechanisms by which cancer-associated fibroblasts contribute to tumour angiogenesis is the release of stromal cell-derived factor (SDF)-1, which leads to the recruitment of bone-marrow cells in the tumour vasculature (Fig. 1)[81]. A current focus of research is the role of myeloid cells of the monocyte/macrophage lineage in mediating multiple pathways leading to tumour progression and angiogenesis (reviewed in ref. 82). The hypoxic tumour microenvironment may lead to upregulation of chemokines and other factors (for example, interleukin-8, angiopoietins, basic firoblast growth factor (bFGF) and hepatocyte growth factor (HGF) from tumour-infiltrating macrophages, which may facilitate angiogenesis, invasion and immune evasion of tumours[82]. Further potential mechanisms of escape are integrin-signalling-mediated events, as there is growing evidence to support positive interactions between integrins and a variety of RTKs involved in tumour invasion, metastasis and angiogenesis[83].

Furthermore, recent studies suggest that in some cases endothelial cells associated with tumours are not a genetically stable, non-transformed, compartment (as long assumed) and instead may provide a further mechanism of resistance to antiangiogenic therapies. Hida *et al.* described cytogenetical abnormalities in murine endothelial cells isolated from human tumour xenografts (aneuploidy, multiple abnormal centrosomes), although the mechanism for the acquisition of such alterations remains unknown[84]. Also, Streubel *et al.* reported that a significant percentage of the endothelial cells in human B-cell lymphomas harbour lymphoma-specific chromosomal abnormalities, suggesting that endothelial cells in B-cell lymphomas may be in part tumour related[85].

Pro-angiogenic therapies

The possibility that pharmacological treatments for disorders characterized by inadequate tissue perfusion may become available is attractive, as often there are no effective alternatives to surgical reconstruction procedures. Many angiogenic factors are active in a variety of animal models of coronary or limb ischaemia (reviewed in refs 86, 87). On the basis of these preclinical data, several angiogenic factors (VEGF-A, VEGF-C, FGF-1, FGF-2 and FGF-4) have been tested in patients with myocardial or limb ischaemia[87]. Although promising results were initially reported in small open-label trials, none of the placebo-controlled trials so far conducted with recombinant proteins or gene therapy has yielded convincingly positive results[88,89]. The placebo effect in these trials proved to be considerably greater than initially suspected. It is conceivable that young and otherwise healthy animals are able to mount an effective endogenous angiogenic response to vascular injury that can be further augmented. However, endothelial cells in patients with extensive atherosclerotic disease may be in part refractory to angiogenic factors[86,87]. Interestingly, more recent studies suggest that a therapy using a 'master switch' such as HIF might provide advantages relative to single angiogenic factors[90]. A more persistent exposure to angiogenic factors than that achieved in early trials may also lead to better results.

Transplantation of angiogenic cells from bone marrow or peripheral blood to treat ischaemic disorders, as an alternative to angiogenic factors, has also generated significant interest[91]. Different strategies have been employed, including the use of bone-marrow cells and either whole peripheral mononuclear cells or purified $CD34^+$ cells, after bone-marrow stimulation with cytokines such as GM-CSF[91]. The first randomized study testing this hypothesis consisted of an intramuscular injection of autologous bone-marrow-derived mononuclear cells in patients with limb ischaemia[92]. This therapy promoted vascular proliferation and resulted in a significant increase in transcutaneous oxygen pressure, reduced rest pain and increased pain-free walking time in 22 patients with leg ischaemia, relative to injection of peripheral mononuclear cells[92]. Additional small trials in coronary or limb ischaemia patients have reported some improvement in angina and walking time after transplantation of blood cells[89]. Large placebo-controlled studies are required to verify these findings and assess any long-term benefit.

These results highlight a concern regarding possible delayed side effects of long-term antiangiogenic therapy. If bone-marrow-derived cells contribute significantly to vessel integrity and repair, can we expect increases in certain adverse cardiovascular events in cancer patients — especially in older patients with subclinical cardiovascular disease — who are successfully treated with antiangiogenic therapies over prolonged periods?

Conclusions and perspectives

There is now proof that an antiangiogenic approach, when combined with chemotherapy, results in increased survival in patients with advanced malignancies. It could be argued that the clinical benefit and impact would be greater if the therapy were initiated at earlier stages of malignancy, possibly in the adjuvant setting, in agreement with many preclinical data. However, lengthy clinical trials will be required to test this hypothesis. Very recent studies suggest that there is reason to be optimistic and that such studies are warranted. A targeted therapy such as trastuzumab given in combination with chemotherapy to patients with early stage Her2-positive breast cancer results in a 52% reduction in the risk of disease recurrence, compared with those patients who received chemotherapy alone, after four years in the study[93]. This is a substantial reduction in the risk of cancer recurrence.

It is important to obtain reliable markers to monitor the activity of antiangiogenic drugs. So far, the absence of such biomarkers may have impaired clinical development of various antiangiogenic drugs. Circulating endothelial cells and their progenitor subset and MRI dynamic measurement of vascular permeability/flow in response to angiogenesis inhibitors are potential candidates, although their long-term predictive value remains to be established[57,67,94].

Inhibition of lymphangiogenesis has been proposed as a strategy to reduce lymphatic metastasis. Work over the past few years has elucidated the role of the VEGF-C/VEGFR-3 pathway in the regulation of normal and tumour-associated lymphangiogenesis (see p. 946). Therefore, combining traditional antiangiogenic agents with inhibitors of lymphangiogenesis may provide a powerful anti-cancer approach.

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Recent studies have provided evidence for the existence of at least one tissue-specific angiogenic factor (EG-VEGF) expressed by a steroidogenic gland[95]. Highly vacularized endocrine malignancies such as Leydig cell tumours express particularly high levels of EG-VEGF mRNA[96], raising the possibility that EG-VEGF inhibition may have a role in the treatment of such tumours. It remains to be established whether other tissue-specific angiogenic pathways exist.

Furthermore, an emerging concept in solid-tumour biology is that of tumour 'stem' cells. It has been argued that this minor self-renewing subpopulation is absolutely crucial to progressive and sustained cancer growth, as opposed to the bulk non-stem-cell population[97,98]. This may explain why rapid and significant tumour shrinkage induced by various anti-cancer drugs such as cytotoxic chemotherapy may have little or no impact on prolonging survival: the 'wrong' population is being eliminated. By contrast, antiangiogenic drugs, which generally do not cause rapid tumour shrinkage, if any shrinkage at all, may nevertheless have a survival benefit if they block the stem-cell population from inducing or exploiting angiogenesis necessary for their growth. Indeed, there is already a hint that the stem-cell subpopulation in breast cancer may be more adept at promoting angiogenesis than its non-stem-cell counterpart[99]. ■

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