DISCOVERY AND DEVELOPMENT OF BEVACIZUMAB, AN ANTI-VEGF ANTIBODY FOR TREATING CANCER

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The existence of factors that stimulate blood vessel growth, thereby recruiting a neovascular supply to nourish a growing tumour, was postulated many decades ago, although the identification and isolation of these factors proved elusive. Now, vascular endothelial growth factor (VEGF), which was identified in the 1980s, is recognized as an essential regulator of normal and abnormal blood vessel growth. In 1993, it was shown that a monoclonal antibody that targeted VEGF results in a dramatic suppression of tumour growth *in vivo*, which led to the development of bevacizumab (Avastin; Genentech), a humanized variant of this anti-VEGF antibody, as an anticancer agent. The recent approval of bevacizumab by the US FDA as a first-line therapy for metastatic colorectal cancer validates the ideas that VEGF is a key mediator of tumour angiogenesis and that blocking angiogenesis is an effective strategy to treat human cancer.

CASE HISTORY

ENDOTHELIAL CELLS
The main type of cell in the inside lining of blood vessels, lymph vessels and the heart.

The observation that tumour growth can be accompanied by increased vascularity was reported more than a century ago (for a review, see REF. 1). However, it was not until 1939 that Ide and colleagues first postulated the existence of a tumour-derived blood-vessel-growth stimulating factor that might serve to provide a neovascular supply to the growing tumour[2]. A few years later, Algire *et al.* proposed that "the rapid growth of tumour transplants is dependent upon the development of a rich vascular supply", on the basis of the observation that local increases in blood-vessel density precede rapid tumour growth[3]. The field was then quiet until the 1960s, when experiments by Greenblatt and Shubik[4], and Ehrmann and Knoth[5], provided early evidence that tumour angiogenesis was mediated by diffusible factors produced by tumour cells.

In 1971, Folkman proposed that anti-angiogenesis might be an effective anticancer strategy[6]. On the basis of this pioneering hypothesis, Folkman and collaborators initiated efforts aimed at the isolation of a 'tumour angiogenesis factor' from human and animal tumours in the early 1970s[7]. In 1978, Gullino also suggested that blocking angiogenesis could prevent cancer[8]. Subsequently, the angiogenic effects of a variety of factors (for example, epidermal growth factor (EGF), transforming growth factor (TGF)-α, TGF-β, tumour-necrosis factor-α (TNF-α) and angiogenin) were described[9]. However, although these factors promoted angiogenesis in several bioassays, none was shown to function physiologically[10].

Most of the attention was directed towards two widely distributed and potent ENDOTHELIAL-CELL mitogens and angiogenic factors: acidic and basic fibroblast growth factors (aFGF and bFGF). The purification to homogeneity, sequencing and cDNA cloning of the FGFs was reported in the mid-1980s[11]. A surprising finding was that the genes for both aFGF and bFGF do not encode a conventional secretory signal peptide. However, as previously noted, earlier studies suggested that tumour angiogenesis was mediated by diffusible molecules[4,5]. Furthermore, several studies indicated that immunoneutralization of FGF had little or no effect on tumour angiogenesis[12,13], suggesting that key regulators of angiogenesis remained to be identified.

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It is now known that vascular endothelial growth factor (VEGF, also referred to as VEGF-A) is one such key regulator of angiogenesis, and the role of the VEGF gene family in the regulation of angiogenesis has been intensively investigated for more than a decade[1]. The VEGF family includes the prototype member VEGF-A, placenta growth factor (PlGF)[14], VEGF-B[15], VEGF-C[16] and VEGF-D[17]. Compelling evidence indicates that whereas assembly and maturation of the vessel wall are highly complex processes requiring the coordinated action of angiopoietins, platelet-derived growth factor B (PDGF-B) and other factors[18], VEGF-A action constitutes a rate-limiting step in normal and pathological blood vessel growth[19]. Importantly, VEGF-C and VEGF-D regulate lymphatic angiogenesis[20], emphasizing the unique role played by this gene family in controlling growth and differentiation of several anatomic components of the vascular system. The purpose of this article, however, is to briefly overview the biology of VEGF, and then to focus on the path from the identification of VEGF and the establishment of its key role in tumour angiogenesis to the first approval by the US FDA of a therapeutic developed to target tumour angiogenesis — the humanized anti-VEGF monoclonal antibody bevacizumab (Avastin; Genentech).

Identification of VEGF

In 1983, Senger *et al.* reported the partial purification from the conditioned medium of a guinea-pig tumour cell line of 'vascular permeability factor' (VPF), a protein that induced vascular leakage in the skin[21]. However, because VPF was not isolated and sequenced, this factor remained molecularly unknown at that time.

In 1989, our laboratory reported the isolation of 'vascular endothelial growth factor' (VEGF), an endothelial-cell-specific mitogen, from medium conditioned by bovine pituitary follicular cells[22]. The amino-terminal amino-acid sequence of VEGF did not match any known protein in available databases[22]. Subsequently, Connolly *et al.*, following up on the work by Senger and collaborators, independently reported the isolation and sequencing of VPF[23]. cDNA cloning of VEGF, reported by our group[24], and of VPF by Connolly's group[25], showed that VEGF and VPF were the same molecule. This was surprising, considering that other endothelial-cell mitogens such as FGF do not increase vascular permeability.

Properties of the VEGF isoforms

VEGF has significant homology to the A and B chains of PDGF[24]. The gene encoding human VEGF-A is organized in eight exons, separated by seven introns[26,27]. Alternative exon splicing results in the generation of four principal isoforms — $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ — that have 121, 165, 189 and 206 amino acids, respectively, following signal-sequence cleavage[24]. $VEGF_{165}$, the predominant isoform, lacks the residues encoded by exon 6, whereas $VEGF_{121}$ lacks the residues encoded by exons 6 and 7. Less frequent splice variants have been also reported, including $VEGF_{145}$, $VEGF_{183}$, $VEGF_{162}$ and $VEGF_{165b}$ (reviewed in REF. 19).

Alternative splicing regulates the bioavailability of VEGF[28,29]. $VEGF_{121}$ fails to bind HEPARIN and is a freely diffusible protein; $VEGF_{165}$ is secreted, but a significant fraction remains bound to the cell surface and the extracellular matrix, by virtue of its heparin-binding properties. The highly basic $VEGF_{189}$ is almost completely bound to the extracellular matrix[28,29]. There is now much evidence to indicate that $VEGF_{165}$ is the most physiologically relevant isoform[30,31]. Also, extracellular proteolysis can have a major role in regulating VEGF bioavailability. Plasmin is able to cleave $VEGF_{165}$ or $VEGF_{189}$ and release a bioactive product consisting of the first 110 amino-terminal amino acids[32]. Given the importance of plasminogen activation during physiological and pathological angiogenesis[33], this mechanism can be especially significant in regulating the activity and bioavailability of VEGF when remodelling occurs and in response to cues from the micro-environment. Furthermore, as discussed later in this article, proteolysis of VEGF mediated by matrix metalloproteinase-9 (MMP9) might be responsible for the angiogenic switch in some tumours[34].

A well-established action of VEGF is to promote the growth of vascular endothelial cells derived from arteries, veins and lymphatics (for a review, see REF. 35). VEGF induces a potent angiogenic response in a variety of *in vivo* models[24,36]. Furthermore, as previously noted, VEGF increases vascular permeability and this property underlies important roles of this molecule in inflammation and in other pathological conditions[37]. In this context, VEGF also induces the expression of several adhesion molecules in the endothelium that regulate leukocyte adhesion during inflammation[38].

In vitro, VEGF prevents endothelial-cell apoptosis induced by serum starvation, an activity mediated by the phosphatidylinositol 3′ kinase (PI3K)/Akt pathway[39]. Also, VEGF induces expression of the anti-apoptotic proteins BCL2 and A1 in endothelial cells[40]. VEGF-dependence has been demonstrated in endothelial cells of newly formed, but not of established, vessels within tumours[41,42]. Coverage by PERICYTES seems to be one of the key events resulting in endothelial loss of VEGF dependence[42].

It is important to emphasize that although endothelial cells are the primary targets of VEGF, several studies have reported mitogenic/survival effects on certain non-endothelial cell types as well, including nerve cells[43].

VEGF receptors

There are two VEGF receptor tyrosine kinases (RTKs): VEGFR1, also known as Flt-1[44,45]; and VEGFR2, also known as Flk-1 or KDR[46–48] (see FIG. 1). Embryonic lethality following inactivation of VEGFR1 or VEGFR2 demonstrated the crucial role of both receptors in the development of the vascular system[49,50].

There is now a consensus that VEGFR2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF (for a more extensive review of the biological and signalling properties of the VEGF receptors, see REF. 31). Although the functions of VEGFR1 are complex, it seems that this molecule is not directly

HEPARIN
Naturally occurring acidic glycosaminoglycan. Heparin-like moieties are common in proteoglycans in the cell surface and extracellular matrix.

PERICYTES
Support cells of capillaries (believed by many to be the equivalent of smooth muscle cells in larger vessels).

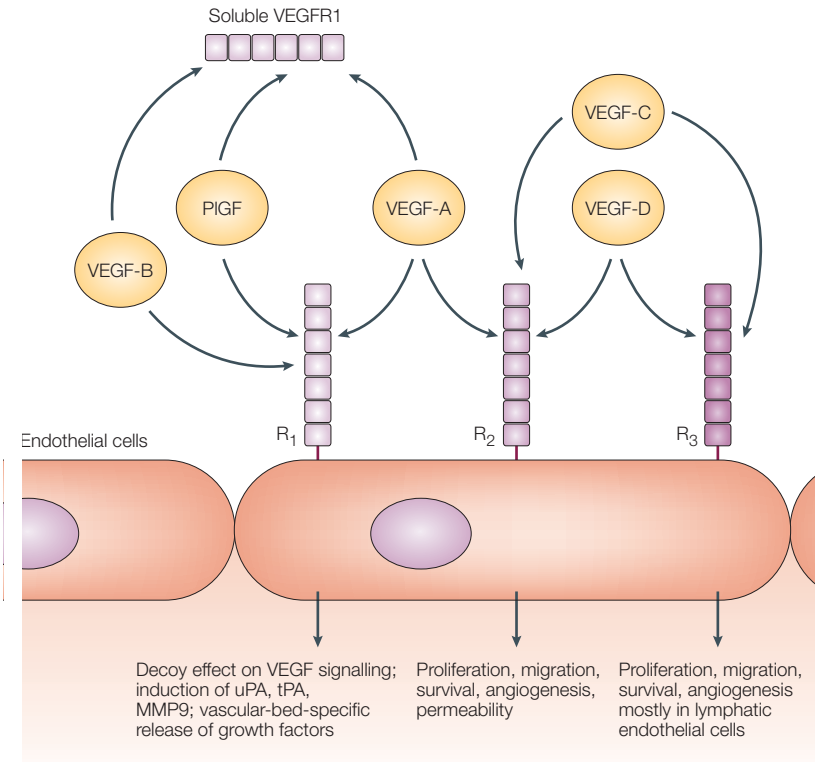


Figure 1 | **Role of the VEGF receptor tyrosine kinases in endothelial cells.** VEGFR1 (shown as R_1) and VEGFR2 (shown as R_2) are expressed on the surface of blood endothelial cells. By contrast, VEGFR3 (shown as R_3) is largely restricted to lymphatic endothelial cells. VEGF-A binds to both VEGFR1 and VEGFR2, whereas PlGF and VEGF-B interact only with VEGFR1. VEGF-C and VEGF-D bind VEGFR3, but following proteolytic processing they might bind VEGFR2. There is evidence that VEGFR2 is the major mediator of endothelial cell mitogenesis, survival and microvascular permeability. By contrast, VEGFR1 does not mediate an effective mitogenic signal in endothelial cells and it might, especially during early embryonic development, perform an inhibitory role by sequestering VEGF and preventing its interaction with VEGFR2. VEGFR1, however, regulates the expression of a variety of genes in the endothelium, such as MMP9 and certain growth factors. MMP, matrix metalloproteinase; PlGF, placenta growth factor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

implicated in mitogenesis and angiogenesis. Under some circumstances, it might function as a 'decoy' receptor that sequesters VEGF and prevents its interaction with VEGFR2[51]. However, growing evidence supports the idea that VEGFR1 has important roles in haematopoiesis[52,53]; in the recruitment of monocytes and other bone marrow-derived cells that might also be incorporated in the tumour vasculature[54,55]; in the induction of matrix metalloproteinases[56]; and in the paracrine release of growth factors from endothelial cells[57]. These activities of VEGFR1 can be important in various pathophysiological situations, including tumour growth and metastasis and inflammation, such that blockade of VEGFR2 alone might be insufficient to achieve a maximal therapeutic benefit in such conditions[58].

Neuropilin 1 (NP1), a molecule that had been previously shown to bind the collapsin/semaphorin family and implicated in neuronal guidance, is also a receptor for the heparin-binding isoforms of VEGF[59]. NP1 seems to present $VEGF_{165}$ to VEGFR2 in a manner that potentiates VEGFR2 signalling[59].

Regulation of VEGF gene expression

Oxygen tension has a key role in regulating the expression of a variety of genes, including *VEGF*[60]. VEGF mRNA expression is induced by exposure to low pO_2 in a variety of pathophysiological circumstances, and it is now well established that hypoxia-inducible factor 1 (HIF1) is a major mediator of hypoxic responses[61]. A link between the product of the von Hippel-Lindau (VHL) tumour-suppressor gene and HIF1-dependent responses has been established (for a review, see REF. 62). The gene encoding VHL is inactivated in patients with von Hippel-Lindau disease, an autosomal dominant neoplasia syndrome characterized by capillary haemangioblastomas in retina and cerebellum, and in most sporadic clear-cell renal carcinomas[63]. Earlier studies indicated that a function of the VHL protein is to provide negative regulation of VEGF and other hypoxia-inducible genes[64]. HIF1 is constitutively activated in VHL-deficient renal-cell carcinoma cell lines[65]. One of the functions of VHL is to be part of a ubiquitin ligase complex that targets HIF subunits for proteasomal degradation following covalent attachment of a poly-ubiquitin chain[66,67]. Oxygen promotes the hydroxylation of HIF at a proline residue, a requirement for the association with VHL[66,67]. Recently, a family of prolyl hydroxylases related to the *Egl-9* gene product from *Caenorhabditis elegans* were identified as HIF prolyl hydroxylases[60,68].

Oncogenic mutations or amplification of *RAS* lead to VEGF upregulation[69–71]. Mutations in the WNT signalling pathway that are frequently associated with pre-malignant colonic adenomas result in upregulation of VEGF[72]. Interestingly, VEGF is upregulated in polyps of Apc knockout (Apc(Delta716)) mice, a model for human familial adenomatous polyposis[73]. In both benign and malignant mouse intestinal tumours, stromal expression of cyclooxygenase-2 (COX2) results in elevated prostaglandin E_2 (PGE_2) levels, which in turn stimulate expression of the cell-surface prostaglandin receptor EP_2, followed by induction of VEGF and angiogenesis[73–75].

VEGF as a regulator of physiological angiogenesis

VEGF is essential for normal embryonic vasculogenesis and angiogenesis. Inactivation of a single VEGF allele in mice results in embryonic lethality[76,77]. VEGF inhibition during the early neonatal period results in growth arrest, endothelial-cell apoptosis and lethality, due primarily to kidney failure[78,79]. VEGF is also essential for endochondral bone formation, a fundamental mechanism for longitudinal bone growth. VEGF inhibitors suppress this process in rodents and in primates[80,81]. Importantly, this effect is fully reversible on interruption of the anti-VEGF treatment[80,81]. Angiogenesis is a key aspect of normal cyclical ovarian and endometrial function[82]. VEGF mRNA expression is temporally and spatially related to the proliferation of blood vessels in the ovary of numerous species[83,84]. Administration of VEGF inhibitors delays follicular development[85] and suppresses luteal angiogenesis in rodents[86] and in primates[81,87,88].

Role of VEGF in tumour angiogenesis

VEGF expression in human tumours. *In situ* hybridization studies have demonstrated VEGF mRNA expression in many human tumours. These include lung[89], breast[90], gastrointestinal tract[91], renal[92] and ovarian carcinomas[93]. However, the expression of VEGF seems to be variable, not only among different tumour types, but also within the same tumour. In glioblastoma multiforme and other tumours with significant necrosis, the expression of VEGF mRNA is highest in hypoxic tumour cells adjacent to necrotic areas[94,95]. A tumour type with particularly high VEGF expression is renal cell carcinoma. Interestingly, inactivating VHL mutations occur in ~50% of renal carcinomas[63]. However, as already pointed out, VEGF upregulation in tumours is not only linked to hypoxia and VHL mutations; a number of transforming events (for example, *RAS* mutations) might also result in increased VEGF expression.

VEGF neutralization in animal models of cancer. In 1993, our laboratory reported that a mouse anti-human VEGF monoclonal antibody called A.4.6.1 exerted a potent inhibitory effect on the growth of several tumour cell lines in nude mice, whereas the antibody had no effect on the tumour cells *in vitro*[96]. The cell lines tested were the A673 rhabdomyosarcoma, the G55 glioblastoma and the SK-LMS-1 leyomiosarcoma. The growth inhibition ranged between 70% and >90%[96]. These findings represented the first direct evidence in support of the hypothesis that tumour growth was angiogenesis-dependent. Subsequently, many other tumour cell lines were shown to be inhibited *in vivo* by the same anti-VEGF monoclonal antibody[97–101].

Tumour growth inhibition has since been demonstrated by numerous laboratories using many other anti-VEGF approaches. These include a retrovirus-delivered DOMINANT-NEGATIVE VEGFR2 mutant[102], small-molecule inhibitors of VEGFR2 signalling[103–105], antisense oligonucleotides targeting VEGF[106,107], anti-VEGFR2 antibodies[108] and soluble VEGF receptors[109–111].

Tumour cells usually represent the main source of VEGF, but tumour-associated stroma is also an important site of VEGF production, possibly in a tumour-type-dependent fashion[109,112,113]. The growth of a variety of human tumour cell lines in nude mice is substantially reduced, but not completely suppressed, by anti-human VEGF monoclonal antibodies[96]. Administration of a chimeric soluble VEGF receptor (mFlt(1-3)-immunoglobulin G (IgG)), which effectively binds both human and mouse VEGF, results in nearly complete suppression of tumour growth, accompanied by dramatic tumour-cell necrosis[109]. Similar results were obtained using a variant soluble receptor referred to as VEGF-trap[111]. Therefore, inhibitors that target both human and mouse VEGF are expected to show greater efficacy in a hybrid system, such as human tumour XENOGRAFTS in nude mice, relative to inhibitors that only block human VEGF[109].

Cre-*LoxP*-mediated gene targeting has shown that VEGF inactivation suppresses tumour angiogenesis in a genetic model of insulinoma[114]. Furthermore, at least in this model, MMP9-mediated proteolytic events determine an angiogenic switch, mediated by enhancement of the activity of low, constitutive levels of VEGF that become available to bind VEGFR2[34,115].

Several studies have shown that combining anti-VEGF treatment with chemotherapy[116] or radiation therapy[117,118] results in greater antitumour effects than either treatment alone. An issue that is now being debated is the mechanism of such potentiation, and a variety of hypotheses — which are not mutually exclusive — have been put forward. Klement *et al.* proposed that chemotherapy, especially when delivered at low-dose, preferentially damages endothelial cells and the blockade of VEGF blunts a key survival signal for endothelial cells, thereby amplifying the antitumour-cell effects of chemotherapy[116]. Jain proposed that anti-angiogenic therapy 'normalizes' the tumour vasculature, leading to pruning of excessive endothelial cells and perivascular cells, reduction in vessel TORTUOSITY, and drop in interstitial pressure and consequent improved oxygenation and delivery of chemotherapy to tumour cells[119]. These effects are accompanied by a reduction in permeability of macromolecules[41,120]. Most recently, Willett *et al.* have shown that VEGF blockade with bevacizumab decreases tumour perfusion, vascular volume, microvascular density, interstitial fluid pressure and the number of viable circulating endothelial and progenitor cells in colorectal cancer patients[121]. Surprisingly, these studies have also shown that permeability to small molecules actually increases following VEGF blockade[121].

Development of bevacizumab

Binding characteristics. In 1997, we reported the HUMANIZATION of the mouse anti-VEGF Mab A.4.6.1[122]. By site-directed mutagenesis of a human antibody framework, the residues involved in the six complementarity-determining regions, and also several framework residues, were changed to murine counterparts. The humanized anti-VEGF monoclonal antibody (rhuMab VEGF; bevacizumab; Avastin) bound VEGF with affinity very similar to that of the original antibody (K_d ~0.5 nM). In common with its mouse counterpart, bevacizumab binds to and neutralizes all human VEGF-A isoforms and bioactive proteolytic fragments. The binding epitope of bevacizumab has been defined by crystal structure analysis of a Fab–ligand complex[123]. This analysis predicts that Gly88 in human VEGF is essential for binding bevacizumab and this residue also underlies the species specificity of bevacizumab binding, since a serine residue is found in mouse and rat VEGF at the corresponding position. Bevacizumab does not neutralize other members of the VEGF gene family, such as VEGF-B or VEGF-C. The pharmacokinetic properties of bevacizumab in several species have been previously described and are consistent with a typical humanized monoclonal antibody[124]. The terminal half-life of bevacizumab in humans is 17–21 days. Importantly, no evidence of antibody response to bevacizumab has been found in any clinical trials so far performed, verifying the success of the humanization.

DOMINANT-NEGATIVE
A defective protein that retains interaction capabilities and so distorts or competes with normal proteins, inhibiting their function.

XENOGRAFTS
Tumour specimens can be grown in immunocompromised rodents to provide tumour models with many of the complexities of human tumours.

HUMANIZATION
A technique used to circumvent the immunogenicity of murine monoclonal antibodies for human therapy. In the simplest case, the complementary determining regions of a mouse monoclonal antibody are transferred to a human antibody that therefore acquires the binding characteristics of the original murine antibody. The amino-acid sequence of the humanized antibody is 93–95% human.

TORTUOSITY
The characteristic serpiginous appearance of newly formed and tumour-associated vessels. In tumour vessels, tortuosity is believed to be a hallmark of defective structural properties.

Table 1 | **Efficacy results of a Phase II study of bevacizumab in colorectal cancer***

Endpoint	Control group‡ (*n* = 36)	Bevacizumab group§ 5 mg per kg every two weeks (*n* = 35)	Bevacizumab group§ 10 mg per kg every two weeks (*n* = 33)
Median time to disease progression (months)	5.2	9.0	7.2
Objective response rate	6 (17%)	14 (40%)	8 (24%)
Median duration of survival (months)	13.6	17.7	15.2

*Data based on REF. 135. ‡The control group received 5-fluorouracil/leucovorin. §The treatment arms received 5-fluorouracil/leuvovorin plus bevacizumab at the doses of 5 or 10 mg per kg every two weeks (see text).

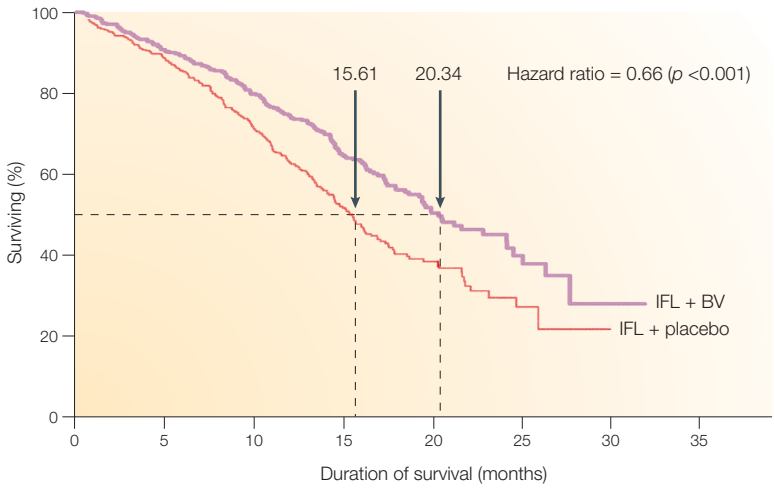
Inhibition of tumour growth in preclinical models: comparison with other protein VEGF inhibitors. Bevacizumab inhibits the growth of human tumour cell lines in nude mice, achieving a maximal inhibition at the dose of 1–2 mg per kg twice weekly[122]. Half-maximal inhibition required 0.1–0.5 mg per kg doses. As previously noted, the magnitude of the inhibition is inversely related to the content of stromal-derived mouse VEGF within the tumour xenograft. In tumours with high human/mouse VEGF ratio, the inhibition can exceed 90%[97,109]. DC101, an extensively used monoclonal antibody targeting mouse VEGFR2, required significantly higher doses than bevacizumab (20–40 mg per kg twice weekly) to achieve maximal tumour growth inhibition[108]. As previously mentioned, it is possible that blockade of VEGFR2 alone is insufficient for a full therapeutic effect, considering that important activities are mediated by VEGFR1[58].

Interestingly, the *in vivo* dose-response of bevacizumab also compares favourably with that of other VEGF inhibitors that have higher binding affinity for VEGF *in vitro*, such as soluble receptors. Several years ago, our laboratory described Flt(1-3)IgG, a Fc fusion with the first three Ig-like domains of VEGFR1[125]. The binding affinity of Flt(1-3)IgG is similar to that of native VEGFR1 for VEGF (K_d ~5–20 pM). This molecule effectively inhibits VEGF across species[80,86,109]. However, it required daily administrations of doses of 10–25 mg per kg to achieve maximal VEGF inhibition[109]. More recently, Holash *et al.* described a hybrid Fc construct in which domain 2 of VEGFR1 is joined to domain 3 of VEGFR2 (VEGF-trap)[111]. The binding specificity of the VEGF-trap is expected to be similar to that of Flt(1-3)IgG because domain 3 of VEGFR2 is insufficient to confer VEGFR2-specific binding[125,126]. This and other modifications resulted in increased half-life such that the VEGF-trap can be administered to mice twice weekly. However, like Flt(1-3)IgG, it requires doses of 10–25 mg per kg to induce a maximal effect[127,128]. It is possible that not only the longer half-life of bevacizumab, but also other variables, such as biodistribution, stability of binding and so on, can offset the affinity advantage of soluble receptors.

Safety evaluation. Safety evaluation studies of bevacizumab were conducted in *Macaca fascicularis* (cynomolgus monkey), a species in which bevacizumab is expected to be pharmacologically active, considering the complete identity between human and cynomolgus VEGF isoforms at the protein level[129]. Following administration of bevacizumab for four or thirteen weeks, young adult cynomolgus monkeys exhibited a physeal dysplasia characterized by a dose-related increase in hypertrophied chondrocytes and inhibition of vascular invasion of the growth plate, which is very similar to the growth-plate lesion observed in mice treated with Flt(1-3)-IgG[80]. Other expected effects of prolonged bevacizumab administration were suppression of angiogenesis in the female reproductive tract, resulting in decreased ovarian and uterine weights, and an absence of corpora lutea. Both the growth-plate and ovarian changes were reversible with cessation of treatment. Importantly, no other treatment-related effects were observed following bevacizumab administration at doses up to 50 mg per kg[81].

Clinical trials. In January 1997, Genentech filed an Investigational New Drug Application (IND) for bevacizumab, and Phase I clinical trials were initiated in April 1997. These Phase I studies showed that bevacizumab as a single agent was relatively non-toxic and that adding bevacizumab to standard chemotherapy regimens did not significantly exacerbate chemotherapy-associated toxicities[130,131]. In 1998, five Phase II studies were initiated in different tumour types: single-agent bevacizumab was tested in hormone-refractory metastatic prostate cancer[132], relapsed metastatic breast cancer[133] and in renal-cell cancer that had progressed following therapy with interleukin-2 (IL-2)[134]. Bevacizumab was combined with standard first-line chemotherapy in metastatic colorectal cancer[135] and stage IIIb/IV non-small-cell lung cancer (NSCLC)[136]. The most encouraging efficacy results were seen when bevacizumab was combined with chemotherapy in colorectal cancer and NSCLC, and when used as a single agent in renal-cell cancer. To date, Phase III trials are either ongoing (in NSCLC and renal-cell cancer) or have been completed (in colorectal cancer) in these tumour types. The following are summaries of the clinical trial results so far in renal-cell and colorectal cancer, the most advanced of the bevacizumab programmes.

The Phase II trial in renal-cell cancer was sponsored by the National Cancer Institute (NCI) and was a randomized, double-blind, placebo-controlled trial of single-agent bevacizumab in subjects with metastatic renal-cell cancer that progressed following treatment with high-dose IL-2 (REF. 134). Bevacizumab was given at doses of 3 and 10 mg per kg every two weeks. With 116



Number of subjects at risk

IFL + BV	402	362	320	178	73	20	1	0
IFL + placebo	411	363	292	139	51	12	0	0

Figure 2 | **Duration of survival in a Phase III study of previously untreated patients with metastatic colorectal cancer in two arms of the study*.** IFL + BV = irinotecan, 5-fluorouracil/leucovorin + bevacizumab; IFL + placebo = irinotecan, 5-fluorouracil/leucovorin + placebo. The design of the study is described in the text. *Data based on REF. 138.

patients randomly assigned to treatment groups (40 to placebo, 37 to low-dose antibody and 39 to high-dose antibody), there was a significant prolongation of the time to progression of disease in the high-dose antibody group as compared with the placebo group (hazard ratio, 2.55; $p < 0.001$, median increased from 2.5 to 4.8 months). Minimal toxic effects were seen, with hypertension and asymptomatic proteinuria predominating.

The Phase II colorectal cancer trial was a randomized, open-label study to evaluate the efficacy and safety of bevacizumab combined with 5-fluorouracil (FU)/leucovorin (LV) chemotherapy in subjects with previously untreated metastatic colorectal cancer[135]. A total of 104 subjects were randomized to the three treatment arms: 36 subjects to the 5-FU/LV-alone arm, 35 subjects to 5-FU/LV + 5 mg per kg bevacizumab every two weeks, and 33 subjects to 5-FU/LV + 10 mg per kg bevacizumab every two weeks. Venous thromboembolism was the most significant adverse event; hypertension, proteinuria and epistaxis were other potential safety concerns. The efficacy results for this trial are shown in TABLE 1. Interestingly, the lower-dose bevacizumab group (5 mg per kg every two weeks) fared better than the higher dose (10 mg per kg every two weeks) arm. This finding is at variance with the renal-cell cancer trial, which showed a dose-responsive enhancement of efficacy[134]. The reasons for this difference are not clear and might reflect some imbalances in randomization that resulted in more patients with poor prognostic factors in the high-dose arm[135]. Alternatively, the lower dose of bevacizumab might have resulted in a better 'normalization' of the tumour vasculature, with improved delivery of chemotherapy to the tumour cells, whereas the higher dose could have led to more advanced regression of blood vessels[119].

During the conduct of this Phase II trial, the addition of irinotecan to bolus 5-FU/LV (known as the IFL regimen) was shown to prolong survival and was therefore considered to be the new standard first-line treatment for metastatic colorectal cancer in the United States[137]. For this reason, the control chemotherapy in the Phase III study was chosen to be the IFL regimen. The Phase III colorectal cancer trial was a large, randomized, double-blind, active-controlled, three-arm study to evaluate the efficacy and safety of bevacizumab in combination with bolus-IFL chemotherapy or 5-FU/LV chemotherapy as first-line therapy for previously untreated metastatic colorectal cancer[138]. A total of 923 subjects were randomized into the three treatment arms in this study: 411 subjects in arm 1 (bolus-IFL + placebo), 402 subjects in arm 2 (bolus-IFL + bevacizumab) and 110 subjects in arm 3 (5-FU/LV + bevacizumab). Enrollment in the third arm was discontinued early, as per protocol, when the data-monitoring committee assessed that the safety profile of IFL + bevacizumab was acceptable. On the basis of the Phase II results[135], bevacizumab was administered at the dose of 5 mg per kg once every two weeks. The addition of bevacizumab to bolus-IFL resulted in a significant increase in overall survival, with a 34% reduction in the hazard of death ($p = 0.00004$, see FIG. 2). Median survival was increased from 15.6 months in the bolus-IFL + placebo arm to 20.3 months in the bolus-IFL + bevacizumab arm (FIG. 2). Similar increases were seen in progression-free survival (6.2 versus 10.6 months, $p < 0.00001$), response rate (34.8% versus 44.8%, $p = 0.0036$) and duration of response (7.1 versus 10.4 months). The clinical benefit of bevacizumab, as measured by survival, progression-free survival and objective response, was seen in all pre-specified subject subgroups, including those defined by age, sex, performance status, location of primary tumour, number of organs involved and duration of metastatic disease.

Smaller, open-label Phase I and II clinical trials had identified a number of adverse events, including thrombosis, bleeding, proteinuria and hypertension as potential bevacizumab-related toxicities. Unexpectedly, the rates of several of these adverse events — thrombosis, major bleeding and proteinuria — were not significantly higher among subjects receiving bolus-IFL + bevacizumab compared with those receiving bolus-IFL alone[138]. Two expected bevacizumab-related toxicities were increased in the IFL + bevacizumab arm: hypertension and epistaxis. The episodes of epistaxis were short-lived and did not require medical attention. The incidence of Grade 3 hypertension (requiring treatment with oral antihypertensive medications) increased from 2.3% in the IFL group to 11.0% in the IFL + bevacizumab group, but was easily managed by standard medication. There were no cases of Grade 4 hypertension (hypertensive encephalopathy) in this study, and there is evidence that hypertension resolves on discontinuation of bevacizumab. One new potential bevacizumab-related toxicity seen in this trial was gastrointestinal perforation. These events were uncommon and had variable clinical presentations, ranging from a

Table 2 | **VEGF inhibitors in cancer clinical trials**

Agent	Description	Company	Development status
Bevacizumab (Avastin)	Humanized monoclonal antibody (VEGF-A)	Genentech	FDA approved
PTK787	RTK inhibitor (VEGFR1, VEGFR2)	Novartis	Phase III
Bay 43-9006	RAF kinase inhibitor (also several RTKs)	Bayer/Onyx	Phase III
SU11248	RTK inhibitor (several RTKs)	Pfizer	Phase I/II
AG 013676	RTK inhibitor (several RTKs)	Pfizer	Phase II
ZD6474	RTK inhibitor (VEGFR-1, VEGFR-2)	AstraZeneca	Phase II
VEGF-trap	Soluble receptor	Regeneron	Phase I/II
Anti-VEGFR2	Monoclonal antibody	ImClone	Phase I

RTK, receptor tyrosine kinase; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

perforated stomach ulcer to bowel obstruction associated with colonic perforation. Additional studies are required to establish whether such toxicity is truly related to bevacizumab.

Other VEGF inhibitors in clinical trials

Besides bevacizumab, many other VEGF inhibitors are being clinically pursued. TABLE 2 lists some of the most clinically advanced molecules. A variety of small-molecule RTK inhibitors targeting the VEGF receptors are being developed. The most advanced is PTK787, an orally available molecule developed by Novartis/Schering[104] that is in Phase III trials for colorectal cancer. The success of imatinib mesylate (Glivec/Gleevec; Novartis) has validated the idea that it is possible to develop clinically useful small-molecule RTK inhibitors[139]. However, the path towards the development of an effective VEGF RTK inhibitor has not been straightforward, and it has been difficult to strike a balance between efficacy, pharmacokinetic characteristics and safety. A first-generation molecule, SU5416, failed to demonstrate efficacy in a controlled Phase III trial in colorectal cancer patients in combination with chemotherapy, in spite of earlier encouraging results[140]. Inhibitors targeting several RTKs are being also developed, such as SU11248 and AG 013676, which inhibit VEGFRs, PDGFR, c-kit and Flt-3[141]. Although these molecules have shown significant efficacy in preclinical models and even evidence of activity in early trials, it remains to be established whether their toxicity profile will be acceptable. In the case of SU11248, the observed toxicity (fatigue, nausea, vomiting, diarrhoea, hair depigmentation and so on) prevented continuous drug administration and dosing holidays were required. It is unclear whether the toxicity is partially or completely mechanism-based. Interestingly, Bay 43-9006, which was initially identified as a RAF kinase inhibitor[142], has subsequently been shown to inhibit several RTKs, including VEGFRs, and at least some of its anticancer activity might be due to this property. This molecule is now in Phase III trials for metastatic renal-cell carcinoma.

An alternative anti-VEGF approach at early-stage clinical development is represented by the VEGF-trap[111] (see previous discussion). It has been proposed that fusions between the constant region of IgG and the extracellular domains of two distinct receptor components represent an advantage over antibodies because they can result in higher binding affinity[143]. This concept, however, remains to be validated. It is also possible that the junctions between the various structural elements in such multi-component molecules can generate an immune response.

An additional VEGF inhibitor is pegaptanib sodium (Macugen; Eyetech/Pfizer), an APTAMER that recognizes the heparin-binding domain of VEGF and inhibits the activity of intact $VEGF_{165}$, but not $VEGF_{121}$ or proteolytic fragments of VEGF[144]. Pegaptanib sodium has been developed for intraocular administration. Preliminary results of a Phase III study with pegaptanib sodium in patients with wet age-related macular degeneration (AMD) indicate reduced vision loss compared with placebo[145]. In addition, ranibizumab (Lucentis; Genentech/ Novartis), a high-affinity Fab variant of bevacizumab[146], is in Phase III trials for the treatment of wet AMD.

Conclusions and perspectives

For the first-line treatment of metastatic colorectal cancer, the addition of bevacizumab to bolus-IFL chemotherapy conferred a clinically meaningful and statistically significant benefit for all study endpoints, including overall survival, progression-free survival and response rate, and was associated with an acceptable side-effect profile. Importantly, a series of potential safety signals identified in small open-label trials did not materialize in placebo-controlled Phase II[134] or Phase III[138] studies. The improvement in survival attributable to bevacizumab is similar or greater than that observed in any Phase III trial for the treatment of colorectal cancer[147]. These positive results occurred in spite of the limited availability of a new active agent in colorectal cancer, oxaliplatin, during the conduct of this trial. In conclusion, bevacizumab, used in combination with intravenous 5-FU-based chemotherapy, should be considered a new standard option for the treatment of first-line metastatic colorectal cancer. On 26 February 2004, bevacizumab was approved by the FDA as a first-line treatment for metastatic colorectal cancer.

The role of bevacizumab in other tumour types and settings is currently under investigation, including ongoing Phase III clinical trials in NSCLC, renal-cell cancer, metastatic breast cancer and colorectal cancer that has progressed following first-line chemotherapy. A Phase III trial involving patients with advanced, heavily pretreated metastatic breast cancer showed that adding bevacizumab to capecitabine chemotherapy did not improve progression-free survival[148]. Encouraging Phase II trial results have been seen using bevacizumab in combination with chemotherapy in pancreatic cancer[149] and NSCLC[136], with interferon-α in melanoma[150], with an EGFR antagonist in NSCLC[151] and renal cell cancer (J. Haisworth, unpublished data); with radiotherapy in rectal cancer[121] and as a single agent in ovarian cancer (R. Burger, unpublished data).

APTAMER
Oligonucleotide ligand selected *in vitro* to bind specific proteins.

As mentioned above, bevacizumab did not result in a survival benefit in patients with refractory metastatic breast cancer when added to capecitabine, as a third-line therapy[148]. It is unclear at present whether such differences in response to bevacizumab reflect a different biology/angiogenic profile between breast and colorectal cancer or simply a reduced response in more advanced disease. Also, progression eventually occurs in many colorectal cancer patients, raising the question of what might mediate angiogenic escape after VEGF inhibition, although the possibility that a different dosage/regimen of bevacizumab might achieve even greater efficacy cannot be ruled out. Different angiogenic mechanisms might be differentially important at various stages of neoplastic progression, and some evidence suggests that VEGF might be especially important in the initial stages[152].

Recent studies have suggested that pericyte recruitment by the tumour vasculature, a process primarily dependent on PDGFR-β signalling, is a mechanism of resistance in late-stage tumours to therapies that only target VEGF[153]. These findings suggest that combination therapies that target both VEGF and PDGF might be promising.

Reliable markers that can predict which patients are more likely to respond to anti-VEGF therapy would be of utmost importance, but so far such markers have been rather elusive. However, recent studies suggest that dynamic contrast-enhanced MRI assessing acute or short-term changes in tumour permeability and vascularity following administration of PTK787 in Phase I studies in patients with metastatic colorectal cancer might represent a biomarker[154]. It remains to be established, however, whether such changes predict any long-term clinical benefit or survival, and therefore whether this marker is useful for identifying patients that will respond to the treatment. Interestingly, Willett *et al.* have shown that administration of bevacizumab reduces the number of circulating endothelial cells and increases the fraction of tumour endothelial cells with pericyte coverage (reflecting dropout of immature endothelial cells) in colorectal cancer patients, potentially providing novel biomarkers[121]. In this context, it is also interesting to point out that the complexity of the actions of VEGF (mitogenesis, angiogenesis, endothelial survival, induction of metalloproteinases and growth factors, regulation of permeability/flow, recruitment of endothelial progenitor cells and so on) raises the question of which effects have the greatest therapeutic relevance to the clinical efficacy of bevacizumab. It is conceivable that the relative contribution of such activities might vary depending on the stage of the malignancy (early versus advanced) or the tumour type, and also on whether bevacizumab is administered as a single agent or in combination with chemotherapy. In the case of monotherapy (for example, in renal-cell carcinoma), mitogenesis/angiogenesis, as well as endothelial-cell survival, are likely to be dominant. By contrast, the potentiation of chemotherapy by bevacizumab is likely to rely on sensitizing the endothelium to cytotoxic agents[116] or on improving delivery of chemotherapy to tumour cells following pruning and remodelling of the tumour vasculature[119].

The potential clinical utility of VEGF inhibition in oncology is not limited to solid tumours. There is growing evidence that VEGF and VEGF receptors are expressed by a variety of leukaemias and other haematological malignancies, indicating that inhibition of VEGF or VEGFR signalling might have a role in the treatment of such conditions[155]. At present, several clinical trials are testing these hypotheses.

As already mentioned, trials in patients with AMD are already at the Phase III stage, using a Fab variant of bevacizumab. In addition, the potential use of bevacizumab in several other non-oncology indications, such as rheumatoid arthritis, psoriasis, endometriosis and cerebral oedema, deserves consideration.

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Competing interests statement
The authors declare competing financial interests: see Web version for details.

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