Monoclonal antibody strategies to block angiogenesis

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Antibodies represent a unique class of therapeutics because of their high specificity towards a defined target antigen. Recent clinical success with antibody-based cancer therapeutics has led to an increase in the clinical development of these agents. Antibody therapeutics offer a promising approach for inhibiting new blood vessel formation (angiogenesis), which is associated with a variety of diseases, including cancer. In this review we will focus on angiogenesis-related mechanisms targeted by antibody-based therapeutics, with an emphasis on those studies where pre-clinical *in vivo* data is available.

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▼ Vasculogenesis is the assembly of the first blood vessels in an embryo from endothelial precursors. All subsequent new blood vessels, including those required for wound healing or tumor growth, form by a process called angiogenesis[1]. Many attempts have been made to find and develop effective angiogenesis inhibitors for therapeutic purposes. Although many of the known angiogenesis inhibitors have been found serendipitously, their underlying mechanism(s) of action are often poorly understood.

Our understanding of the complex series of events that collectively are referred to as angiogenesis has improved dramatically over the past decade. These include the migration of endothelial stem cells, the migration and invasion of endothelial cells, the proliferation of endothelial cells, the organization of endothelial cells into tubular structures, the formation of circulatory systems (anastomosis), the maturation of vessels, and vessel regression. New information about how each of these steps participate along the angiogenesis pathway, and the molecules responsible for these events, has led to a variety of novel, and increasingly mechanism-based, approaches for the development of angiogenesis inhibitors.

Among the many potential mechanism-based approaches are those that interfere with endothelial cell growth and adhesion. Several strategies have been devised to block these processes with reasonable specificity. Additional mechanisms, for example, the inhibition of metalloproteinase activity, which ostensibly blocks invasion and migration of endothelial cells, and the inhibition of intracellular signaling pathways to interfere with cell growth and survival, have also been targeted. However, these approaches are not necessarily endothelial-cell specific. The development of various angiogenesis inhibitors as therapeutics for disease has led to several clinical trials (Table 1). Of the trials listed in Table 1, three are for monoclonal antibodies (mAb). Kohler and Milstein first described the generation of mAb 25 years ago. Initially there was a great deal of optimism for the potential of mAb in the clinic. First, in 1982, a complete clinical response of a B-cell lymphoma patient treated with a monoclonal anti-idiotype antibody was reported[2]. This was followed by the launch of several antibody-based companies and the FDA approval of OKT3 for the treatment of acute allograft rejection. However, this early enthusiasm was short-lived after a series of unsuccessful clinical trials and problems with the immunogenicity of the antibodies, toxicity and high production costs. Nevertheless, during the past five years mAb have made a comeback, led in part by newly developed molecular antibody engineering methods to produce chimeric, humanized and fully human mAb. Since 1994, the FDA has approved eight therapeutic mAb, with over 70 mAb currently in clinical trials[3].

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Table 1. Angiogenesis inhibitors in clinical trials for cancer

Trial	Mechanism
Phase I	
Vitaxin	Integrin antagonist
IMC-1C11	Monoclonal antibody to VEGFR2
EMD121974	Small-molecule integrin antagonist
Endostatin	Induces endothelial cell apoptosis *in vivo*
BMS275291	Synthetic MMP inhibitor
SU6668	TK-inhibitor: blocks VEGFR, FGFR, and PDGFR
Phase II	
CAI	Inhibitor of calcium influx
Squalamine	Inhibits Na^{2+}/H^{+} exchanger
COL-3	Synthetic MMP inhibitor; tetracycline derivative
Interleukin-12	Induces interferon
Anti-VEGF Ab	Monoclonal antibody to VEGF
FTK787	TK-inhibitor: blocks VEGFR2
Phase III	
SU5416	TK-inhibitor: blocks VEGFR
Thalidomide	Unknown
Marimastat	Synthetic MMP inhibitor
Neovastat	Natural MMP inhibitor and VEGF antagonist
Interferon-α	Inhibition of bFGF and VEGF production
IM862	Unknown

Abbreviations: Ab, antibody; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; MMP, matrix metalloprotease; FGFR, fibroblast growth factor receptor; PDGFR, platelet-derived growth factor receptor; TK, tyrosine kinase; bFGF, basic fibroblast growth factor.

This article will review known efforts to develop mAb that inhibit angiogenesis and related diseases, with an emphasis on tumor angiogenesis. We exclude from this discussion studies that focus on polyclonal antibodies, diagnostic antibodies, and mAb that have not yet been shown to be effective *in vivo*.

Targeting the mechanisms that regulate endothelial cell growth

Many growth factors, cytokines and other regulatory proteins have been identified that stimulate endothelial cell growth either directly or indirectly by interfering with endothelial cell growth mechanisms (Fig. 1). Among these, basic fibroblast growth factor (bFGF, FGF-2) and vascular endothelial growth factor (VEGF) have received by far the most attention, mainly because of high efficacy (FGF-2), exquisite specificity (VEGF) and an apparent central regulatory role in angiogenesis (VEGF). Consequently, considerable effort has been invested in generating and testing inhibitory mAb against these growth factors and/or their receptors. This section of the review will focus on these antibodies.

The VEGF pathway

VEGF is a key regulator of vasculogenesis during embryonic development and angiogenic processes during adult life such as wound healing, diabetic retinopathy, rheumatoid arthritis, psoriasis, inflammatory disorders, tumor growth and metastasis[4]. VEGF is a strong inducer of vascular permeability, stimulator of endothelial cell migration and proliferation, and is an important survival factor for newly formed blood vessels. VEGF binds to and mediates its activity mainly via two tyrosine-kinase receptors, VEGFR-1-(flt1) and VEGFR-2 (KDR in humans and flk1 in mice). Targeted deletion of genes encoding VEGF, VEGFR-1 or VEGFR-2 in mice is lethal to the embryo, demonstrating the physiological importance of the VEGF pathway in blood vessel formation. Mice lacking even a single VEGF allele die prior to birth from vascular abnormalities. VEGFR-1-null embryos fail to develop normal vasculature because of the defective formation of vascular tubes. Interestingly, inactivation of VEGFR-1 by truncation of the tyrosine-kinase domain does not impair embryonic angiogenesis, suggesting that signaling via the VEGFR-1 receptor is not important for development of the vasculature in the embryo[5]. VEGFR-2-deficient mice have impaired blood-island (origin of first hoemopoietic cells in an embryo) formation and lack mature endothelial cells.

Several growth factors related to VEGF have now been identified: VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PlGF)[6]. VEGF-B and PlGF bind exclusively to VEGFR-1, VEGF-E is specific for VEGFR-2, and VEGF-C and VEGF-D can bind to VEGFR-2 and another receptor, VEGFR-3 (flt4), which is expressed predominantly on lymphatic endothelium. Additional distinct biological functions might be attributed to the complexity in binding specificity between various VEGF and receptor combinations. Furthermore, some VEGF ligands (i.e. VEGF and PlGF) and receptors (i.e. VEGFR-1 and VEGFR-2) might form homo- or hetero-dimers that bind differentially to various VEGF or VEGF-receptor family members and signal through different pathways[7]. The angiogenic processes that these VEGF and VEGF-receptor combinations control are yet to be determined.

Numerous studies have shown that overexpression of VEGF and VEGFR-2 is strongly associated with invasion and metastasis in human malignant disease[7]. VEGF is expressed at high levels in various types of human and mouse tumors and is strongly upregulated under hypoxic conditions such as those associated with rapidly growing tumors. The importance of VEGF and VEGFR-2 in tumor angiogenesis has been directly demonstrated in studies where VEGF or VEGFR-2 is inhibited through either antisense, antibody, soluble receptor or kinase inhibitors[8].

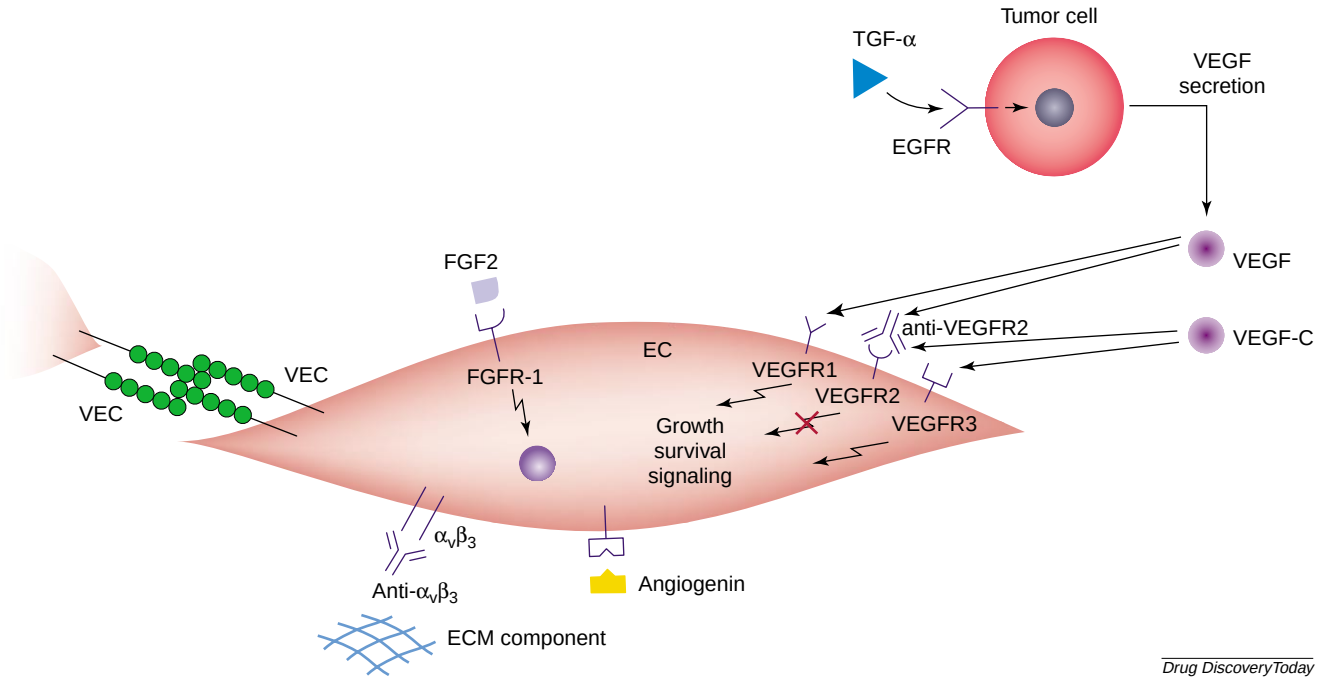

Figure 1. The interaction of cytokines, growth factors and other regulatory proteins in the process of tumor angiogenesis. This figure shows several points at which the multistep process of tumor angiogenesis could potentially be inhibited by monoclonal antibodies. Tumor cells express vascular endothelial growth factor (VEGF), which upregulates endothelial growth and migration by binding to several VEGF-receptor isoforms (VEGFR1, VEGFR2 and VEGFR3). Additional growth factors expressed by tumor cells such as transforming growth factor-α (TGF-α) and its receptor, the epidermal growth factor receptor, can regulate VEGF expression by tumor cells. Monoclonal antibodies raised to these growth factors or their receptors, therefore, provide a means of inhibiting tumor cell angiogenesis. Similarly, fibroblast growth factors (FGF) are potent endothelial cell mitogens that activate FGF-receptor isoforms (e.g. FGFR-1) and offer another target for monoclonal antibody therapy. Other approaches include using monoclonal antibodies to target the adhesins responsible for endothelial cell interactions, such as integrins (e.g. $\alpha_v\beta_3$) and VE-cadherin (VEC), in addition to secreted endothelial-cell proteins such as angiogenin. Abbreviations: TGF-α, transforming growth factor-α; EC, endothelial cell.

The importance of VEGF and its receptors in tumor angiogenesis suggests that blockade of this pathway by mAb therapy would be a useful therapeutic strategy for inhibiting angiogenesis and tumor growth. To this end, antibodies that neutralize VEGF, VEGFR-2 and, more recently, VEGFR-1 and VEGFR-3, have been developed and have shown potent anti-angiogenic and anti-tumor activities.

Anti-VEGF antibodies

In 1993, Kim and coworkers reported that an anti-human VEGF antibody, A4.6.1, efficiently inhibited the growth of three human xenograft tumors in nude mice models[9]. This finding provided the first evidence that inhibition of an endogenous angiogenic factor could result in suppression of tumor growth. Subsequently, tumor inhibition was observed in a variety of human xenograft tumors treated with the anti-VEGF antibody including carcinomas of colorectal[10], prostate[11], and ovarian[12] origin. Intravital videomicroscopy has provided further evidence that anti-VEGF antibody treatment not only resulted in reduction of tumor vascular permeability[13], but caused almost complete suppression of tumor angiogenesis[14].

A humanized version of A.4.6.1, rhuMab-VEGF (IgG1; Genentech, South San Francisco, CA, USA), inhibits VEGF-induced proliferation of endothelial cells *in vitro* and tumor growth *in vivo* with potency and efficacy similar to that of the parent murine antibody[15]. Toxicological studies in primates have shown that rhuMab-VEGF is safe even after prolonged treatment, and its effects are limited to inhibition of angiogenesis in the female reproductive tract and induction of growth-plate dysplasia in animals that have not completed statural growth[16]. The results of a Phase I trial in cancer patients showed that rhuMab-VEGF is safe and also suggested some biological activity of the antibody as a single agent therapy[a].

[a] Gordon, M.S. *et al.* Phase I trial of recombinant humanized monoclonal anti-vascular endothelial growth factor (anti-VEGF mAb) in patients (PTS) with metastatic cancer. *American Society of Clinical Oncology 34th Annual Meeting, 16–19 May , 1998, Los Angeles, CA, USA,* Abstract 809

Preliminary results from three Phase II clinical trials with rhuMab-VEGF antibody in cancer patients have been reported[b]. In the first open-label Phase II trial, 99 patients with advanced metastatic non-small-cell lung cancer were randomized into three arms receiving either carboplatin or paclitaxel alone, or the same doses of carboplatin or paclitaxel in combination with rhuMab-VEGF antibody at 7.5 mg kg^{-1} or 15 mg kg^{-1}. The response rates were 21.9% (7/32 patients) and 40% (14/35 patients) in the low-dose and high-dose antibody and chemotherapy combination arms, respectively, compared with a response rate of 31.3% (10/32 patients) in the chemotherapy alone arm. Time to disease progression was 3.9 and 7 months in the low-dose and high-dose antibody and chemotherapy combination arms, respectively, compared with 6 months after chemotherapy alone. In this trial, six patients treated with the rhuMab-VEGF antibody experienced sudden, serious pulmonary hemorrhage with fatal outcome in four patients. All patients that died had squamous cell carcinoma, which typically occurs in larger airways. Other adverse events seen more frequently in the combination arms included nosebleeds and hypertension.

In the second open-label Phase II trial, 104 patients with metastatic colorectal cancer were randomized into three arms receiving either 5-fluorouracil (5-FU) or leucovorin alone, or the same doses of 5-FU or leucovorin in combination with rhuMab-VEGF antibody at 5 mg kg^{-1} or 10 mg kg^{-1}. The response rates were 40% (14/35 patients) and 24% (8/33 patients) in the low-dose and high-dose antibody and chemotherapy combination arms, respectively, compared with a response rate of 17% (6/36 patients) in the chemotherapy alone arm. Time to disease progression was 9 and 7.2 months in the low-dose and high-dose antibody and chemotherapy combination arms, respectively, compared with 5.2 months after chemotherapy alone. Adverse events included frequent nosebleeds, and incidents associated with thrombosis and hypertension. It is unclear why patient response rates were significantly better in the low-dose rhuMab-VEGF arm of the study compared with the high-dose group. Additional clinical trials are needed to determine whether this observation is associated with the type of carcinoma (i.e. colon) or the chemotherapeutic drug (5-FU) used in this study[c].

In another open-label Phase II trial, a total of 59 breast cancer patients who had all relapsed following at least one chemotherapy treatment for metastatic disease, were given rhuMab-VEGF antibody at either 3 mg kg^{-1} or 10 mg kg^{-1}. Interim results showed that objective responses were seen in 5/59 patients (8.5%), including one partial response among 18 patients in the 3 mg kg^{-1} group, and one complete response plus three partial responses among 41 patients in the 10 mg kg^{-1} group[d].

A second anti-VEGF antibody, MV833 (Protein Design Labs, Maintain View, CA, USA), has also been shown to inhibit human xenograft tumor growth in animal models[17]. The humanized version of MV833, HuMV833 (IgG4), is currently in a Phase I clinical trial designed to establish its dose-limiting toxicity, maximum tolerated dose and optimum biological dose. Cohorts of three patients were treated with 0.3, 1, 3 and 10 mg kg^{-1} on days 1, 15, 22 and 29. Patient enrollment is actively ongoing in this trial[e].

Another anti-VEGF antibody, 2C3, an antibody that specifically blocks the interaction of VEGF with VEGF-R2 but not with VEGF-R1, has been shown to block VEGF-induced vascular permeability *in vivo*, and to inhibit the growth of human xenograft tumors in mouse models[18].

Apart from antibodies to VEGF, antibodies to other members of the VEGF family, such as VEGF-C and VEGF-D, are also being explored for therapeutic application. For example, an antibody to VEGF-D was shown to block the interaction of VEGF-D with both VEGF-R2 and VEGF-R3, and to neutralize the mitogenic effect of VEGF-D on human endothelial cells[19].

Anti-VEGF receptor antibodies

Anti-flk1 antibody Scientists at ImClone (New York, NY, USA) have developed anti-VEGFR-2 mAb generated against the extracellular domain of the receptor[7]. These mAb function as potent antagonists for VEGF binding, VEGFR-2 signaling and VEGF-induced endothelial cell growth *in vitro*. Early proof-of-concept studies were performed by developing a rat anti-mouse flk1 mAb, referred to as DC101 (Ref. 20). The neutralizing DC101 mAb has been studied

[b] DeVore, R.F. *et al.* A randomized Phase II trial comparing rhuMAb VEGF (recombinant humanized mAb to vascular endothelial cell growth factor) plus Carboplatin/Paclitaxel (CP) to CP alone in patients with stage IIIB/IV NSCLC. *American Society of Clinical Oncology 36th Annual Meeting, 20–23 May, 2000, New Orleans, LA, USA*, Abstract 1896

[c] Bergsland, E. *et al.* A randomized Phase II trial comparing rhuMAb VEGF (recombinant humanized mAb to vascular endothelial cell growth factor) plus 5-fluorouracil/leucovorin (FU/LV) to FU/LV alone in patients with metastatic colorectal cancer. *American Society of Clinical Oncology 36th Annual Meeting, 20–23 May, 2000, New Orleans, LA, USA*, Abstract 939

[d] Sledge, G. *et al.* A Phase II trial of single-agent rhuMAb VEGF (recombinant humanized mAb to vascular endothelial cell growth factor) in patients with relapsed metastatic breast cancer. *American Society of Clinical Oncology 36th Annual Meeting, 20–23 May, 2000, New Orleans, LA, USA*, Abstract 5C

[e] Jayson, G.C. *et al.* Anti-VEGF antibody HuMV833: An EORTC-biological treatment development group Phase I toxicity, pharmacokinetic and pharmacodynamic trial. *The 11th NCI-EORTC-AACR Symposium on New Drugs in Cancer Therapy, 7–10 November, 2000, Amsterdam, The Netherlands*, Abstract 270

extensively in mouse models of angiogenesis, mouse tumors and human tumor xenografts[7,21–23], demonstrating potent anti-angiogenic and anti-tumor activity in these models. In addition, DC101 treatment inhibited the dissemination and growth of metastases following removal of the primary tumor. Typically, treatment of human xenograft tumors with DC101 results in complete cessation of tumor growth. No relapse of tumors has been observed in several tumor models with continued administration of DC101 for >200 days. Withdrawal of DC101 treatment in the various models tested resulted in the regrowth of tumors with similar kinetics to that of control groups.

Histological examination of DC101-treated tumors showed evidence of decreased microvessel density, tumor cell apoptosis, decreased tumor cell proliferation and extensive tumor necrosis[22,24]. The reduction in microvessels occurred rapidly, that is, after 1–2 weeks of treatment with DC101, and reached a peak after 14–21 days of treatment. DC101 treatment inhibits endothelial cell proliferation and induces endothelial cell apoptosis, which leads to vessel regression. Approximately two weeks following DC101 treatment, areas of necrosis developed in tumors, which gradually increased as antibody treatment continued. The decrease in tumor cell proliferation and increase in necrosis in DC101-treated tumors probably reflects the lack of new vasculature that is needed to supply the rapidly growing tumor mass.

It should be noted that no toxicity was observed in long-term treatment experiments of tumor-bearing animals. Autopsy of DC101-treated mice revealed no abnormalities in the organs of these mice including the heart, intestine, kidney, liver, lung and spleen. These findings are important, because low levels of VEGFR-2 expression are present on the endothelium of some normal tissues and are required for normal angiogenic processes[4]. Indeed, DC101 treatment has an impact on normal angiogenesis that is associated with reproduction and bone formation. The lack of toxicity observed during DC101 therapy might be because of the limited dependence of resting endothelium on VEGFR-2 stimulation. By contrast, tumor angiogenesis is expected to be more dependent on the upregulation and function of VEGFR-2 and its effect on tumor vasculature, and is thus more susceptible to anti-VEGFR-2 blockade.

Combination therapy using anti-VEGF2 mAb and either chemotherapeutic drugs or radiation could be a useful strategy, because the use of these therapies alone is unable to completely eradicate tumors. In this regard, DC101 treatment has been shown to potentiate the effects of chemotherapeutic agents such as paclitaxel, cyclophosphamide and gemcitabine[25,f,g]. In addition, DC101 treatment combined with radiotherapy also showed an enhanced anti-tumor response[26]. Because VEGF has been shown to act as a survival factor for endothelial cells in response to radiation or anti-neoplastic drugs[27], anti-VEGFR-2 therapy might block this protective effect on proliferating tumor vasculature. Kerbel and colleagues have tested this hypothesis, where DC101 was combined with chronic, low-dose vinblastine treatment of human neuroblastoma xenografts in athymic mice[28]. Remarkably, this treatment regimen resulted in complete tumor regression of large, established tumors, which was sustained for more than six months. These data support the notion that anti-VEGFR-2 treatment potentiates the anti-vascular effects of low-dose chemotherapy on proliferating tumor endothelium.

Anti-KDR antibody The proof-of-principle studies with the DC101 antibody clearly demonstrate that blockade of VEGFR-2 is an attractive strategy for the treatment of cancer. Because DC101 does not cross-react with human VEGFR-2 KDR, a panel of mAb directed against KDR was produced, using both the traditional hybridoma method and the antibody phage-display technique[7]. This led to a lead candidate, IMC-1C11, which is a mouse–human chimeric IgG1 antibody derived from a single-chain Fv isolated from a mouse antibody phage-display library[29,30]. IMC-1C11 binds to both soluble and cell-surface expressed KDR with high affinity (K_d = ~300 pM), and competes with radiolabeled VEGF for binding to KDR-expressing human endothelial cells[30]. The binding epitope(s) for IMC-1C11 are located within the first three N-terminal extracellular Ig-like domains of the receptor, the same domains that encompass the binding site for VEGF[31]. IMC-1C11 strongly blocks VEGF-induced phosphorylation of both KDR and mitogen-activated protein kinase (MAPK) p42 and p44, and inhibits VEGF-stimulated mitogenesis of human endothelial cells[30]. Cross-species examination revealed that IMC-1C11 cross-reacts with VEGFR-2 expressed on the endothelial cells of monkeys and dogs, but not with that expressed in rat and mouse. In a canine retinopathy model, IMC-1C11 significantly inhibited retinal neovascularization induced by a high concentration of oxygen in newborn dogs (G. Lutty, unpublished data). Finally,

[f] Shrader, M. *et al.* Blockade of VEGF-receptor (flk-1) function +/– gemcitabine as therapy for primary pancreatic cancer and metastasis growing orthotopically in nude mice. *American Association for Cancer Research 91st Annual Meeting, 1–5 April, 2000, San Francisco, CA, USA,* Abstract 1952

[g] Huber, J. *et al.* (2000) Vascular endothelial growth factor receptor (VEGFR-2) antibody therapy combined with conventional chemotherapy inhibits growth of established tumors in mice. *American Association for Cancer Research 91st Annual Meeting, 1–5 April, 2000, San Francisco, CA, USA,* Abstract 3613

IMC-1C11 strongly inhibits proliferation and migration of certain types of human leukemia (including cell lines and primary leukemia cells), and significantly prolonged the survival of immunodeficient mice inoculated with human leukemic cells[32].

Immunohistochemistry staining of 37 normal human tissues revealed that IMC-1C11 only binds to selected endothelial cells of the placenta and the corpus luteum of the ovary and, occasionally, to endothelial cells of the mammary gland and skin. Toxicological studies in cynomolgus monkeys demonstrated that a twice-weekly intravenous bolus injection of IMC-1C11 at doses of 1, 3, or 10 mg kg^{-1} $dose^{-1}$ over 26 days for a total of eight doses, was well tolerated. There were no treatment-related clinical adverse signs, changes in body weight or ocular, hematological or clinical biochemical abnormalities. Further, no macroscopic or microscopic changes were observed in any of 44 organs or tissues examined from each animal[h]. Taken together, these results suggest that IMC-1C11 has potential clinical application in the treatment of cancer and other diseases where pathological angiogenesis is involved. IMC-1C11 is currently in Phase I clinical trials in patients with metastatic colorectal cancer (Albert LoBuglio, University of Alabama Medical Center, Birmingham, AL, USA).

Anti-VEGFR-1 antibody The role of VEGFR-1 in tumor angiogenesis is considerably less clear than that of VEGFR-2. A recent study demonstrated that ribozyme-specific targeting of VEGFR-1 in a mouse model resulted in the inhibition of angiogenesis, tumor growth and metastasis[33]. Interestingly, anti-VEGFR-2 ribozyme treatment in this study did not significantly inhibit the growth of lung metastases. Neutralizing antibodies to the human form of VEGFR-1 have been reported and were shown to inhibit receptor signaling and migration in response to VEGF stimulation[34]. However, the effect of anti-VEGFR-1 mAb on tumor angiogenesis has not been investigated, probably because of the lack of neutralizing antibodies against mouse VEGFR-1. A neutralizing anti-mouse VEGFR-1 mAb (MF1) was used to investigate whether blockade of VEGFR-1 would lead to inhibition[i] of angiogenesis and tumor growth in mouse models. *In vivo*, MF1 significantly inhibits neovascularization in a Matrigel™ plug assay and the mouse corneal-pocket assay (in which angiogenesis is induced on the cornea). In a human tumor A431 xenograft model, treatment with MF1 resulted in a significant inhibition of tumor angiogenesis and tumor growth. In contrast to treatment with DC101, tumor growth was not completely inhibited by treatment with the MF1 antibody. Histological examination of MF1-treated tumors showed decreased microvessel density and extensive tumor necrosis, similar to that found in studies with the DC101 antibody. However, the mechanism(s) whereby VEGFR-1 exerts its influence on tumor angiogenesis remain unresolved. Are there distinct mechanisms mediated by VEGFR-1 and VEGFR-2 in tumor angiogenesis? Does VEGFR-1 act through a homodimer or a VEGFR-1–VEGFR-2 heterodimer to promote tumor angiogenesis? Additional studies with neutralizing antibodies to VEGFR1 are underway to address these questions.

Anti-VEGFR-3 antibody VEGFR-3 has been identified as a receptor that is induced in all endothelial cells during early embryogenesis, whereas in normal adult tissues it appears to be expressed only by adult lymphatic endothelium and not by vascular endothelium[35,36]. However, recent studies have shown that VEGFR-3 is expressed on activated endothelium and, more specifically, is upregulated on tumor vasculature in mouse models[37]. A neutralizing antibody (AFL4), generated against the mouse form of VEGFR-3, was reported to inhibit angiogenesis and tumor growth in mouse models[37]. A prominent feature observed in AFL4-treated mice was micro-hemorrhage within the tumor tissue. Scanning electron microscopy of AFL4-treated tumor tissues revealed the disruption of the endothelial cell lining of postcapillary venules and collapse of proximal vessels. The stage at which blockade of VEGFR-3 inhibits tumor angiogenesis and whether it is different from blockade of VEGFR-1 or VEGFR-2 is still unclear. The absence of secondary and tertiary vascular branches in AFL4-treated mice suggests a role for VEGFR-3 in the remodeling process of tumor-induced angiogenesis. These findings suggest that VEGFR-3 might be another target in the VEGF/VEGF-receptor family for antibody-based therapy of cancer.

Anti-FGF-2 antibodies

Fibroblast growth factors (FGFs), in particular FGF-2, have long been recognized as highly potent endothelial-cell mitogens and angiogenesis-inducing agents. FGF-2 is produced by a wide variety of cell types and accumulates in the extracellular matrix of most tissues where it remains sequestered in an inactive form. FGF activity is thought to

[h] Zhu, Z. *et al*. Safety profile in primates and anti-angiogenic activity in murine and canine models of a chimeric antibody directed against the vascular endothelial growth factor receptor 2. *American Society of Clinical Oncology 36th Annual Meeting, 20–23 May, 2000, New Orleans, LA, USA*, Abstract 1793

[i] Wu, Y *et al*. Inhibition of tumor growth and angiogenesis in a mouse model by a neutralizing anti-Flt-1 monoclonal antibody. *American Society of Cancer Research 92nd Annual Meeting, 24–28 March, 2001, New Orleans, LA, USA*, Abstract 4436

be regulated by enzymes that degrade extracellular matrix components, thus liberating active growth factor. FGF-2 predominantly activates the FGF-receptor type 1 (FGFR1) present on endothelial cells and most other cell types, including most tumor cells, which commonly results in either increased cell proliferation, migration or survival. There is an abundance of evidence indicating that increased FGF activity is correlated with increased angiogenesis, both physiological and pathological, as well as tumor growth.

Given the evidence of FGF involvement in angiogenesis and tumor growth, blocking FGF activity with mAb has long been considered a potential therapeutic strategy and various investigators have reported *in vivo* results using anti-FGF-2 antibodies. A neutralizing mAb, GD2, has been shown in several animal models to inhibit angiogenesis induced by cytokines[38], FGF-2 (Ref. 39**)** or tumor tissue[40]. GD2 was also shown to inhibit chondrosarcoma growth in the rat[40]. Another antibody, 3H3, was reported to inhibit angiogenesis in gastrointestinal ulcers and to delay ulcer healing[41] as well as inhibiting the growth of tumors after implantation of tumorigenic transformed-cells into nude mice[42]. Other anti-bFGF antibodies were shown to inhibit glioma neovascularization and tumor growth in an intracranial rat glioma model[43] as well as inhibiting bFGF-induced healing of cryo-injured skin grafts[44].

Collectively, these data provide strong evidence that anti-FGF-2 antibodies block angiogenesis in a variety of experimental settings. Some of these data also show that such antibodies can inhibit tumor growth. It is, therefore, perhaps surprising that anti-FGF antibodies have not been more thoroughly evaluated for their therapeutic potential as anti-tumor agents. It is not clear whether anti-FGF antibodies block tumor growth by an anti-angiogenic mechanism, or by interfering directly with FGF-driven tumor cell growth. However, regardless of the mechanism of action, anti-FGF-2 antibodies could be effective agents to interfere with the growth of most tumors that depend on vascularization and/or FGF-2-regulated tumor-cell division. The same should apply for anti-FGF receptor antibodies (FGFR1) that appear not to have been investigated so far. However, it should be noted that FGF-2 receptors are expressed on many different cell types (e.g. neurons) and, therefore, it remains to be seen if anti-FGF-2 tumor therapeutic strategies would be encumbered with significant adverse effects.

Anti-EGF receptor antibodies

The epidermal growth factor receptor (EGFR) and ErbB2/neu (HER-2) tyrosine kinase receptors contribute to the proliferation, metastasis and survival of solid tumors. Recently, these receptors have been implicated in regulating processes within the microenvironment of human tumors, including that of angiogenesis[27]. This hypothesis has been supported by studies showing that the anti-EGFR mAb, IMC-C225 (Ref. 45), caused a dose-dependent inhibition of VEGF protein expression in EGFR-positive A431 human epidermoid carcinoma cells, and inhibited angiogenesis of these tumors in a mouse model[46]. *In vivo*, suppression of VEGF expression by IMC-C225 treatment was accompanied by a significant reduction in the number of tumor blood vessels. Similarly, treatment of HER-2-positive tumor cells with the anti-HER-2 mAb, 4D5, inhibited VEGF production in a dose-dependent manner. The anti-angiogenic effect of the anti-EGFR antibody IMC-C225 has recently been confirmed in several studies of human tumor xenografts including pancreatic carcinoma[47], transitional cell carcinoma of the bladder[48], squamous-cell carcinoma[49] and colorectal carcinoma[50]. These findings suggest that antibodies against HER-2 and EGFR such as Herceptin and IMC-C225, respectively, could have therapeutic use not only by their ability to suppress the growth and survival of tumors cells, but also via their ability to inhibit the process of tumor angiogenesis.

Targeting endothelial cell adhesion

Endothelial cells are highly dependent on appropriate interactions with their environment. During migration and invasion, new vessel-forming endothelial cells must be able to adhere to certain extracellular matrix components for anchorage and survival. Endothelial cells must also be able to recognize each other, for the purpose of assembling and maintaining vascular tubular structures. Finally, endothelial cells need to interact with other cells, such as pericytes and smooth muscle cells to form mature blood vessels, or certain inflammatory cells that can activate endothelial cells and initiate the angiogenic process. Several cell-surface proteins have been identified on endothelial cells that facilitate their adhesion and contact with other cells. Prominent endothelial-cell adhesion molecules include:

- Certain integrins (e.g. $\alpha_v\beta_3$, $\alpha_v\beta_5$), which are involved in endothelial cell interactions with extracellular matrix components such as fibrin or fibronectin, and cadherins;
- VE-cadherin, which takes part in homophilic binding between endothelial cells, enabling tube formation by the formation of adherens junctions;
- Occludin, which is involved in endothelial tight junctions;
- EphrinB2 and its receptor Eph4, which are thought to play a role in arteriole–venule recognition;
- E-selectin, which is involved in leukocyte binding; and
- Other adhesion molecules with less well-defined function, such as platelet/endothelial-specific cell adhesion molecule (PECAM).

It is conceivable that some prominent angiogenesis inhibitors, such as angiostatin and endostatin, function by inhibiting the vital adhesive functions of endothelial cells. Many adhesion molecules are specifically expressed on endothelial cells (VE-cadherin), or upregulated on activated endothelium ($\alpha_v\beta_3$) and are, therefore, thought to be choice anti-angiogenesis targets. However, the rationale and effects of adhesion inhibitors on angiogenesis are generally less well-understood than those of endothelial cell growth inhibitors. Several mAb have been generated to validate some of these targets, as outlined in the following section.

Anti-integrin antibodies ($\alpha_v\beta_3$ integrin)

Of the various integrins reported to be relevant for angiogenesis, integrin $\alpha_v\beta_3$ has been most extensively studied. Integrin $\alpha_v\beta_3$, is specifically expressed on endothelial cells, upregulated on activated endothelium, and thought to be required for angiogenesis[51]. Integrin $\alpha_v\beta_3$ serves as an adhesion receptor for extracellular matrix components (e.g. vitronectin, fibrin) providing a means for attachment and thus, survival of endothelial cells in provisional matrices of tissues undergoing neovascularization. Ligand-binding triggers intracellular signaling via $\alpha_v\beta_3$ to survival mechanisms[52] and has also been shown to play a role in the activation of VEGFR-2 (Ref. 53). Other integrins reported to be involved in angiogenesis are integrin $\alpha_v\beta_5$ (Ref. 54) and certain endothelial-cell integrins containing the β_1 integrin subunit[55]. It is interesting to note that blood vessels in mice with a deleted integrin α_v gene develop normally[56], although results from antibody studies (discussed in the following section) suggest that interference with the function of α_V integrin would have detrimental effects.

A mAb against human $\alpha_v\beta_3$ integrin, LM609, has been shown to interfere with angiogenesis in the quail embryo by preventing proper maturation of newly formed blood capillaries[57]. LM609 also inhibits angiogenesis and invasiveness of human vessels in the SCID mouse/human skin transplant tumor model, and growth of human breast cancer in transplanted human skin[58]. The antibody induces apoptosis of endothelial cells in newly forming blood vessels but not in pre-existing vessels of the chick chorioallantoic membrane (CAM), resulting in regression of human tumors transplanted into the CAM[59]. In corneal and CAM models, LM609 appears to block FGF-induced angiogenesis, whereas an antibody against $\alpha_v\beta_5$ inhibits VEGF-induced angiogenesis[54].

A humanized form of LM609, Vitaxin, is currently undergoing clinical testing. Vitaxin was originally developed by Ixsys (San Diego, CA, USA), and is now licensed to Medimmune (Gaithersburg, MD, USA). Results of several Phase I studies have been reported. Vitaxin is tolerated at doses up to 4 mg kg^{-1}, given as an intravenous infusion once a week. In one study[j], groups of three patients were treated with doses of 10, 50 and 200 mg. The antibody was well tolerated (four patients had grade 2 adverse events, mainly fever). Pharmacokinetic data suggested a half-life of about one week with saturable clearance at the highest dose, and sustainable circulating antibody levels of 5 μg ml^{-1} with a dose of 200 mg per patient given every three weeks. In another study[k], 15 patients with leiomyosarcomas were treated with 0.25 mg kg^{-1} Vitaxin once weekly for up to six months. Although a previous Phase I study of patients with various cancers had suggested a response from a leiomyosarcoma patient, this study provided no objective evidence of efficacy. The antibody was generally well tolerated (grade 3 or 4 toxicities in 2 of 15 patients). Additional studies are, therefore, required to determine an appropriate patient population and efficacious dose and treatment schedule.

Anti-VE-cadherin antibody

Cadherins represent a rapidly growing superfamily of cell adhesion molecules that display a common feature of calcium-dependent homotypic interactions[60]. Cadherin-mediated selective cell–cell recognition and adhesion are crucial for 'sorting' and maintaining the integrity of tissues. They are transmembrane glycoproteins that typically contain five ectodomains and a short, highly conserved cytoplasmic portion that anchors the cadherin to the cytoskeleton. The ectodomains mediate homotypic cell–cell interactions and a series of signaling events relevant to cell growth, survival and cadherin clustering.

VE-cadherin (VE-cad) is an endothelial-cell specific cadherin that mediates adherens junction-formation between endothelial cells[61]. Accumulating evidence implicates VE-cad in several aspects of vascular biology related to angiogenesis, most notably, endothelial cell assembly into tubular structures[61,62]. VE-cad-null-mouse embryos exhibit severely impaired assembly of vascular structures that results in embryonic lethality at day E9.5, implicating VE-cad as an important mediator in developmental angiogenesis. Its highly restricted distribution and unique

[j] Posey, J. *et al.* A pilot trial of Vitaxin, an anti-angiogenic humanized antibody in patients with advanced solid tumors. *The 1999 AACR/NCI/EORTC International Conference on Molecular Targets and Cancer Therapeutics, Washington, DC, USA, 16–19 November, 1999,* Abstract 97

[k] Patel, S. *et al.* A pilot study of an angiogenesis inhibitor Vitaxin in patients with advanced leiomyosarcomas. *American Society of Clinical Oncology 36th Annual Meeting, 20–23 May, 2000, New Orleans, LA, USA,* Abstract 2202

biological function distinguish VE-cad as a potential target for endothelial-cell specific events, such as angiogenesis.

ImClone recently began developing anti-VE-cad antibodies for testing as anti-angiogenic agents. Blocking VE-cad is a novel anti-angiogenic strategy in which the assembly of capillary structures is inhibited. Proof-of-principle of this concept has been established by showing that anti-VE-cad antibodies inhibit adherens junction-formation and endothelial-cell tube-formation. Most significantly, it has been demonstrated that an antibody (BV13) inhibits angiogenesis, tumor growth and metastasis in several mouse models[63]. These findings indicate that VE-cad plays a crucial role in post-natal angiogenesis, and validate VE-cad as a potential target for anti-angiogenic therapy.

It is important to note that BV13 has potent *in vivo* antitumor activity at lower doses (50 μg dose^{-1}) than other anti-angiogenic antibodies, such as those that block the functions of VEGF, VEGF receptor or $\alpha_v\beta_3$, which must be given at tenfold higher doses[9,22,58]. However, higher doses of BV13 resulted in increased vascular permeability and edema in the lung followed by the death of some animals within 24–48 h (Ref. 64). This permeability effect of BV13 on normal tissues is not entirely unexpected, because VE-cad is expressed equally in tumor and normal vasculature and BV13 binds to vessels in normal tissues as well as tumor vasculature[64]. Therefore, anti-VE-cad antibodies might not only prevent the formation of adherens junctions in nascent vasculature (junction formation) but might also interfere with established adherens junctions and thus cause increased permeability of the affected vasculature (junction disruption).

These results indicate, therefore, that antibody BV13 would not be an appropriate agent for therapeutic use because of its disrupting activity on existing adherens junctions. Future studies will aim to identify a more desirable VE-cad inhibitor that preferentially affects ongoing angiogenesis. Assuming that VE-cad-mediated adhesion is as complex as that of other classical cadherins, which involve multiple adhesive contacts between multiple ectodomains[60], it might be possible to target regions of the VE-cad protein responsible for one or more steps in the formation of adherens junctions, but which are not accessible or influenced once adherens junctions have formed. It remains to be determined whether such antibodies can be identified.

Miscellaneous targets

Anti-angiogenin antibody

Angiogenin is a secreted angiogenic protein with structural and functional homology to members of the pancreatic RNase superfamily. It is expressed in a variety of cell types, including tumor cells, and its expression is positively correlated with angiogenic conditions such as those in tumor growth and female reproductive function. Angiogenin has various activities related to angiogenesis, such as the stimulation of endothelial cell proliferation, invasion and vascular tube formation. However, its mechanism of action is not well understood. Angiogenin has been reported to stimulate endothelial cell proliferation via a receptor-mediated mechanism, and to activate multiple signaling pathways; however, other mechanisms might be more relevant to its angiogenesis-inducing properties. Angiogenin has been shown to be specifically internalized by endothelial cells. Perhaps its most angiogenesis-relevant activity is its modest, but essential, tRNA-specific ribonuclease activity, which results in ribosome inactivation and protein-synthesis inhibition. Angiogenin antagonists (antibodies, aptamers and antisense agents) have been shown to inhibit angiogenesis *in vivo*.

Fett and coworkers generated a mouse mAb to human angiogenin, designated 26-2F, which has potent blocking activity ($IC_{50} = 1.6 \times 10^{-9}$ M) and inhibits angiogenesis in the CAM assay[65]. The antibody also delayed the growth of HT-29 human colon carcinoma, A549 human lung adenocarcinoma and HT-1080 fibrosarcoma tumors in athymic mice[66,67]. The antibody appears to act specifically by neutralizing angiogenin activity and has no cytotoxic activity against tumor cells. The same investigators have also created a human–mouse chimeric form of antibody 26-2F, presumably as a clinically suitable antibody, and have shown that it is equipotent to mAb 26-F in inhibiting tumor growth and metastasis formation in a human breast-cancer xenograft nude-mouse model[68]. To date, there is no information suggesting that the chimeric mAb 26-2F is being tested in the clinic.

Anti-MIF antibody

Macrophage inhibitory factor (MIF) is a multifunctional cytokine essential for macrophage and T cell activation, and involved in autocrine growth of certain B cell lymphomas. Recent evidence suggests that MIF might be an interesting target for inhibitors of neovascularization, although its role in angiogenesis is still controversial[69]. In support of this hypothesis it was demonstrated that neutralizing anti-MIF mAb can inhibit endothelial cell proliferation *in vitro*, new blood vessel formation in the Matrigel™ implant angiogenesis model, and the growth and vascularization of a mouse B-cell lymphoma in syngeneic mice[69]. Interestingly, MIF was found to be expressed in endothelial cells but not in lymphoma cells. Additional studies are needed to determine whether MIF is a valid target for anti-angiogenic therapy.

Antibody immunoconjugates

Antibodies that target antigens expressed by tumor cells have been coupled to cytotoxic agents to direct these agents to cancer cells and induce cancer-cell death. These immunoconjugates can be used in a similar fashion to target tumor vasculature, provided that the target antigen is expressed on tumor vasculature with sufficient selectivity compared with normal vasculature or other normal tissues. Currently, the specificity of this targeting approach to tumor vasculature remains the major obstacle in the practical use of anti-angiogenesis immunoconjugates.

The concept of using antibodies to deliver cytotoxic agents to tumor vasculature was first tested by Thorpe and collaborators[70,71] who used a mouse tumor model in which an artificially expressed target, major histocompatibility complex (MHC) class II, was expressed on the tumor endothelial cells. An MHC class II antibody–ricin-A-chain conjugate was shown to cause complete occlusion of the tumor vasculature and dramatic regression of large, established tumors[70]. By contrast, a conventional anti-tumor cell immunotoxin of equivalent *in vitro* potency produced only weak and transient anti-tumor effects. This approach was further validated by using an antibody coupled to truncated tissue factor. Intravenous injection of this immunoconjugate to mice with large tumors caused the formation of thrombosis within the tumor vessels and resulted in complete tumor regressions in 38% of the mice[71].

Endoglin (CD105) is a vascular-specific marker that has been tested as a target for anti-angiogenic immunoconjugate therapy. Endoglin is a homodimeric endothelial-cell-surface protein that functions as a low-affinity transforming growth factor-β (TGF-β) receptor, whose expression level on endothelial cells correlates with the proliferative state of the cells[72]. Overexpression of endoglin in the tumor vasculature of a variety of tumor types has been reported (e.g. brain tumors, melanoma, breast carcinoma, cervical cancer and lung carcinoma). Anti-endoglin mAb have been shown to inhibit the proliferation of human endothelial cells *in vitro*, possibly via a urokinase-related mechanism[73,74], and thus, could be used as function-blocking agents. However, investigators have focused on the use of anti-endoglin immunoconjugates for anti-angiogenic therapy. Anti-endoglin antibody, K4-2C10, coupled to ricin-A chain, was used to treat human breast-cancer xenografts in SCID mice[74]. Long-lasting complete inhibition of tumor growth was observed in all mice treated with the immunoconjugate, whereas the same dose of unconjugated antibody showed no anti-tumor effect[74,75]. Another anti-endoglin immunoconjugate based on the TEC11 antibody has also been evaluated[76].

TES-23 is another endothelial-cell directed mAb that was obtained by immunizing mice with endothelial cells derived from a rat tumor. TES-23 was coupled to radioactive iodine and the ^{125}I–TES-23 immunoconjugate was shown to localize to tumor tissue and to suppress the growth of KMT-17 solid tumors[77]. Histopathological examination revealed the apoptotic death of tumor endothelial cells, thrombus formation in affected tumor blood vessels, and subsequent vascular collapse.

Finally, an interesting anti-angiogenic targeting approach focuses on the ED-B domain of the oncofetal fibronectin B, an alternative splice variant of fibronectin that is present in the stroma of tumor tissues and is expressed in neoplastic, but not normal, blood vessels[78]. Radiolabeled high affinity mAb against the ED-B fibronectin domain have been shown to accumulate in tumor tissue with high selectivity[79]. This antibody has been used in imaging studies[80] but might also be of use for the therapeutic targeting of tumor vasculature.

Conclusions

This review describes the current status of antibody-based approaches towards inhibition angiogenesis. Undoubtedly, additional antibodies against novel angiogenic targets will rapidly emerge in the future. Several new targets for anti-angiogenesis antibody development are already available. For example, antibodies targeting the angiopoietins or their receptor tie-2 could be used to target vessel stability, antibodies against endothelial cell-specific ligand–receptor systems like ephrinB2 and Eph4, or Notch and Notch ligand could target endothelial-specific cell–cell recognition systems, and finally, antibodies against additional angiogenesis factors or their receptors (e.g. hepatocyte growth factor, interleukin-8) could interfere with vascular growth systems. It seems probable that improved techniques of vascular targeting with immunoconjugates will also become available in the future.

Antibodies represent a unique class of therapeutics because of their high specificity towards a defined antigen. Recent commercial success in antibody-based cancer therapeutics, including Rituxan™ (IDEC Pharmaceuticals – Genentech), Herceptin™ (Genentech) and Mylotarg™ (Wyeth-Ayerst, Princeton, NJ, USA) has greatly revitalized interest in the area. In fact, antibody therapeutics has rapidly grown from the ideal of 'magic-bullet' to practical cancer therapy in the past decade. Traditional obstacles in antibody therapy, such as the immunogenicity of rodent-derived antibodies and the difficulty of producing antibodies in sufficient quantity and quality for commercial application, are being rapidly superseded by the advancement in antibody-engineering technologies. Antibody

chimerization and humanization have greatly reduced the immunogenicity of murine antibodies, thus making repeated-dosing schedules in the clinic a reality. With the availability of human antibody transgenic mice and human antibody phage-display libraries, wholly human antibodies with desired specificities can be readily isolated. Further, the development in new recombinant techniques for genetically manipulating antibodies has enabled us to tailor-make antibody molecules with predefined characteristics such as size, valency and multi-specificity to suit the intended application. Finally, achievements in technologies related to antibody production, such as high-level mammalian expression systems and antibody production in transgenic plants and animals, will make large-scale antibody production more feasible and economical than ever. Taken together, this leads us to anticipate that a large amount of antibody-based therapeutics against angiogenesis targets will enter clinical development in the future.

References

1 Risau, W. (1997) Mechanisms of angiogenesis. *Nature* 386, 671–674
2 Miller, R.A. *et al.* (1982) Treatment of B-cell lymphoma with monoclonal anti-idiotype antibody. *New Engl. J. Med.* 306, 517–522
3 Glennie, M.J. and Johnson, P.W.M. (2000) Clinical trials of antibody therapy. *Immunol. Today* 21, 403–410
4 Ferrara, N. (1999) Molecular and biological properties of vascular endothelial growth factor. *J. Mol. Med.* 77, 527–543
5 Hiratsuka, S. *et al.* (1998) Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc. Natl. Acad. Sci. U. S. A.* 95, 9349–9354
6 Veikkola, T. and Alitalo, K. (1999) VEGFs, receptors and angiogenesis. *Semin. Canc. Biol.* 3, 211–220
7 Witte, L. *et al.* (1998) Monoclonal antibodies targeting the VEGF receptor-2 (Flk1/KDR) as an anti-angiogenic therapeutic strategy. *Cancer Metastasis Rev.* 17, 155–161
8 Zhu, Z. and Witte, L. (1999) Inhibition of tumor growth and metastasis by targeting tumor-associated angiogenesis with antagonists to the receptors of vascular endothelial growth factor. *Invest. New. Drugs* 17, 195–212
9 Kim, K.J. *et al.* (1993) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature* 362, 841–844
10 Warren, R.S. *et al.* (1995) Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J. Clin. Invest.* 95, 1789–1797
11 Borgstrom, P. *et al.* (1998) Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma micro tumors *in vivo*. *Prostate* 35, 1–10
12 Mesiano, S. *et al.* (1998) Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization. *Amer. J. Pathol.* 153, 1249–1256
13 Yuan, F. *et al.* (1996) Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor/vascular permeability factor antibody. *Proc. Natl. Acad. Sci. U. S. A.* 93, 14765–14770
14 Borgstrom, P. *et al.* (1996) Complete inhibition of angiogenesis and growth of microtumours by anti-vascular endothelial growth factor neutralizing antibody: novel concepts of angiostatic therapy from intravital videomicroscopy. *Cancer Res.* 56, 4032–4039
15 Presta, L.G. *et al.* (1997) Humanization of an anti-vascular endothelial growth factor mAb for the therapy of solid tumors and other disorders. *Cancer Res.* 57, 4593–4599
16 Ryan, A.M. *et al.* (1999) Preclinical safety evaluation of rhuMAbVEGF, an antiangiogenic humanized mAb. *Toxicol. Pathol.* 27, 78–86
17 Asano, M. *et al.* (1998) An anti-human VEGF mAb, MV833, that exhibits potent anti-tumor activity *in vivo*. *Hybridoma* 17, 185–190
18 Brekken, R.A. *et al.* (2000) Selective inhibition of vascular endothelial growth factor (VEGF) receptor 2 (KDR/Flk-1) activity by a monoclonal anti-VEGF antibody blocks tumor growth in mice. *Cancer Res.* 60, 5117–5124
19 Achen, M.G. *et al.* (2000) MAb to vascular endothelial growth factor-D block its interactions with both VEGF receptor-2 and VEGF receptor-3. *Eur. J. Biochem.* 267, 2505–2515
20 Rockwell, P. *et al.* (1995) *In vitro* neutralization of vascular endothelial growth factor activation of Flk-1 by a monoclonal antibody. *Mol. Cell. Diff.* 3, 91–109
21 Skobe, M. *et al.* (1997) Halting angiogenesis suppresses carcinoma cell invasion. *Nat. Med.* 3, 1222–1227
22 Prewett, M. *et al.* (1999) Anti-VEGF receptor (Flk-1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. *Cancer Res.* 59, 5209–5218
23 Yoshiji, H. *et al.* (1999) KDR/Flk-1 is a major regulator of vascular endothelial growth factor-induced tumor development and angiogenesis in murine hepatocellular carcinoma cells. *Hepatology* 30, 1179–1186
24 Bruns, C.J. *et al.* (2000) Vascular endothelial growth factor is an *in vivo* survival factor for tumor endothelium in a murine model of colorectal liver metastases. *Cancer* 89, 488–499
25 Inoue, K. *et al.* (2000) Treatment of human metastatic transitional cell carcinoma of the bladder in a murine model with the anti-vascular endothelial growth factor receptor monoclonal antibody DC101 and paclitaxel. *Clin. Canc. Res.* 6, 2635–2643
26 Kozin, S.V. *et al.* Vascular Endothelial Growth Factor Receptor-2 blockade selectively increases radiation-induced cure rate of human tumor xenografts. *Cancer Res.* (in press)
27 Kerbel, R.S. *et al.* (2000) 'Accidental' anti-angiogenic drugs: anti-oncogene directed signal transduction inhibitors and conventional chemotherapeutic agents as examples. *Eur. J. Canc.* 36, 1248–1257
28 Klement, G. *et al.* (2000) Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J. Clin. Invest.* 105, R15–R24
29 Zhu, Z. *et al.* (1998) Inhibition of vascular endothelial growth factor-induced receptor activation with anti-kinase insert domain-containing receptor single-chain antibodies from a phage display library. *Cancer Res.* 58, 3209–3214
30 Zhu, Z. *et al.* (1999) Inhibition of vascular endothelial growth factor induced mitogenesis of human endothelial cells by a chimeric anti-kinase insert domain-containing receptor antibody. *Cancer Lett.* 136, 203–213
31 Lu, D. *et al.* (2000) Identification of the residues in the extracellular region of KDR important for interaction with vascular endothelial growth factor and neutralizing anti-KDR antibodies. *J. Biol. Chem.* 275, 14321–14330
32 Dias, S. *et al.* (2000) Autocrine stimulation of VEGFR-2 activates human leukemic cell growth and migration. *J. Clin. Invest.* 106, 511–521
33 Pavco, P.A. *et al.* (2000) Antitumor and antimetastatic activity of ribozymes targeting the messenger RNA of vascular endothelial growth factor receptors. *Clin. Canc. Res.* 5, 2094–2103
34 Kanno, S. *et al.* (2000) Roles of two VEGF receptors, Flt-1 and KDR, in the signal transduction of VEGF effects in human vascular endothelial cells. *Oncogene* 19, 2138–2146
35 Kaipainen, A. *et al.* (1995) Expression of the fms-like tyrosine kinase FLT4 gene becomes restricted to endothelium of lymphatic vessels during development. *Proc. Natl. Acad. Sci. U. S. A.* 92, 3566–3570

36 Wilting, J. *et al.* (1997) The avian VEGF receptor homologues Queck1 and Queck2 during quail embryonic development: expression in blood-vascular and lymphatic endothelial and non-endothelial cells. *Cell Tissue Res.* 288, 207–223

37 Kubo, H. *et al.* (2000) Involvement of vascular endothelial growth factor receptor-3 in maintenance of integrity of endothelial cell lining during tumor angiogenesis. *Blood* 96, 546–553

38 Hu, D. *et al.* (1994) Inhibition of angiogenesis in rats by IL-1 receptor antagonist and selected cytokine antibodies. *Inflammation* 18, 45–58

39 Reilly, T. *et al.* (1989) MAb directed against basic fibroblast growth factor which inhibit its biological activity *in vitro* and *in vivo*. *Biochem. Biophys. Res. Commun.* 164, 736–743

40 Coppola, G. *et al.* (1997) Effect of intraperitoneally, intravenously and intralesionally administered monoclonal anti-beta-FGF antibodies on rat chondrosarcoma tumor vascularization and growth. *Anticancer Res.* 17, 2033–2039

41 Satoh, H. *et al.* (1997) Role of endogenous basic fibroblast growth factor in the healing of gastric ulcers in rats. *Jpn. J. Pharmacol.* 73, 59–71

42 Hori, A. *et al.* (1991) Suppression of solid tumor growth by immunoneutralizing mAb against human basic fibroblast growth factor. *Cancer Res.* 51, 6180–6184

43 Nemati, M. *et al.* (1996) Inhibition of angiogenesis and growth of malignant gliomas in the athymic nude rat model: immunotherapy against basic fibroblast growth factor. *Zentralbl. Neurochir.* 57, 12–19

44 Lees, V. and Fan, T.P. (1994) A freeze-injured skin graft model for the quantitative study of basic fibroblast growth factor and other promoters of angiogenesis in wound healing. *Br. J. Plast. Surg.* 47, 349–359

45 Waksal, H.W. (1999) Role of an anti-epidermal growth factor receptor in treating cancer. *Cancer Metastasis Rev.* 18, 427–436

46 Petit, A.M. *et al.* (1997) Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells *in vitro* and *in vivo*: angiogenic implications for signal transduction therapy of solid tumors. *Amer. J. Pathol.* 151, 1523–1530

47 Bruns, C.J. *et al.* (2000) Epidermal growth factor receptor blockade with C225 plus gemcitabine results in regression of human pancreatic carcinoma growing orthotopically in nude mice by antiangiogenic mechanisms. *Clin. Canc. Res.* 6, 1936–1948

48 Perrotte, P. *et al.* (2000) Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin. Canc. Res.* 5, 257–265

49 Huang, S.M. and Harari, P.M. (2000) Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. *Clin. Canc. Res.* 6, 2166–2174

50 Ciardiello, F. *et al.* (2000) Antiangiogenic and antitumor activity of anti-epidermal growth factor receptor C225 monoclonal antibody in combination with vascular endothelial growth factor antisense oligonucleotide in human GEO colon cancer cells. *Clin. Canc. Res.* 6, 3739–3747

51 Brooks, P. *et al.* (1994) Requirement of vascular integrin $\alpha_v\beta_3$ for angiogenesis. *Science* 264, 569–571

52 Stromblad, S. and Cheresh, D. (1996) Cell adhesion and angiogenesis. *Trends Cell. Biol.* 6, 462–468

53 Soldi, R. *et al.* (1999) Role of $\alpha_v\beta_3$ integrin in the activation of vascular endothelial growth factor receptor-2. *EMBO J.* 18, 882–892

54 Friedlander, M. *et al.* (1995) Definition of two angiogenic pathways by distinct α_v integrins. *Science* 270, 500–502

55 Kubota, Y. *et al.* (1996) CDw49b/CD29 integrin complex mediates the differentiation of human endothelial cells into capillary-like structures *in vitro*. *J. Dermatol. Sci.* 12, 36–43

56 Bader, B. *et al.* (1998) Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all αv integrins. *Cell* 95, 507–519

57 Drake, C. *et al.* (1995) An antagonist of integrin $\alpha_v\beta_3$ prevents maturation of blood vessels during embryonic neovascularization. *J. Cell. Sci.* 108, 2655–2661

58 Brooks, P. *et al.* (1995) Anti-integrin $\alpha_v\beta_3$ blocks human breast cancer growth and angiogenesis in human skin. *J. Clin. Invest.* 96, 1815–1822

59 Brooks, P. *et al.* (1994) Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* 79, 1157–1164

60 Gumbiner, B.M. (1996) Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 84, 345–357

61 Dejana, E. *et al.* (1999) Vascular endothelial (VE)-cadherin: only an intercellular glue. *Exp. Cell. Res.* 252, 13–19

62 Carmeliet, P. *et al.* (1999) Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* 98, 147–157

63 Liao, F. *et al.* (2000) Monoclonal antibody to vascular endothelial (VE)-cadherin is a potent inhibitor of angiogenesis, tumor growth and metastasis. *Cancer Res.* 60, 6805–6810

64 Corada, M. *et al.* (1999) Vascular endothelial-cadherin is an important determinant of microvascular integrity *in vivo*. *Proc. Natl. Acad. Sci. U. S. A.* 96, 9815–9820

65 Fett, J. *et al.* (1994) A mAb to human angiogenin. Inhibition of ribonucleolytic and angiogenic activities and localization of the antigenic epitope. *Biochemistry* 33, 5421–5427

66 Olson, K. *et al.* (1994) A mAb to human angiogenin suppresses tumor growth in athymic mice. *Cancer Res.* 54, 4576–4579

67 Olson, K. *et al.* (1995) Angiogenin antagonists prevent tumor growth *in vivo*. *Proc. Natl. Acad. Sci. U. S. A.* 92, 442–446

68 Piccoli, R. *et al.* (1998) Chimeric anti-angiogenin antibody cAb 26-2F inhibits the formation of human breast cancer xenografts in athymic mice. *Proc. Natl. Acad. Sci. U. S. A.* 95, 4579–4583

69 Chesney, J. *et al.* (1999) An essential role for macrophage migration inhibitory factor (MIF) in angiogenesis and the growth of a murine lymphoma. *Mol. Med.* 5, 181–191

70 Burrows, F.J. and Thorpe, P.E. (1993) Eradication of large solid tumors in mice with an immunotoxin directed against tumor vasculature. *Proc. Natl. Acad. Sci. U. S. A.* 90, 8996–9000

71 Huang, X. *et al.* (1997) Tumor infarction in mice by antibody-directed targeting of tissue factor to tumor vasculature. *Science* 275, 547–550

72 Miller, D.W. *et al.* (1999) Elevated expression of endoglin, a component of the TGF-β-receptor complex, correlates with proliferation of tumor endothelial cells. *Intl. J. Cancer* 81, 568–572

73 Maier, J.A. *et al.* (1997) *In vitro* inhibition of endothelial cell growth by the antiangiogenic drug AGM1470 (TNP470) and the anti-endoglin antibody TEC11. *Anticancer Drugs* 8, 238–244

74 Seon, B.K. *et al.* (1997) Long-lasting complete inhibition of human solid tumors in SCID mice by targeting endothelial cells of tumor vasculature with antihuman endoglin immunotoxin. *Clin. Canc. Res.* 3, 1031–1044

75 Matsuno, F. *et al.* (1999) Induction of lasting complete regression of preformed distinct solid tumors by targeting the tumor vasculature using two new anti-endoglin mAb. *Clin. Canc. Res.* 5, 371–382

76 Thorpe, P. and Burrows, F. (1995) Antibody-directed targeting of the vasculature of solid tumors. *Breast Cancer Res. Treat.* 36, 237–251

77 Ohizumi, I. *et al.* (1999) Suppression of solid tumor growth by a mAb against tumor vasculature in rats: involvement of intravascular thrombosis and fibrinogenesis. *Intl. J. Cancer* 82, 853–859

78 Carnemolla, B. *et al.* (1996) Phage antibodies with pan-species recognition of the oncofoetal angiogenesis marker fibronectin ED-B domain. *Intl. J. Cancer* 68, 397–405

79 Viti, F. *et al.* (1999) Increased binding affinity and valence of recombinant antibody fragments lead to improved targeting of tumoral angiogenesis. *Cancer Res.* 59, 347–352

80 Berndorff, D. *et al.* (1999) A high-affinity human antibody that targets tumoral blood vessels. *Blood* 94, 192–198