Therapeutic Anti-VEGF Antibodies

S. Lien and H.B. Lowman(✉)

Abstract Vascular endothelial growth factor (VEGF-A) is a key cytokine in the development of normal blood vessels as well as the development of vessels in tumors and other tissues undergoing abnormal angiogenesis. Here, we review the molecular engineering of two humanized antibodies derived from a common mouse anti-VEGF antibody – bevacizumab, a full-length IgG1 approved for the treatment of specified cancer indications, and ranibizumab, an affinity-matured antibody Fab domain approved for use in age-related macular degeneration (AMD). In clinical trials and as FDA-approved therapeutics, these two anti-VEGF antibodies, bevacizumab (Avastin® anti-VEGF antibody) and ranibizumab (Lucentis® anti-VEGF antibody), have demonstrated therapeutic utility in blocking VEGF-induced angiogenesis.

H.B. Lowman
Antibody Engineering, Protein Engineering, and Immunology Departments, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, USA
e-mail: hbl@gene.com

Y. Chernajovsky, A. Nissim (eds.) *Therapeutic Antibodies. Handbook of Experimental Pharmacology 181.*

1 Introduction

The idea of a tumor-derived blood vessel growth stimulating factor was first postulated in 1939 due to observations of a strong neovascular response induced by transplanted tumors (Ide et al. 1939). The authors proposed that the induction of such a factor, and hence newly developed vasculature, would allow rapidly growing tumors to receive nutrients. It was not until 1989, however, that this factor was cloned from medium conditioned by bovine pituitary cells (Ferrara and Henzel 1989). The factor was named vascular endothelial growth factor (VEGF), in recognition of its potent mitogenic effects on endothelial cells.

2 VEGF in Angiogenesis

VEGF, also known as VEGF-A, is a homodimeric glycoprotein of 36–46 kDa with significant homology to the A and B chains of placental-derived growth factor, PDGF (Leung et al. 1989). The VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor, PlGF. Each of these proteins represents a distinct gene product with distinct receptor interactions, as opposed to the various isoforms of VEGF-A described below. While VEGF-A availability is the rate-limiting step in normal and pathological blood vessel growth (Ferrara et al. 2003), VEGF-C and VEGF-D regulate lymphatic angiogenesis (Stacker et al. 2002). The current review will focus on inhibition of VEGF-A activity, henceforth referred to as VEGF.

The human VEGF gene is organized into eight exons (Houck et al. 1991; Tischer et al. 1991). Alternative splicing results in four main VEGF isoforms: VEGF121 (i.e., VEGF-A isoform with residues 1–121), VEGF165, VEGF189, and VEGF206 (Leung et al. 1989), which differ in their bioavailability. VEGF121, which lacks the heparin-binding domain, is a freely diffusible protein. VEGF189 and VEGF206 are nearly completely retained in the extracellular matrix (Houck et al. 1992; Park et al. 1993). VEGF165 is secreted but remains bound to the cell surface and the extracellular matrix due to its heparin binding ability. The bound isoforms may be released by heparin or heparinase, or by plasmin cleavage at the C-terminus. Plasmin cleavage generates a bioactive fragment consisting of the first 110 amino acids (VEGF110; Houck et al. 1992). There is now much evidence to suggest that VEGF165 is the most physiologically relevant isoform (reviewed in Ferrara (2004)).

Expression of VEGF is induced by a variety of factors. As its biology would suggest, VEGF mRNA is upregulated in conditions of hypoxia (Dor et al. 2001). Increased VEGF signaling can occur in the hypoxic environment of aberrant tumor vasculature, or due to mutation of other elements in the hypoxia induced pathway such as hypoxia-inducible factor 1, the von Hippel-Lindau tumor suppressor gene, the PTEN tumor suppressor gene, and/or the Forkhead transcription factor FOXO4. VEGF mRNA transcription and stability is also influenced by other growth factors, hormones, and oncogenes, including estrogen, nitric oxide, FGF, PDGF, TNF-alpha, EGF, IL-1 alpha, IL-6, Ras, and wnt (reviewed in Ferrara (2004)).

Signaling occurs through two VEGF receptor tyrosine kinases: Flt-1/VEGFR1 (de Vries et al. 1992; Shibuya et al. 1990) and Flk-1/KDR/VEGFR2 (Millauer et al. 1993; Quinn et al. 1993; Terman et al. 1992). Growing evidence supports the idea that VEGFR1 plays an important role in hematopoiesis while VEGFR2 on endothelial cells is the major mediator of the mitogenic, migratory, survival, angiogenic, and vascular permeability enhancing effects of VEGF (reviewed in Ferrara (2004)). Neuropilin 1 and neuropilin 2, molecules previously shown to bind the collapsin/semaphorin family, are also receptors for the heparin-binding isoforms of VEGF. Neuropilin 1 is thought to present VEGF165 to VEGFR2 in a manner that potentiates VEGFR2 signaling (Soker et al. 1998).

3 Production and Characterization of an Anti-VEGF Antibody: A4.6.1

In 1971, Judah Folkman proposed that anti-angiogenesis might be an effective anticancer strategy (Folkman 1971). Subsequently, human VEGF-A was identified (Ferrara and Henzel 1989), and it was demonstrated that VEGF was upregulated in many human cancers, including glioblastoma, colorectal cancer, nonsmall-cell lung cancer, renal cell cancer, pancreatic cancer, ovarian cancer, acute myeloid leukemia, multiple myeloma, Hodgkin's disease, and Non-Hodgkin's Lymphoma (reviewed in Ranieri et al. (2006)). The numbers of malignancies in which VEGF levels have been correlated with survival, as well as the demonstration that the growth of tumors beyond 0.2–2 mm depends on angiogenesis (Gimbrone et al. 1972), underlines the widespread utility of an agent capable of preventing VEGF signaling.

To address this need, Ferrara and coworkers generated murine monoclonal antibodies using recombinant human VEGF165 as the immunogen (Kim et al. 1992). They isolated four antibodies of IgG1 isotype with high affinity for human VEGF (K_D's of 0.4–2.2 nM). Competition binding experiments revealed that the antibodies could be divided into two classes: antibodies A3.13.1 and B2.6.2 recognized one epitope, while antibodies A4.6.1 and B4.3.1 recognized another. Since A4.6.1 and B2.6.2 had the highest affinities to VEGF, they were characterized further.

Subsequent experiments showed that B2.6.2 recognized a discontinuous epitope, whereas A4.6.1 appeared to recognize a continuous epitope. Furthermore, A4.6.1 bound VEGF121, VEGF165, and VEGF189, while B2.6.2 bound only VEGF165 and VEGF189. The antibodies also differed in their ability to inhibit VEGF activity. A4.6.1 was a far more effective inhibitor of VEGF activity in an in vitro bovine adrenal cortex endothelial cell proliferation assay, an in vivo vascular permeability assay, and an in vivo embryonic chicken angiogenesis assay (Kim et al. 1992). The results suggested that A4.6.1 had potent VEGF neutralizing activities.

The utility of this antibody in pathological models was demonstrated by treatment of immunodeficient mice bearing human tumor cell line xenografts (Borgstrom et al. 1996; Kim et al. 1993; Melnyk et al. 1996; Warren et al. 1995), and by treatment of cynomolgous monkeys with laser-induced retinal ischemia (Adamis et al.

1996). Treatment with as little as 0.05 mg ($\sim$2 mg kg^{-1}) of A4.6.1, given twice weekly intraperitoneally, was enough to inhibit growth of human rhabdomyosarcoma, glioblastoma multiforme, leiomyosarcoma (Kim et al. 1993), and colon carcinoma cell lines (Warren et al. 1995). Treatment with 0.1 mg twice weekly resulted in decreased tumor burden in a liver metastatic model of colon carcinoma (Warren et al. 1995) and reduction of metastasis in an epidermoid carcinoma model (Melnyk et al. 1996). Results were similar regardless of whether the antibody treatment was initiated at the time of tumor implantation or 1 week later (Kim et al. 1993). Treatment of the cell lines with A4.6.1 in vitro had no effect on growth, demonstrating that the effect was not due to autocrine VEGF activity or direct cytotoxicity of the antibody (Kim et al. 1993; Melnyk et al. 1996; Warren et al. 1995).

Ocular neovascularization, a characteristic of diabetic retinopathy and age-related macular degeneration (AMD), is associated with leakage and bleeding of vessels within the subretinal space. Intraocular levels of VEGF have been shown to correlate temporally, spatially, and quantitatively with new blood vessel formation (Alon et al. 1995; Stone et al. 1995). Furthermore, intraocular VEGF levels are elevated in diabetic retinopathy, iris revascularization, and retinopathy of prematurity (Adamis et al. 1994; Aiello et al. 1994; Malecaze et al. 1994). When iris neovascularization was induced in cynomolgous monkeys by laser retinal vein occlusion, 5 of 8 control eyes developed symptoms within 4–7 days. In contrast, iris neovascularization was not seen in any of the eight eyes treated with A4.6.1 (Adamis et al. 1996). Collectively, these results suggested that VEGF did indeed play a large role in ocular neovascularization and that anti-VEGF treatment might be a useful approach to therapy.

4 Humanization and the Development of Bevacizumab

Murine-derived antibodies are generally not used for human therapeutic purposes due to their potential immunogenicity. Antibody A4.6.1 was therefore humanized by a process of CDR grafting and framework mutations (Presta et al. 1997). This procedure had been successfully performed previously for trastuzumab, an anti-HER2 antibody (Carter et al. 1992). CDR residues were identified based on sequence (Kabat et al. 1991) and structural (Chothia et al. 1989) hypervariability and grafted into the consensus sequence of the human heavy chain subgroup III and light chain subgroup kI immunoglobulin variable-domain frameworks (Fig. 1; Kabat et al. 1991) ELISA assays of the CDR-grafted antibody showed that it was 1,000-fold reduced in binding to VEGF compared to the original antibody. Comparisons of the human and murine framework residues and the use of judicious mutations resulted in a humanized antibody with seven framework residue mutations in the heavy chain variable region and one framework residue mutation in the light chain variable region as compared to the human consensus sequence (Table 1, Fig. 2). The humanized antibody (bevacizumab) had an affinity within twofold of the parent antibody, A4.6.1 (Fig. 3), but showed no reduction in VEGF bioactivity (Presta et al. 1997).

```
                  10        20          30          40
MB1.6       DIQLTQSPSSLSASVGDRVTITC [SASQDISNYLN] WYQQKP
              *
Hu2.0       DIQMTQSPSSLSASVGDRVTITC [SASQDISNYLN] WYQQKP
             **      *    * *
A4.6.1      DIQMTQTTSSLSASLGDRVIISC [SASQDISNYLN] WYQQKP
             **      *    * *
Fab 1       DIQMTQSPSSLSASVGDRVTITC [SASQDISNYLN] WYQQKP

Fab 12      DIQMTQSPSSLSASVGDRVTITC [SASQDISNYLN] WYQQKP
              *
Y0317       DIQLTQSPSSLSASVGDRVTITC [SASQDISNYLN] WYQQKP
              *                    *   *     *
hum ki      DIQMTQSPSSLSASVGDRVTITC [RASQSISNYLA] WYQQKP

                 50          60        70        80
MB1.6       GKAPKLLIY [FTSSLHS] GVPSRFSGSGSGTDYTLTISSLQP
                                                 *
Hu2.0       GKAPKLLIY [FTSSLHS] GVPSRFSGSGSGTDFTLTISSLQP
            **** *                           **    * *
A4.6.1      DGTVKVLIY [FTSSLHS] GVPSRFSGSGSGTDYSLTISNLEP
            **** *                           **    * *
Fab 1       GKAPKLLIY [FTSSLHS] GVPSRFSGSGSGTDFTLTISSLQP
              *
Fab 12      GKAPKVLIY [FTSSLHS] GVPSRFSGSGSGTDFTLTISSLQP

Y0317       GKAPKVLIY [FTSSLHS] GVPSRFSGSGSGTDFTLTISSLQP
            *     **   *
hum ki      GKAPKLLIY [AASSLES] GVPSRFSGSGSGTDFTLTISSLQP

                              90   100
MB1.6       EDFATYYC [QQYSTVPWT] FGQGTKVEIKR

Hu2.0       EDFATYYC [QQYSTVPWT] FGQGTKVEIKR
              *                   *   *
A4.6.1      EDIATYYC [QQYSTVPWT] FGGGTKLEIKR
              *                   *   *
Fab 1       EDFATYYC [QQYSTVPWT] FGQGTKVEIKR

Fab 12      EDFATYYC [QQYSTVPWT] FGQGTKVEIKR

Y0317       EDFATYYC [QQYSTVPWT] FGQGTKVEIKR
                        ***
hum ki      EDFATYYC [QQYNSLPWT] FGQGTKVEIKR
```

Fig. 1 (a) Light chain amino acid sequences of MB1.6, Hu2.0, A4.6.1, Fab-1, Fab-12, Y0317, and human consensus sequences of light chain subgroup kappa I (hum kI) and heavy chain subgroup III (hum III). CDR loops are enclosed in brackets. Asterisks denote differences between sequences. Residue numbering is according to Kabat et al. (1991)

```
                10        20          30          40
MB1.6      EVQLVESGGGLVQPGGSLRLSCAAS [GYTFTNYGMN] WIRQA
                                                  *
Hu2.0      EVQLVESGGGLVQPGGSLRLSCAAS [GYTFTNYGMN] WVRQA
            *   *  ** *   *** *  *                 *
A4.6.1     EIQLVQSGPELKQPGETVRISCKAS [GYTFTNYGMN] WVKQA
            *   *  ** *   *** *  *                 *
Fab 1      EVQLVESGGGLVQPGGSLRLSCAAS [GYTFTNYGMN] WVRQA
                                       *  *
Fab 12     EVQLVESGGGLVQPGGSLRLSCAAS [GYTFTNYGMN] WVRQA
                                       *  *
Y0317      EVQLVESGGGLVQPGGSLRLSCAAS [GYDFTHYGMN] WVRQA
                                      ** ** * *
hum III    EVQLVESGGGLVQPGGSLRLSCAAS [GFTFSSYAMS] WVRQA

                     50  a      60          70        80
MB1.6      PGKGLEWVG [WINTYTGEPTYAADFKR] RFTISADTSSNTVYL
                                           * * *  *
Hu2.0      PGKGLEWVG [WINTYTGEPTYAADFKR] RFTISRDNSKNTLYL
           * *                            * *** ** *
A4.6.1     PGKGLKWMG [WINTYTGEPTYAADFKR] RFTFSLETSASTAYL
           * *                            * *** ** *
Fab 1      PGKGLEWVG [WINTYTGEPTYAADFKR] RFTISRDNSKNTLYL
                                           * * *  * *
Fab 12     PGKGLEWVG [WINTYTGEPTYAADFKR] RFTFSLDTSKSTAYL

Y0317      PGKGLEWVG [WINTYTGEPTYAADFKR] RFTFSLDTSKSTAYL
                 *  * **** ***  *** *     * * *  * *
hum III    PGKGLEWVS [VISGDGGSTYYADSVKG] RFTISRDNSKNTLYL

             abc      90         100abcde         110
MB1.6      QMNSLRAEDTAVYYCAK [YPHYYGSSHWYFDV] WGQGTLVTVSS
                             *
Hu2.0      QMNSLRAEDTAVYYCAR [YPHYYGSSHWYFDV] WGQGTLVTVSS
            *** ***   * *  *                   *  *
A4.6.1     QISNLKNDDTATYFCAK [YPHYYGSSHWYFDV] WGAGTTVTVSS
            *** ***   * *  *                   *  *
Fab 1      QMNSLRAEDTAVYYCAR [YPHYYGSSHWYFDV] WGQGTLVTVSS
                             *
Fab 12     QMNSLRAEDTAVYYCAK [YPHYYGSSHWYFDV] WGQGTLVTVSS
                               *   *
Y0317      QMNSLRAEDTAVYYCAK [YPYYYGTSHWYFDV] WGQGTLVTVSS
                            *  **** ***
hum III    QMNSLRAEDTAVYYCAR [----------FDY] WGQGTLVTVSS
```

Fig. 1 (b) Heavy chain amino acid sequences of the variable domains

The crystal structure of the Fab portion of bevacizumab was solved in complex with VEGF (Fig. 4; Muller et al. 1998), elucidating the importance of the framework mutations as well as the specificity of the antibody. In the heavy chain variable domain, residues 49, 69, 71, and 78 (residue numbers are indicated in the numbering system of Kabat) were buried or partially buried and affected binding by influencing

Table 1 Humanized anti-VEGF Fab constructs generated during bevacizumab development[1]

Variant	Template	Changes	Purpose
Chim-Fab	Chimeric Fab	Murine variable, human constant domains	Standard transfer of variable domains
Fab-1	Human FR	CDR swap:murine CDRs in human FR with VH:S49G[2]	Humanization starting point
Fab-2	–	Chim-Fab light chain, Fab-1 heavy chain	See effect of heavy chain CDR swap
Fab-3	–	Fab-1 light chain, Chim-Fab heavy chain	See effect of light chain CDR swap
Fab-4	Fab-1	VH: R71L VH: N73T	CDR H2 conformation framework change
Fab-5	Fab-4	VL: L46V	VL-VH interface
Fab-6	Fab-5	VH: L78A	CDR H1 conformation
Fab-7	Fab-5	VH: I69F	CDR H2 conformation
Fab-8	Fab-5	VH: I69F VH: L78A	CDR H2 conformation CDR H1 conformation
Fab-9	Fab-8	VH: G49A	CDR H2 conformation
Fab-10	Fab-8	VH: N76S	Framework change
Fab-11	Fab-10	VH: K75A	Framework change
Fab-12	Fab-10	VH: R94K	CDR H3 conformation

[1] Data taken from Presta et al. (1997)
[2] The human subgroup III heavy chain consensus sequence was defined as having Ser at position 49; however, Ala and Gly are also commonly found in human antibody sequences at this position; the murine A4.6.1 sequence contained G49

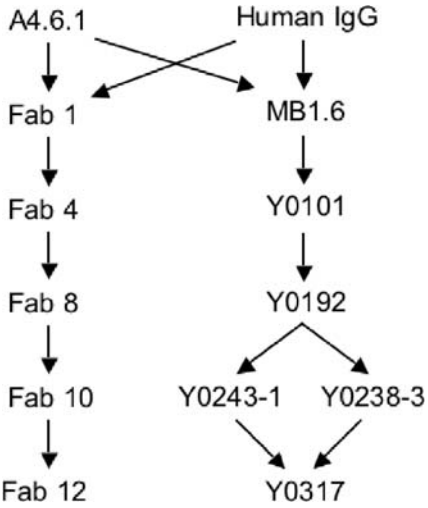

Fig. 2 Significant clones during humanization to bevacizumab and ranibizumab

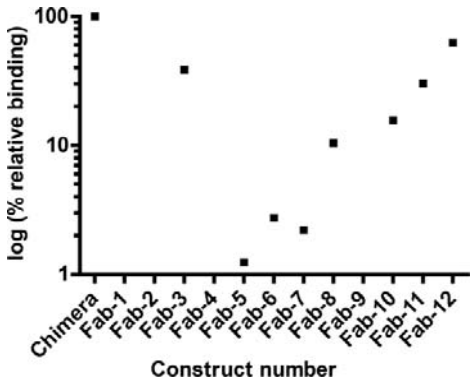

Fig. 3 Binding affinities during antibody A4.6.1 humanization leading to bevacizumab. Approximate changes in affinity of the humanized anti-VEGF Fab constructs relative to the binding of the chimeric Fab as measured by ELISA (Presta et al. 2007) are plotted against construct number. VEGF binding was undetectable for Fabs −1, −2, −4, and −9

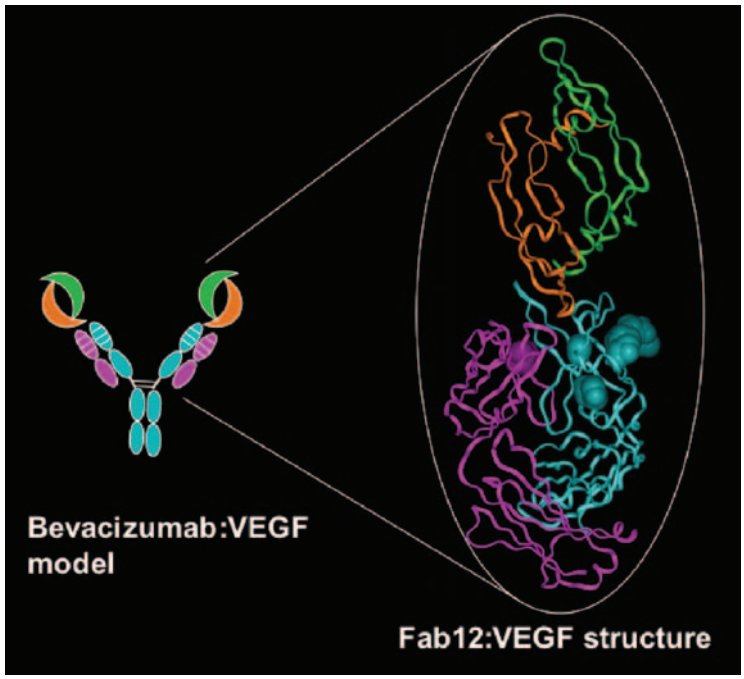

Fig. 4 Bevacizumab structure. The relationship of this Fab:VEGF complex to that of the full-length bevacizumab IgG in complex with VEGF is shown at *left*. At *right* is shown a ribbon diagram of the Fab fragment Fab-12 (heavy chain in *cyan*, light chain in *magenta*), in complex with the dimer form of VEGF-A (fragment 8–109, with one monomer in *orange* and the other in *green*) from the crystal structure of Muller et al. (1998) (PDB accession number 1BJ1). Framework residues that were changed during humanization are shown in space-filling form (spherical atoms): L46V in the light chain, and I69F, R71L, N73T, N76S, L78A, and R94K in the heavy chain

CDR loop conformations. Residues 73 and 76 were located in a non-CDR loop adjacent to CDRs H1 and H2 and interacted directly with VEGF. A lysine was required at H94, instead of the usual human arginine. The presence of a lysine allowed favorable packing interactions with two tyrosines, Y27 and Y32 of CDR H1. Both Y27 and Y32 are important for VEGF binding, as demonstrated by alanine-scanning mutagenesis. In the light chain variable domain, only L46 had to be changed to the murine residue (Val). In the Fab structure, residue 46 is buried and interacts directly with CDR H3, suggesting that it is required to maintain the conformation of this loop.

Alanine-scanning mutagenesis of VEGF revealed that the epitope for antibody binding overlapped the epitope for binding to VEGFR1 (Muller et al. 1998; Wiesmann et al. 1997). Bevacizumab, therefore, prevents VEGF bioactivity by steric hindrance of receptor binding. Alanine-scanning also revealed the basis for the species specificity of bevacizumab binding. G88 of human VEGF is bound in a deep pocket formed by the sidechains of residues from CDR L3, CDR H2, and CDR H3 (Muller et al. 1998). Murine VEGF contains a serine at position 88 and is therefore unable to bind bevacizumab due to steric hindrance.

4.1 Preclinical Studies with Bevacizumab

Following humanization, the anti-VEGF activity of bevacizumab was compared directly to that of A4.6.1. The two antibodies were shown to be equipotent in in vitro bovine capillary endothelial cell proliferation assays. Furthermore, both antibodies efficiently suppressed the growth of human rhabdomyosarcoma and breast carcinoma xenografts in nude mice at 0.5 and 5 mg kg^{-1} doses (Presta et al. 1997). Other preclinical studies of bevacizumab, as a single agent or in combination with cytotoxic therapies, are reviewed in Gerber and Ferrara (2005).

The pharmacokinetics of bevacizumab after intravenous administration have been studied in several species and are consistent with those of other humanized antibodies. Bevacizumab is cleared from circulation in a similar manner to endogenous antibodies. The terminal elimination half-life was 1–2 weeks in all species tested (Lin et al. 1999). Safety studies were performed in cynomolgus monkeys (Ryan et al. 1999). This species was chosen since cynomolgus monkey VEGF is identical to human VEGF at the protein level (Shima et al. 1996). After administration of bevacizumab for 4–13 weeks, young adult cynomolgus monkeys showed apparent mechanism-of-action-related effects such as physeal dysplasia and suppression of angiogenesis in the female reproductive tract. Both effects were reversible with cessation of treatment. No other treatment-related effects were seen, even at doses up to 50 mg kg^{-1} (Ryan et al. 1999).

The pharmacokinetics, ocular tissue distribution, and safety of the Fab fragment of bevacizumab (known as Fab-12) have also been studied following intravitreal administration. ^{125}Iodine labeling studies in rhesus monkeys showed that the intravitreal half-life of a Fab fragment of bevacizumab was 3.2 days, compared with 5.6 days for a full-length antibody (trastuzumab). The Fab reached the retinal pigment

epithelial layer within 1 h and was detectable within this layer for up to 7 days. In contrast, the full-length antibody was unable to penetrate the inner limiting membrane of the retina. Systemic exposure to the full-length antibody was variable but low, whereas the Fab fragment was not detected in the plasma at any time point. No adverse treatment-related effects were noted in this study (Mordenti et al. 1999).

4.2 Clinical Studies with Bevacizumab

Phase I clinical trials of bevacizumab began in 1997. FDA approval was first granted on the 26th of February 2004, following a successful phase III trial for treatment of metastatic colorectal cancer. In a randomized controlled trial of 813 patients with first-line metastatic colorectal cancer, the median duration of survival was 20.3 months in patients who received bevacizumab plus chemotherapy, compared to 15.6 months in those patients receiving chemotherapy alone (Hurwitz et al. 2004). FDA approval was then granted on the 11th of October 2006, for use of bevacizumab in combination with carboplatin and paclitaxel chemotherapy in metastatic, nonsquamous, nonsmall-cell lung cancer. Combination therapy resulted in a 25% improvement in survival compared to chemotherapy alone (Sandler and Herbst 2006). Many other clinical trials are currently under way, for indications including renal cell cancer, metastatic breast cancer, and cervical cancer (reviewed in Ranieri et al. 2006). Bevacizumab has the potential to significantly improve the standard of patient treatment in a wide variety of indications.

The most common side effects of bevacizumab treatment are hypertension, proteinuria, bleeding, and thrombosis (Zondor and Medina 2004). In most cases, hypertension during treatment can be managed with antihypertensive medications. Patients with proteinuria have been generally asymptomatic. Bleeding, thrombosis, and complications with wound healing are the most significant side effects of bevacizumab therapy. Importantly, there were no reported incidents of patients developing antibodies to bevacizumab (Ferrara 2004), suggesting that the humanization of A4.6.1 was successful.

5 Humanization and the Development of Ranibizumab

Preclinical and clinical studies show that VEGF is involved in the ocular neovascularisation associated with age-related macular degeneration (AMD) and diabetic retinopathy (Ferrara and Alitalo 1999). Anti-VEGF therapy is a promising new treatment for these conditions, particularly with the development of ranibizumab. Ranibizumab is a Fab fragment of an anti-VEGF antibody distinct from bevacizumab. Its smaller size allows penetration into the retina (Gaudreault et al. 2005) and more rapid clearance from the circulation than a full length antibody (cf. Gaudreault et al. 2005, Lin et al. 1999). Ranibizumab also has a higher affinity

for VEGF than does bevacizumab (Chen et al. 1999; Ferrara et al. 2004), allowing it to more efficiently inhibit VEGFR binding before it is cleared.

Clearance of a VEGF inhibitor is likely to reduce the incidence of treatment-related side effects. There is evidence that VEGF has multiple important roles in vivo. Safety studies in cynomolgus monkeys demonstrated that VEGF plays a part in bone growth, cyclic endometrial development, and placental vascularization (Ryan et al. 1999). VEGF has also been shown to be important in wound healing and psoriasis (Detmar et al. 1995), monocyte chemotaxis (Clauss et al. 1990), B cell production (Hattori et al. 2001), and neuronal function (Storkebaum et al. 2004). Given these findings and the known side effects of anti-VEGF therapy (hypertension, thrombotic events, and proteinuria), the use of an agent with rapid systemic clearance is particularly important for elderly or wound-healing compromised patients.

The origin of ranibizumab was A4.6.1, the same murine anti-human VEGF antibody that gave rise to bevacizumab. However, the humanization processes for bevacizumab and ranibizumab were quite different (Fig. 2). While bevacizumab was the product of site-directed mutagenesis of a CDR graft from A4.6.1, humanization to ranibizumab involved both phage display and site-directed mutagenesis. Initially, the CDRs of A4.6.1 were grafted into a phage-displayed Fab construct, hu2.0 (Fig. 1), with the C-terminal region of the Fab fused to a portion of the gene-3 protein of bacterophage M13 (Baca et al. 1997). Framework-region libraries were constructed and panned to yield a humanized Fab, MB1.6 (Fig. 1; Table 2), that bound VEGF with greater than 125-fold improved affinity than the CDR graft. Introduction of a single additional mutation, L46V, selected by rational design further improved

Table 2 Humanized anti-VEGF Fab constructs generated during ranibizumab development

Variant	Template	Changes
Chim-Fab	Chimeric Fab	Murine variable, human constant domains
Fab 12[1]	Human FR	VL: L46V VH: I69F, R71L, N73T, N76S, L78A, R94K
Hu2.0[2]	Human FR Fab phage vector	CDR swap with VH:S49G (see Table 1) and T221L in the C_H1 domain for fusion to M13 phage gene-3 protein
MB1.6[2]	Hu2.0	VL: M4L, F71Y VH: V37I, R71A, N73T, K75S, L78V, R94K
Y0101[3]	MB1.6	VL: L46V, Y71F VH: I37V, I69F, A71L, S75K, N76S, V79A
Y0192[3]	Y0101	VL: S24R, S26N, Q27E, D28Q, I29L VH: M34I
Y0243-1[4]	Y0192	VH: T28D, N31H, I34M
Y0238-3[4]	Y0192	VH: H97Y, S100aT
Y0317[4]	Y0192	VL: R24S, N26S, E27Q, Q28D, L29I VH: T28D, N31H, I34M, H97Y, S100aT

[1] Data taken from Presta et al. (1997)
[2] Data taken from Baca et al. (1997)
[3] Data taken from Muller et al. (1998)
[4] Data taken from Chen et al. (1999)

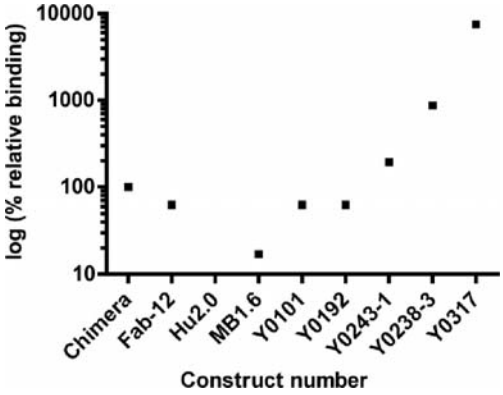

Fig. 5 Binding affinities during antibody MB1.6 affinity maturation leading to ranibizumab. Approximate changes in affinity of the humanized anti-VEGF Fab constructs relative to the binding of the chimeric Fab as measured by BIAcore studies (Baca et al. 1997; Muller et al. 1998; Chen et al. 1999) are plotted against construct number

the affinity to within sixfold of a human/murine chimera of A4.6.1 (Baca et al. 1997). Additional framework changes yielded MB1.6, which had similar affinity to Fab-12 and was used as the basis of clone Y0101 (Table 2; Muller et al. 1998). Y0101 was a phage displayed Fab with framework mutations identified during the humanization of bevacizumab (Presta et al. 1997), and two mutations from MB1.6 (Baca et al. 1997). Unfortunately, this variant expressed poorly. Further phage display efforts produced clone Y0192, which was significantly improved in expression (Muller et al. 1998).

Alanine scanning mutagenesis of Y0192 and a crystal structure highlighted the importance of the heavy-chain CDRs H1, H2, and H3 for VEGF binding (Muller et al. 1998). Directed phage display libraries were therefore created and panned, with resulting marked improvements in affinity (Table 2; Fig. 5). Mutations isolated from CDR H1 and H3 libraries were combined to create ranibizumab, also known as Y0317 or rhuFAb V2, a Fab with greater than 100-fold affinity improvement over Fab 12 as measured by surface plasmon resonance. Cell proliferation assays were used to confirm the increased biological potency of the affinity matured antibody (Chen et al. 1999).

The binding epitopes of Fab 12 and Y0317 were shown to be similar by affinity measurements of VEGF alanine scanning mutants and crystal structures of the Fab:VEGF complexes (Fig. 4, Fig. 6) (Chen et al. 1999; Muller et al. 1998). One notable difference was the presence of two additional hydrogen bonds in the complex between VEGF and Y0317 due to the CDR H3 mutation H97Y in the latter. The tyrosine substitution resulted in exclusion of a water molecule present in the Fab 12 structure, an increase in buried surface area, and additional hydrogen bonds, all of which may contribute to increased binding energy and slower complex-dissociation kinetics. The H97Y substitution was indeed shown to be the amino acid substitution contributing the largest portion of the increase in binding affinity to VEGF. Thus, the

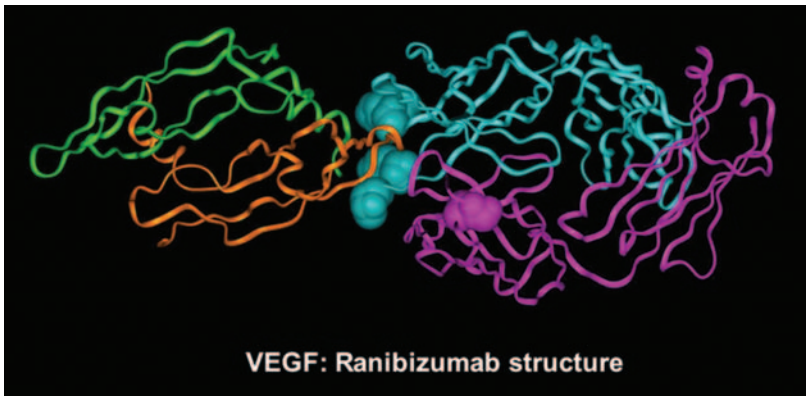

Fig. 6 Ranibizumab structure. The structure of ranibizumab (Y0317 Fab; heavy chain in *cyan*; light chain in *magenta*) in complex with VEGF (fragment 8-109; with one monomer in *orange* and the other in *green*) is shown based on the crystal structure reported in Chen et al. (1999) (PDB accession number 1CZ8). Heavy-chain CDR residues changed during affinity maturation of the Y0101 parental antibody are shown in space-filling form: T28D and N31H in CDR-H1 and H97Y and S100aT in CDR-H3. Also shown in space-filling form is the site M4L in the light chain variable framework region, which differs from bevacizumab. The site of one constant-domain change, made for cloning convenience, as compared to bevacizumab is near the C-terminus (bottom of the figure) of the heavy chain. This residue, T221L, is not shown in this figure

structural data correlated well with the 14-fold affinity improvement measured with this single mutation (Chen et al. 1999).

5.1 Preclinical Studies with Ranibizumab

The utility of an anti-VEGF for treatment of choroidal neovascularization has been demonstrated in a number of models (Adamis et al. 1996; Bashshur et al. 2006; Krzystolik et al. 2002; Michels et al. 2005; Rosenfeld et al. 2005a). However, because of concerns about the systemic inhibition of VEGF, the safety and pharmacokinetics of intravitreal injections of ranibizumab were of interest. Ranibizumab delivered intravitreally was demonstrated to penetrate all layers of the rabbit retina (Gaudreault et al. 1999), in contrast to a full length antibody (trastuzumab), which could not penetrate the inner limiting membrane of the retina in rhesus monkeys (Mordenti et al. 1999). Further, pharmacokinetic studies in cynomolgus monkeys showed that after a single intravitreal injection of ranibizumab, the Fab was present at biologically effective retinal levels for about a month, while serum levels were less than 1000th of the ocular levels. Any tissue distribution of ranibizumab was therefore below the limit of detection (Gaudreault et al. 2005). Combination treatment with ranibizumab and verteporfin therapy also demonstrated benefits in nonhuman primates (Husain et al. 2005; Kim et al. 2006). Verteporfin is a small-molecule dye that has been previously used in photodynamic therapy for AMD, in which

the dye is infused systemically and red light is used to activate its cytotoxic action locally on vascular structures in the eye (Schmidt-Erfurth and Michels 2003).

5.2 Clinical Studies with Ranibizumab

A phase 1 study in patients with AMD found that the maximum tolerated dose was 0.5 mg when injected intravitreally. The major dose limiting toxicity was due to intraocular inflammation (Rosenfeld et al. 2005b). However, in a dose-escalating study, clinically significant inflammation was not seen at doses of up to 2 mg per eye. There were no notable differences in clinical outcomes between study groups receiving different maximal doses, but this trial was insufficiently powered to detect small treatment benefits (Rosenfeld et al. 2006a). Significantly, no patients developed antibodies to ranibizumab in either study, suggesting that the substitution of new CDR and framework residues did not create significant new immunogenic determinants (Rosenfeld et al. 2006b, 2005a).

In December 2005, Genentech submitted a Biologics License Application to the FDA for the use of ranibizumab in the treatment of neovascular AMD based on the results of two phase three trials (Rosenfeld et al. 2006b; Rosenfeld et al. 2006c; Brown et al. 2006). In both trials, patients treated with ranibizumab demonstrated significantly better visual acuity than control patients at 1 and 2 year time-points. Adverse events were similar to those seen in earlier trials, with low rates of serious ocular adverse events, although slightly increased rates of myocardial infarction and stroke were noted at higher ranibizumab dose (Rosenfeld et al. 2006b). It is important to note that ranibizumab therapy is the first treatment for neovascular AMD that improves vision for most patients. When compared with verteporfin at 12 months (Brown et al. 2006), ranibizumab treatment led to 94.3–96.4% of patients losing fewer than 15 letters in visual acuity (0.3 and 0.5 mg doses, respectively) vs. 64.3% of patients losing fewer than 15 letters in the verteporfin group. Mean visual acuity increased by 8.5–11.3 letters (low and high doses) in the ranibizumab groups, while it decreased by 9.5 letters in the verteporfin group. Improvement in acuity was stable for at least 2 years. FDA approval for ranibizumab therapy was given on June 30, 2006.

6 Next Generation Anti-VEGF Antibodies

Next generation anti-VEGF antibodies might have improved VEGF affinity or might more efficiently block VEGF activity through binding to a novel epitope. Structural studies (Wiesmann et al. 1997) and alanine scanning mutagenesis (Li et al. 2000; Muller et al. 1997; Pan et al. 2002) show that VEGF receptors 1 and 2 bind similar epitopes on VEGF. Bevacizumab and ranibizumab bind to an epitope that only partially overlaps the receptor binding site. It, therefore, appears that bevacizumab

inhibits receptor binding by steric hindrance (Wiesmann et al. 1997). Thus, it is possible that mutations, which may arise in vivo, could abrogate bevacizumab binding without impacting receptor interaction.

The site of bevacizumab binding can be explained by considering the origin of the antibody. Mice were immunized with human VEGF to produce A4.6.1, the progenitor of bevacizumab. Since self-reactive antibodies are disfavored by immune tolerance in vivo, antibodies raised by the hybridoma technique will tend to bind to regions of the immunizing antigen distinct from that of any corresponding host protein. The sequence of the receptor binding regions of human and mouse VEGF are not completely conserved (Claffey et al. 1992; Leung et al. 1989). Thus, bevacizumab binding is centered around G88 (where the mouse sequence contains a Ser), located at the periphery of the receptor binding epitope (Wiesmann et al. 1997).

To identify a larger set of antibody specificities for a variety of therapeutic targets, phage-displayed synthetic antibody libraries that mimic the diversity of natural human antibodies have been developed at Genentech (Lee et al. 2004). Panning these libraries against VEGF resulted in novel, high affinity, antibodies capable of blocking both murine and human VEGF activity (Liang et al. 2006). Structural and functional analysis of these antibodies in complex with VEGF showed that their binding epitopes closely matched the epitope for VEGFR1 (Fuh et al. (2006)). Therefore, hypothetical mutations in VEGF that prevent the binding of these antibodies may also prevent VEGF:receptor interaction.

An additional desirable quality for new anti-VEGF antibodies is an ability to bind both mouse and human VEGF. This allows testing of the antibodies in a greater variety of model systems before progression to primate studies and address the contribution of host VEGF to xenograft growth. The antibodies isolated from synthetic phage display libraries possess this quality (Liang et al. 2006). Some of these novel antibodies had equivalent anti-VEGF activity to bevacizumab (B20 family of variants), while others had equivalent anti-VEGF activity to ranibizumab (G6 family). These novel reagents therefore not only allow an examination of the effects of host vs. tumor VEGF contribution to tumor growth in mouse models, but also a comparison of epitope and affinity effects upon efficacy and safety in mouse models (Liang et al. 2006; Gerber et al. 2007).

7 Conclusion

Bevacizumab (Avastin® anti-VEGF antibody) is a full-length, high-affinity IgG1 produced through humanization of antibody A4.6.1 from mice immunized with human VEGF. In vivo, bevacizumab may benefit from avidity effects to bind tightly to VEGF, effectively blocking VEGF receptor binding and signaling. This blockade results from steric hindrance of the ligand–receptor interaction since the antibody epitope overlaps – and is not identical to – the epitope of the VEGF receptors VEGFR-1 and VEGFR-2. As an intravenously injected molecule, the full-length antibody is maintained at high concentrations in the blood and binds

circulating VEGF in the vasculature. As a cancer therapeutic, bevacizumab is being investigated in a growing number of oncology indications and in combination with other therapies to more fully define its safety and efficacy. For example, some patients with metastatic colorectal have demonstrated prolonged survival over several years with treatment using bevacizumab in combination with chemotherapy, and the drug has generally remained well tolerated (Hurwitz et al. 2006).

Ranibizumab (Lucentis® anti-VEGF antibody) is the Fab form of Y0317, another humanized antibody variant based on A4.6.1. Ranibizumab was affinity-matured using phage display, and differs at five residues in the variable domains and one residue in the constant domain from bevacizumab. These changes led to ~100-fold higher binding affinity to VEGF and enabled the monomeric (Fab) form of this antibody to very effectively block VEGF binding to its receptors. The lower molecular weight (~48 kDa) of ranibizumab as compared to bevacizumab (~150 kDa) may enhance the activity of this molecule in blocking VEGF within retinal tissue following intraocular injection. At the same time, because of the unique route of delivery, the rapid systemic clearance of the Fab from the circulation allows for the maintenance of therapeutic drug concentrations at the site of disease with low systemic concentrations. Such clearance may be important for limiting possible side effects such as hemorrhagic events that are potentially associated with systemic exposure to VEGF inhibitors (Liew and Mitchell 2007). Ranibizumab is currently being investigated in additional eye diseases and in combination with other therapies, and long-term studies are underway to evaluate further its safety and efficacy.

References

Adamis AP, Miller JW, Bernal MT, D'Amico DJ, Folkman J, Yeo TK, Yeo KT (1994) Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. Am J Ophthalmol 118:445–450

Adamis AP, Shima DT, Tolentino MJ, Gragoudas ES, Ferrara N, Folkman J, D'Amore PA, Miller JW (1996) Inhibition of vascular endothelial growth factor prevents retinal ischemia-associated iris neovascularization in a nonhuman primate. Arch Ophthalmol 114:66–71

Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE et al. (1994) Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 331:1480–1487

Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E (1995) Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat Med 1:1024–1028

Baca M, Presta LG, O'Connor SJ, Wells JA (1997) Antibody humanization using monovalent phage display. J Biol Chem 272:10678–10684

Bashshur ZF, Bazarbachi A, Schakal A, Haddad ZA, El Haibi CP, Noureddin BN (2006) Intravitreal bevacizumab for the management of choroidal neovascularization in age-related macular degeneration. Am J Ophthalmol 142:1–9

Borgstrom P, Hillan KJ, Sriramarao P, Ferrara N (1996) Complete inhibition of angiogenesis and growth of microtumors by anti-vascular endothelial growth factor neutralizing antibody: Novel concepts of angiostatic therapy from intravital videomicroscopy. Cancer Res 56:4032–4039

Brown DM, Kaiser PK, Michels M, Soubrane G, Heier JS, Kim RY, Sy JP, Schneider S (2006) Ranibizumab versus verteporfin for neovascular age-related macular degeneration. New Eng J Med 355:1432–1444

Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME, Shepard HM (1992) Humanization of an anti-p185HER2 antibody for human cancer therapy. Proc Natl Acad Sci USA 89:4285–4289

Chen Y, Wiesmann C, Fuh G, Li B, Christinger HW, McKay P, de Vos AM, Lowman HB (1999) Selection and analysis of an optimized anti-VEGF antibody: Crystal structure of an affinity-matured Fab in complex with antigen. J Mol Biol 293:865–881

Chothia C, Lesk AM, Tramontano A, Levitt M, Smith-Gill SJ, Air G, Sheriff S, Padlan EA, Davies D, Tulip WR et al. (1989) Conformations of immunoglobulin hypervariable regions. Nature 342:877–883

Claffey KP, Wilkison WO, Spiegelman BM (1992) Vascular endothelial growth factor. Regulation by cell differentiation and activated second messenger pathways. J Biol Chem 267:16317–16322

Clauss M, Gerlach M, Gerlach H, Brett J, Wang F, Familletti PC, Pan YC, Olander JV, Connolly DT, Stern D (1990) Vascular permeability factor: A tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. J Exp Med 172:1535–1545

de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT (1992) The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science 255:989–991

Detmar M, Yeo KT, Nagy JA, Van de Water L, Brown LF, Berse B, Elicker BM, Ledbetter S, Dvorak HF (1995) Keratinocyte-derived vascular permeability factor (vascular endothelial growth factor) is a potent mitogen for dermal microvascular endothelial cells. J Invest Dermatol 105:44–50

Dor Y, Porat R, Keshet E (2001) Vascular endothelial growth factor and vascular adjustments to perturbations in oxygen homeostasis. Am J Physiol Cell Physiol 280:C1367–C1374

Ferrara N (2004) Vascular endothelial growth factor: Basic science and clinical progress. Endocr Rev 25:581–611

Ferrara N, Alitalo K (1999) Clinical applications of angiogenic growth factors and their inhibitors. Nat Med 5:1359–1364

Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. Nat Med 9:669–676

Ferrara N, Henzel WJ (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 161:851–858

Ferrara N, Hillan KJ, Gerber HP, Novotny W (2004) Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov 3:391–400

Folkman J (1971) Tumor angiogenesis: Therapeutic implications. N Eng J Med 285:1182–1186

Fuh G, Wu P, Liang WC, Ultsch M, Lee CV, Moffat B, Wiesmann C (2006) Structure-function studies of two synthetic anti-vascular endothelial growth factor Fabs and comparison with the Avastin Fab. J Biol Chem 281:6625–6631

Gaudreault J, Fei D, Rusit J, Suboc P, Shiu V (2005) Preclinical pharmacokinetics of Ranibizumab (rhuFabV2) after a single intravitreal administration. Invest Ophthalmol Vis Sci 46:726–733

Gaudreault J, Webb W, Van Hoy M (1999) Pharmacokinetics and retinal distribution of AMD rhuFab V2 after intravitreal administration in rabbits. AAPS Pharm Sci Suppl 1:2142

Gerber HP, Ferrara N (2005) Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies. Cancer Res 65:671–680

Gerber HP, Wu X, Yu L, Wiesmann C, Liang XH, Lee CV, Fuh G, Olsson C, Damico L, Xie D, Meng YG, Gutierrez J, Corpuz R, Li B, Hall L, Rangell L, Ferrando R, Lowman H, Peale F, Ferrara N (2007) Mice expressing a humanized form of VEGF-A may provide insights into the safety and efficacy of anti-VEGF antibodies. Proc Natl Acad Sci USA 104:3478–3483

Gimbrone MA, Leapmaan SB, Cotran RS, Folkman J (1972) Tumor dormancy in vivo by prevention of neovascularization. J Exp Med 136:261–276

Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M, Hicklin DJ, Zhu Z, Witte L, Crystal RG, Moore MA, Rafii S (2001) Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. J Exp Med 193:1005–1014

Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW (1991) The vascular endothelial growth factor family: Identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol 5:1806–1814

Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N (1992) Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. J Biol Chem 267:26031–26037

Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, Berlin J, Baron A, Griffing S, Holmgren E, Ferrara N, Fyfe G, Rogers B, Ross R, Kabbinavar F (2004) Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Eng J Med 350:2335–2342

Hurwitz HI, Honeycutt W, Haley S, Favaro J (2006) Long-term treatment with bevacizumab for patients with metastatic colorectal cancer: Case report. Clin Colorectal Cancer 6:66–69

Husain D, Kim I, Gauthier D, Lane AM, Tsilimbaris MK, Ezra E, Connolly EJ, Michaud N, Gragoudas ES, O'Neill CA, Beyer JC, Miller JW (2005) Safety and efficacy of intravitreal injection of ranibizumab in combination with verteporfin PDT on experimental choroidal neovascularization in the monkey. Arch Ophthalmol 123:509–516

Ide AG, Baker NH, Warren SL (1939) Vascularization of the Brown Pearce rabbit epithelioma transplant as seen in the transparent ear chamber. Am J Roentgenol 42:891–899

Kabat EA, Wu TT, Perry H, Gottesmann KS, Foeller C (1991) Sequences of proteins of immunological interest. Public Health Service, National Institutes of Health, Bethesda, MD

Kim IK, Husain D, Michaud N, Connolly EJ, Lane AM, Durrant K, Hafezi-Moghadam A, Gragoudas ES, O'Neill CA, Beyer JC, Miller JW (2006) Effect of the intravitreal injection of ranibizumab in combination with verteporfin PDT on normal primate retina and choroids. Invest Ophthalmol Vis Sci 47:357–363

Kim KJ, Li B, Houck K, Winer J, Ferrara N (1992) The vascular endothelial growth factor proteins: Identification of biologically relevant regions by neutralizing monoclonal antibodies. Growth Factors 7:53–64

Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N (1993) Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth in vivo. Nature 362:841–844

Krzystolik MG, Afshari MA, Adamis AP, Gaudreault J, Gragoudas ES, Michaud NA, Li W, Connolly E, O'Neill CA, Miller JW (2002) Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. Arch Ophthalmol 120:338–346

Lee CV, Liang WC, Dennis MS, Eigenbrot C, Sidhu SS, Fuh G (2004) High-affinity human antibodies from phage-displayed synthetic Fab libraries with a single framework scaffold. J Mol Biol 340:1073–1093

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306–1309

Li B, Fuh G, Meng G, Xin X, Gerritsen ME, Cunningham B, de Vos AM (2000) Receptor-selective variants of human vascular endothelial growth factor. Generation and characterization. J Biol Chem 275:29823–29828

Liang WC, Wu X, Peale FV, Lee CV, Meng YG, Gutierrez J, Fu L, Malik AK, Gerber HP, Ferrara N, Fuh G (2006) Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. J Biol Chem 281:951–961

Liew G, Mitchell P (2007) Ranibizumab for neovascular age-related macular degeneration. N Eng J Med 356:747–750

Lin YS, Nguyen C, Mendoza JL, Escandon E, Fei D, Meng YG, Modi NB (1999) Preclinical pharmacokinetics, interspecies scaling, and tissue distribution of a humanized monoclonal antibody against vascular endothelial growth factor. J Pharmacol Exp Ther 288:371–378

Malecaze F, Clamens S, Simorre-Pinatel V, Mathis A, Chollet P, Favard C, Bayard F, Plouet J (1994) Detection of vascular endothelial growth factor messenger RNA and vascular endothelial growth factor-like activity in proliferative diabetic retinopathy. Arch Ophthalmol 112:1476–1482

Melnyk O, Shuman MA, Kim KJ (1996) Vascular endothelial growth factor promotes tumor dissemination by a mechanism distinct from its effect on primary tumor growth. Cancer Res 56:921–924

Michels S, Rosenfeld PJ, Puliafito CA, Marcus EN, Venkatraman AS (2005) Systemic bevacizumab (Avastin) therapy for neovascular age-related macular degeneration twelve-week results of an uncontrolled open-label clinical study. Ophthalmology 112:1035–1047

Millauer B, Wizigmann-Voos S, Schnurch H, Martinez R, Moller NP, Risau W, Ullrich A (1993) High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell 72:835–846

Mordenti J, Cuthbertson RA, Ferrara N, Thomsen K, Berleau L, Licko V, Allen PC, Valverde CR, Meng YG, Fei DT, Fourre KM, Ryan AM (1999) Comparisons of the intraocular tissue distribution, pharmacokinetics, and safety of 125I-labeled full-length and Fab antibodies in rhesus monkeys following intravitreal administration. Toxicol Pathol 27:536–544

Muller YA, Chen Y, Christinger HW, Li B, Cunningham BC, Lowman HB, de Vos AM (1998) VEGF and the Fab fragment of a humanized neutralizing antibody: Crystal structure of the complex at 2.4 Å resolution and mutational analysis of the interface. Structure 6:1153–1167

Muller YA, Christinger HW, Keyt BA, de Vos AM (1997) The crystal structure of vascular endothelial growth factor (VEGF) refined to 1.93 Å resolution: Multiple copy flexibility and receptor binding. Structure 5:1325–1338

Pan B, Li B, Russell SJ, Tom JY, Cochran AG, Fairbrother WJ (2002) Solution structure of a phage-derived peptide antagonist in complex with vascular endothelial growth factor. J Mol Biol 316:769–787

Park JE, Keller GA, Ferrara N (1993) The vascular endothelial growth factor (VEGF) isoforms: Differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol Biol Cell 4:1317–1326

Presta LG, Chen H, O'Connor SJ, Chisholm V, Meng YG, Krummen L, Winkler M, Ferrara N (1997) Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res 57:4593–4599

Quinn TP, Peters KG, De Vries C, Ferrara N, Williams LT (1993) Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. Proc Natl Acad Sci USA 90:7533–7537

Ranieri G, Patruno R, Ruggieri E, Montemurro S, Valerio P, Ribatti D (2006) Vascular endothelial growth factor (VEGF) as a target of bevacizumab in cancer: From the biology to the clinic. Curr Med Chem 13:1845–1857

Rosenfeld PJ, Heier JS, Hantsbarger G, Shams N (2006a) Tolerability and efficacy of multiple escalating doses of ranibizumab (Lucentis) for neovascular age-related macular degeneration. Ophthalmology 113:623–632

Rosenfeld PJ, Moshfeghi AA, Puliafito CA (2005a) Optical coherence tomography findings after an intravitreal injection of bevacizumab (avastin) for neovascular age-related macular degeneration. Ophthalmic Surg Lasers Imaging 36:331–335

Rosenfeld PJ, Schwartz SD, Blumenkranz MS, Miller JW, Haller JA, Reimann JD, Greene WL, Shams N (2005b) Maximum tolerated dose of a humanized anti-vascular endothelial growth factor antibody fragment for treating neovascular age-related macular degeneration. Ophthalmology 112:1048–1053

Rosenfeld PJ, Rich RM, Lalwani GA (2006b) Ranibizumab: Phase III clinical trial results. Ophthalmol Clin North Am 19:361–372

Rosenfeld PJ, Brown DM, Heier JS, Boyer DS, Kaiser PK, Chung CY, Kim RY (2006c) Ranibizumab for neovascular age-related macular degeneration. New Eng J Med 355:1419–1431

Ryan AM, Eppler DB, Hagler KE, Bruner RH, Thomford PJ, Hall RL, Shopp GM, O'Neill CA (1999) Preclinical safety evaluation of rhuMAbVEGF, an antiangiogenic humanized monoclonal antibody. Toxicol Pathol 27:78–86

Sandler A, Herbst R (2006) Combining targeted agents: blocking the epidermal growth factor and vascular endothelial growth factor pathways. Clin Cancer Res 12:4421s–4425s

Schmidt-Erfurth UM, Michels S (2003) Changes in confocal indocyanine green angiography through two years after photodynamic therapy with verteporfin. Ophthalmology 110:1306–1314

Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, Sato M (1990) Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene 5:519–524

Shima DT, Gougos A, Miller JW, Tolentino M, Robinson G, Adamis AP, D'Amore PA (1996) Cloning and mRNA expression of vascular endothelial growth factor in ischemic retinas of Macaca fascicularis. Invest Ophthalmol Vis Sci 37:1334–1340

Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M (1998) Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. Cell 92:735–745

Stacker SA, Achen MG, Jussila L, Baldwin ME, Alitalo K (2002) Lymphangiogenesis and cancer metastasis. Nat Rev Cancer 2:573–583

Stone J, Itin A, Alon T, Pe'er J, Gnessin H, Chan-Ling T, Keshet E (1995) Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. J Neurosci 15:4738–4747

Storkebaum E, Lambrechts D, Carmeliet P (2004) VEGF: Once regarded as a specific angiogenic factor, now implicated in neuroprotection. Bioessays 26:943–954

Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Bohlen P (1992) Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem Biophys Res Commun 187:1579–1586

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA (1991) The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 266:11947–11954

Warren RS, Yuan H, Matli MR, Gillett NA, Ferrara N (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

Wiesmann C, Fuh G, Christinger HW, Eigenbrot C, Wells JA, de Vos AM (1997) Crystal structure at 1.7 Å resolution of VEGF in complex with domain 2 of the Flt-1 receptor. Cell 91:695–704

Zondor SD, Medina PJ (2004) Bevacizumab: an angiogenesis inhibitor with efficacy in colorectal and other malignancies. Ann Pharmacother 38:1258–1264