

DEVELOPMENT OF RANIBIZUMAB, AN ANTI-VASCULAR ENDOTHELIAL GROWTH FACTOR ANTIGEN BINDING FRAGMENT, AS THERAPY FOR NEOVASCULAR AGE-RELATED MACULAR DEGENERATION

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Background: Angiogenesis is a key aspect of the wet form of age-related neovascular (AMD), the leading cause of blindness in the elderly population. Substantial evidence indicated that vascular endothelial growth factor (VEGF)-A is a major mediator of angiogenesis and vascular leakage in wet AMD. VEGF-A is the prototype member of a gene family that includes also PlGF, VEGF-B, VEGF-C, VEGF-D and the orf virus-encoded VEGF-E. Several isoforms of VEGF-A can be generated due to alternative mRNA splicing. Various VEGF inhibitors have been clinically developed. Among these, ranibizumab is a high affinity recombinant Fab that neutralizes all isoforms of VEGF-A. The article briefly reviews the biology of VEGF and then focuses on the path that led to clinical development of ranibizumab.

Results: The safety and efficacy of ranibizumab in the treatment of neovascular AMD have been evaluated in two large phase III, multicenter, randomized, double-masked, controlled pivotal trials in different neovascular AMD patient populations. Combined, the trial results indicate that ranibizumab results not only in a slowing down of vision loss but also in a significant proportion of patients experiencing a clinically meaningful vision gain. The visual acuity benefit over control was observed regardless of CNV lesion type. Furthermore, the benefit was associated with a low rate of serious adverse events.

Conclusions: Ranibizumab represents a novel therapy that, for the first time, appears to have the potential to enable many AMD patients to obtain a meaningful and sustained gain of vision. On June 30 2006, ranibizumab was approved by the US Food and Drug Administration for the treatment of wet AMD.

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Extensive research over the last 2 decades has shown that angiogenesis is a highly complex and coordinated process, requiring the sequential activa-

tion of a series of receptors in endothelial and mural cells by numerous ligands.^{1–3} In addition, several inhibitors of angiogenesis have been identified.⁴ However, in spite of such complexity and potential redundancy, vascular endothelial growth factor (VEGF)–A appears to be required for growth of blood vessels in a variety of normal and pathologic circumstances.⁵

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Mylan v. Regeneron, IPR2021-00881
U.S. Pat. 9,254,338, Exhibit 2055

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VEGF-A is the prototype member of a gene family that also includes placenta growth factor, VEGF-B, VEGF-C, VEGF-D, and the orf virus–encoded VEGF-E.⁶

Diabetes mellitus, central retinal vein obstruction, and retinopathy of prematurity can all be associated with retinal ischemia and intraocular neovascularization, which may result in vitreous hemorrhages, retinal detachment, neovascular glaucoma, and blindness.^{7,8} As early as 1948, Michaelson⁹ hypothesized that the ischemic retina releases an angiogenic “factor X.” Much research has elucidated the role of VEGF-A as a mediator of ischemia-related intraocular neovascularization.^{10–12}

Neovascularization and leakage are also prominent features of the wet form of age-related macular degeneration (AMD).⁸ Definitive clinical trials have demonstrated that VEGF is important in the progression of AMD. In December 2004, the US Food and Drug Administration approved pegaptanib (Macugen; Eyetech/OSI), an aptamer that blocks the 165-amino acid isoform of VEGF-A, for the treatment of wet AMD.^{13,14} In parallel, ranibizumab (Lucentis), a high-affinity recombinant antigen binding fragment (Fab) that neutralizes all isoforms of VEGF-A (codeveloped by Genentech, Inc., and Novartis), has shown efficacy in large randomized phase 3 studies.¹⁵ Ranibizumab was approved by the US Food and Drug Administration for the treatment of wet AMD in June 2006. The main purpose of this article is to briefly review the biology of VEGF and then focus on the path that led to clinical development of ranibizumab, summarizing early clinical findings.

History of VEGF

Independent lines of research contributed to the discovery of VEGF.⁵ In 1983, Senger et al¹⁶ at Beth Israel Hospital in Boston reported the identification in the supernatant of a guinea pig tumor cell line of a permeability-enhancing protein, which was named vascular permeability factor (VPF). However, these efforts did not yield the full purification of the VPF protein. The lack of amino acid sequence information precluded cDNA cloning and elucidation of the identity of VPF for several years.

In 1989, we reported the isolation of an endothelial cell–specific mitogen from the supernatant of bovine pituitary cells, which we named VEGF.¹⁷ The NH₂-terminal amino acid sequence of VEGF did not match any known protein in available databases.¹⁷ Subsequently, Connolly et al¹⁸ at Monsanto Company reported the isolation and sequencing of VPF. By the end of 1989, we reported the isolation of cDNA clones

for bovine VEGF₁₆₄ and three human VEGF isoforms: VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉.¹⁹ The Monsanto group described a human VPF clone, which encoded a protein identical to VEGF₁₈₉.²⁰ These findings indicated that VEGF and VPF were, surprisingly, the same molecule.

VEGF Isoforms

Alternative exon splicing results in the generation of four main VEGF-A isoforms with 121, 165, 189, and 206 amino acids after signal sequence cleavage: VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, and VEGF₂₀₆, respectively.^{21,22} Several additional minor splice variants also have been described.⁶ VEGF₁₆₅ is the predominant isoform. Similar to VEGF₁₆₅, native VEGF is a heparin-binding homodimeric glycoprotein of 45 kd.^{17,23} In contrast, VEGF₁₂₁ lacks heparin-binding properties.²⁴ VEGF₁₈₉ and VEGF₂₀₆ bind to heparin with particularly high affinity, due to the presence of a highly basic 24-amino acid insertion.²⁴ Although VEGF₁₂₁ is a freely diffusible protein, VEGF₁₈₉ and VEGF₂₀₆ are almost completely sequestered in the extracellular matrix. VEGF₁₆₅ has intermediate properties.²⁵ The extracellular matrix–bound isoforms may be released in a diffusible form by plasmin cleavage at the COOH terminus, which generates VEGF₁₁₀, a bioactive fragment consisting of the first 110 NH₂-terminal amino acids.^{24,26} VEGF₁₆₅ may also undergo a similar processing by plasmin.^{24,26}

More recent studies have shown that various matrix metalloproteases (MMPs)—especially MMP-3—can also cleave VEGF₁₆₅ to generate diffusible, non-heparin-binding fragments.²⁷ Proteolytic processing of VEGF₁₆₅ by MMP-3 takes place in sequential steps, with initial cleavage occurring at residues 135, 120, and, finally, 113.²⁷ Thus, the final product of MMP-3 processing, VEGF₁₁₃, is expected to be biologically and biochemically very similar to the plasmin-generated VEGF fragment.

According to recent studies, levels of plasminogen and MMP-3 in the vitreous of patients affected by proliferative retinopathies are higher than those in controls, providing evidence for a prodegradative environment that may locally generate non-heparin-binding VEGF fragments (T.A. Ciulla, L. Yu, J. Lowe, et al, unpublished observation, 2006). In summary, not only alternative RNA splicing but also extracellular proteolysis regulates the activity of VEGF. Proteolytic mechanisms are known to be important during pathologic angiogenesis,²⁸ suggesting that non-heparin-binding, cleaved forms of VEGF-A may play an especially significant role.

Biologic Roles of VEGF-A

VEGF-A promotes growth of vascular endothelial cells from arteries, veins, and lymphatics.²⁹ VEGF-A also induces angiogenesis *in vivo*.^{19,30} Administration of VEGF also induces rapid increases in microvascular permeability in several experimental model systems.³¹ VEGF-A is essential for embryonic vasculogenesis and angiogenesis in the mouse such that inactivation of a single VEGF allele results in embryonic lethality.³² Recent tissue-specific gene targeting studies have also documented the key role of VEGF-A in the development of the choroid vasculature.³³

VEGF-A is also a survival factor for vascular endothelial cells.^{34–36} VEGF prevents endothelial apoptosis induced by serum starvation in a PI 3' kinase/Akt-dependent manner.³⁵ *In vivo*, a marked VEGF dependence has been demonstrated in endothelial cells of newly formed vessels.^{36,37} Coverage by pericytes is one of the key events resulting in loss of VEGF-A dependence.³⁶ Administration of VEGF inhibitors results in changes in the normal and pathologic microvasculature, and fenestrated vessels appear to be especially sensitive to VEGF withdrawal.^{37–39} In tumor-associated vessels, VEGF-A blockade results in “pruning” of excessive endothelial cells and in a series of morphologic and functional changes (including an increase in the proportion of endothelial cells covered by pericytes and reduced permeability), which have been referred to as “normalization.”⁴⁰ VEGF has some effects outside the vascular endothelium. In particular, VEGF has been reported to have nonvascular, neurotrophic effects on the developing retina.⁴¹

Much evidence indicates that VEGF-A is a mediator of tumor angiogenesis.⁶ In 1993, a murine monoclonal antibody (Mab) targeting human VEGF-A (Mab A.4.6.1) was shown to inhibit tumor growth *in vivo*.⁴² Other anti-VEGF agents, including small molecule VEGF receptor tyrosine kinase inhibitors, have also demonstrated antitumor activity.⁴³ Clinical trials with the humanized variant of Mab A.4.6.1,⁴⁴ known as bevacizumab (Avastin; Genentech, Inc.), began in 1997.⁴⁵ Being a full-length immunoglobulin G (IgG) and thus endowed with a long systemic half-life, bevacizumab is usually administered every 2 weeks or 3 weeks. After a phase 3 study showing a survival benefit,⁴⁶ bevacizumab was approved by the US Food and Drug Administration in 2004 as first-line treatment of metastatic colorectal cancer in combination with 5-fluorouracil-based chemotherapy regimens.⁴⁷ In 2006, bevacizumab also was approved by the US Food and Drug Administration for the treatment of relapsed metastatic colorectal cancer in combination with 5-fluorouracil-based chemotherapy regimens.

Clinical trials testing bevacizumab, as well as other VEGF inhibitors, for treatment of several malignancies are ongoing.⁴⁷

VEGF Receptors

VEGF-A binds two highly related receptor tyrosine kinases, VEGF receptor (VEGFR)–1⁴⁸ and VEGFR–2.^{49,50} VEGFR–1 was the first receptor tyrosine kinase to be identified as a VEGFR >1 decade ago,⁵¹ but the precise function of this molecule is still object of debate.⁶ VEGFR–1 functions and signaling properties appear to be different depending on the developmental stage and the cell type (e.g., endothelial vs nonendothelial cells). VEGFR–1 binds not only VEGF-A but also placenta growth factor⁵² and VEGF-B⁵³ and fails to mediate a strong mitogenic signal in endothelial cells. Nonmitogenic functions mediated by VEGFR–1 in the vascular endothelium include the release of growth factors⁵⁴ and the induction of MMP-9.⁵⁵ Furthermore, VEGFR–1 mediates monocyte chemotaxis in response to VEGF-A or placenta growth factor,⁵⁶ and it is believed that VEGF-dependent recruitment of inflammatory cells is largely mediated by VEGFR–1.^{6,57}

There is now agreement that VEGFR–2 is the major mediator of the mitogenic, angiogenic, and permeability-enhancing effects of VEGF.^{6,58} The importance of VEGFR–2 signaling is underscored by lack of vasculogenesis and failure to develop blood islands in VEGFR–2-null mice.⁵⁹

In addition to the two receptor tyrosine kinases, VEGF₁₆₅ (but not VEGF₁₂₁) binds nonsignaling coreceptors, neuropilin 1 and neuropilin 2 (two molecules previously implicated in axon guidance).^{60,61} Neuropilin 1 appears to present VEGF₁₆₅ to VEGFR–2 in a configuration that increases the effectiveness of VEGFR–2-mediated signal transduction.⁶²

Role of VEGF in Intraocular Neovascular Syndromes

The expression of VEGF mRNA is spatially and temporally correlated with neovascularization in several animal models of retinal ischemia.^{34,63,64} This agrees with the notion that VEGF-A gene expression is upregulated by hypoxia, via HIF-dependent transcriptional activation.⁶⁵ In 1994, elevations of VEGF levels in the aqueous and vitreous humor of human eyes with proliferative retinopathy secondary to diabetes and other conditions were first described.^{66,67} Subsequently, animal studies using various VEGF inhibitors, including soluble VEGF receptor chimeric proteins,⁶⁸ Mabs,⁶⁹ antisense oligonucleotides,⁷⁰ and small molecule VEGFR–2 kinase inhibitors,⁷¹ have

directly demonstrated the role of VEGF as a mediator of ischemia-induced intraocular neovascularization.

As already noted, choroidal neovascularization (CNV) and vascular leakage are features of AMD.⁸ AMD is the most common cause of severe, irreversible vision loss in older adults.⁷² AMD is classified into nonexudative (dry) and exudative (wet or neovascular) disease. Although the exudative form accounts for $\approx 10\%$ to 20% of cases, it is responsible for 80% to 90% of the visual loss associated with AMD.⁷³

Early studies demonstrated the immunohistochemical localization of VEGF in surgically resected choroidal neovascular membranes from AMD patients.^{74,75} Interestingly, transgenic mice with increased VEGF expression in photoreceptors were found to develop neovascularization reminiscent of lesions seen in humans with neovascular AMD.⁷⁶ It is unclear whether VEGF upregulation in AMD lesions is hypoxia related.⁷⁷ Much recent evidence indicates that inflammation plays an important role in the pathogenesis of AMD. In particular, a polymorphism in the gene encoding complement factor H, which normally inhibits the alternative complement pathway, has been identified as a significant risk factor for AMD, at least among whites.⁷⁸ Thus, complement activation, inflammation, and subsequent vascular damage in drusen might contribute to increased expression of VEGF-A. Furthermore, several proinflammatory cytokines may upregulate VEGF-A in the microenvironment of CNV lesions.⁶

Intravitreal administration of ranibizumab resulted in marked suppression of neovascularization and leakage in a primate model of CNV.⁷⁹ In addition, the "VEGF-trap," a chimeric soluble VEGF receptor, has been reported to reduce neovascularization in a murine model of CNV.⁸⁰ Interestingly, these studies show that systemic administration of the VEGF-trap inhibits neovascularization by $\approx 75\%$. However, intravitreal administration of the same agent resulted in $\approx 25\%$ inhibition.⁸⁰ This limited efficacy occurred in spite of the high binding affinity of the VEGF-trap for VEGF,⁸¹ and it may be due, at least in part, to the existence of a barrier to the transretinal penetration of large molecules such as the VEGF-trap.⁸²

Path Toward Development of an Anti-VEGF Therapy for AMD

In 1996, after the humanization of murine Mab A.4.6.1 into what was later known as bevacizumab was completed,⁴⁴ cancer was the primary clinical target of our anti-VEGF program. Other conditions were also examined, and among those, neovascular AMD received significant consideration. However, an anti-

VEGF program for AMD would be far from straightforward. By 1996, inhibition studies had provided the proof-of-concept that established the important role of VEGF-A in tumor⁴² and ischemia-related retinal^{68,69} angiogenesis. In contrast, no such direct studies had been done in the setting of CNV, and a role for VEGF-A in AMD was suggested by its immunohistochemical localization in human CNV lesions.^{74,75} Moreover, it was debatable whether the animal models of CNV—typically created by laser-induced injury of Bruch membrane—replicate the pathogenesis of human AMD. Thus, the hypothesis that VEGF is involved in AMD had to rely to a large degree on extrapolating from other disease models, but the magnitude of the clinical problem and the unmet need were vast.

Another key question was how to deliver the inhibitor in such a way that it effectively reaches CNV lesions. We initially considered testing the intravenous administration of bevacizumab. The choroidal capillaries are fenestrated,⁸³ and there is no apparent barrier to the diffusion of systemically administered macromolecules into CNV. Direct experimental evidence that intravenously injected fluoresceinated bevacizumab or control IgG leaks from CNV in a primate model was then obtained.⁸⁴ However, the safety of prolonged systemic exposure to an anti-VEGF agent was a potential concern. Although toxicological studies of young adult monkeys receiving intravenously administered bevacizumab had not revealed any major safety signals,⁸⁵ the possibility that adverse effects might occur in an elderly population such as AMD patients, frequently affected by cardiovascular diseases, could not be ruled out. Furthermore, initial clinical studies of systemic administration of bevacizumab in cancer patients showed hints of serious toxicity, such as bleeding.⁸⁶ Given these unknowns, we concluded that, for the treatment of AMD, intraocular administration of an anti-VEGF agent would probably be safer and thus preferable. Several years later, it became known that systemic bevacizumab given in combination with cytotoxic chemotherapy to cancer patients results in a twofold increase in the incidence of arterial thromboembolic events relative to chemotherapy alone and that age of older than 60 years and a history of thromboembolism are risk factors.⁴⁷

There were significant hurdles to overcome in the development of a local anti-VEGF therapy for AMD, because there was no known precedent of long-term administration of proteins in the human eye. There was very limited information on the ability of intravitreally injected molecules to penetrate across the retinal layers and reach the choroid, but the available

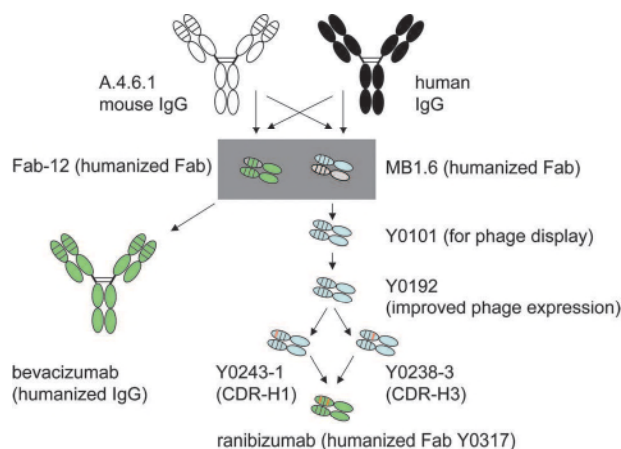


Fig. 1. Genealogy of bevacizumab and ranibizumab. The diagram illustrates steps in the construction of each antibody, beginning with the mouse hybridoma clone A.4.6.1. The different fill colors indicate distinct frameworks for the two parental humanized antigen binding fragments (Fabs) of the two lineages. Each arrow indicates one or more recombinant DNA steps used to construct genes encoding the corresponding immunoglobulin G (IgG) or Fab form of the antibody variants.^{44,93,94,96} The selection process yielded two independent affinity-improved variants, Y0243-1 and Y0238-3. The amino acid changes were combined and introduced in the context of Fab-12 (the Fab variant of bevacizumab) to generate the final product, ranibizumab, as described in the text.

evidence suggested the existence of a size-dependent barrier.⁸⁷ To address these questions, in studies conducted in 1996 and earlier, we compared the abilities of intravitreally injected ¹²⁵I-labeled full-length IgG (molecular weight, 150 kD) and Fab (molecular weight, 48 kD) antibodies to penetrate across the retina of rhesus monkeys⁸⁸ and rabbits (authors' unpublished data). At that time, the humanization of Mab A.4.6.1 had just been completed, and recombinant anti-VEGF antibody (bevacizumab) with a level of purity suitable for intraocular administration was not yet available. Pilot studies had shown that the presence of even low levels of impurities could induce a strong inflammatory response in the primate eye. For primate studies, clinical grade trastuzumab (Herceptin, Genentech, San Francisco, CA), a humanized antibody with a framework very similar to that of bevacizumab, was compared with the Fab version of bevacizumab.⁸⁸ In engineering bevacizumab, development of the Fab preceded assembly of the full-length IgG version⁴⁴; therefore, the bevacizumab Fab, also known as Fab-12 (see Fig. 1 for genealogy of anti-VEGF antibodies), was available. For the rabbit studies, full-length anti-VEGF Mab A.4.6.1 was compared with a mouse/human chimeric Fab. These studies showed that, under the conditions tested, the Fabs were more diffusible than the IgGs; the penetration of the Fabs was rapid and complete, whereas most of the IgG stopped either at the internal limiting membrane

(monkey)⁸⁸ or at the outer plexiform layer (rabbit). These results were entirely consistent with the finding that smaller antibody moieties such as single-chain variable fragments and Fabs have better tissue penetration than IgGs in other contexts.⁸⁹ Furthermore, a study reported several years later by Jackson et al⁸² also strongly supported our earlier conclusion. In a comparative analysis of the ability of FITC-dextran of different molecular weights to diffuse across human, porcine, bovine, and rabbit retinas, it was shown that there is a retinal exclusion limit of ≈ 76 kD for the human retina with similar values for the retinas of the other species examined; the inner and outer plexiform layers were found to be the sites of highest resistance to the diffusion of large molecules.⁸²

In apparent conflict with these findings, Shahar et al⁹⁰ recently reported that intravitreal injection of bevacizumab in the rabbit eye results in penetration throughout the retina (but not within the retinal pigment epithelium or choroid), as assessed by immunohistochemical analysis using an anti-Fc antibody. However, the dose of bevacizumab injected was ≈ 100 times higher than the dose of antibodies administered in the aforementioned primate study.⁸⁸ Furthermore, immunohistochemical analysis is a sensitive but not quantitative technique, and thus, it is very difficult to estimate the amount of bevacizumab that penetrated the retina in the study by Shahar et al.⁹⁰

In addition to greater retinal penetration, a Fab appeared preferable to a full-length IgG also on the basis of other characteristics. For example, the absence of Fc eliminated the possibility of complement-mediated or cell-dependent cytotoxicity that might be triggered by interaction of the antibody with Fc receptors in inflammatory or immune cells.⁹¹ Furthermore, the systemic half-life of a Fab is a few hours, while that of a full-length IgG is up to 3 weeks.⁹² Therefore, any systemic exposure to an anti-VEGF agent would be minimized by using a Fab.

Ranibizumab, A High-Affinity Anti-VEGF Fab

Systemic administration of antibodies (e.g., for cancer therapy) is usually done every week or every 2 weeks; thus, concentrations of antibody in excess to the antigen can be easily maintained. However, intraocular administration presented new challenges. The desire to minimize the frequency of invasive intravitreal injections prompted us to strive to generate a VEGF antagonist with the highest possible biologic potency, ideally able to completely neutralize VEGF-A in the microenvironment of CNV lesions even when the ratio of inhibitor to VEGF is low.

To this end, we chose to engineer a Fab of the

humanized anti-VEGF to further enhance its binding affinity, taking advantage of earlier crystal structure and mutagenesis studies of such Fab and its antigen VEGF.⁹³ Whereas bevacizumab was directly derived from one humanized Fab variant, known as Fab-12 as previously described,⁴⁴ ranibizumab was initially derived from a different humanized anti-VEGF Fab variant, known as MB1.6,^{93,94} through a series of recombinant DNA and phage-display selection steps (Fig. 1). MB1.6 resulted from a parallel effort at Genentech to humanize anti-VEGF Mab A.4.6.1., using a phage-display approach.⁹⁴ Initially, this construct was used to produce a phagemid encoding the variant Y0101, which contained the light-chain mutation M4L and heavy-chain mutation T221L (using the numbering system of Kabat et al⁹⁵ compared with Fab-12). Y0101 in turn was used in Fab phage display to derive an improved Fab for phage expression known as Y0192 with five light-chain (CDR [complementarity-determining region (loops in antibody light- and heavy-chain variable regions involved in antigen binding)]-L1) mutations.⁹³ Y0192 was then used as a template for a series of diversified CDR libraries, which independently yielded two affinity-improved variants: Y0243-1, with substitutions T28D and N31H in the first CDR of the heavy chain, and Y0238-3, with substitutions H97Y and S100aT in the third CDR of the heavy chain.⁹⁶ It is well established that several framework residues within the variable regions are important in supporting the appropriate conformation of the CDR loops, even though they are rarely directly involved in antigen binding.⁹⁷ Previous studies had suggested that the framework of Fab-12, the Fab form of bevacizumab, had a greater degree of optimization relative to the framework of MB1.6 in supporting the CDRs.^{44,94} Thus, the four CDR changes were combined with the light-chain substitution M4L and the heavy-chain cloning site substitution T221L in the context of a construct corresponding to Fab-12. Hence, the final construct, Y0317, later known as rhuFab V2 or ranibizumab, contains five variable domain substitutions and one constant domain substitution (at the C-terminus of the heavy chain) as compared with Fab-12⁹⁶ (Fig. 2).

The affinity improvement of ranibizumab relative to Fab-12 was ≈ 100 -fold. This translated into a 30- to 100-fold increased potency in bioassays measuring human VEGF-induced endothelial cell mitogenesis.⁹⁶ Furthermore, on a molar basis, ranibizumab was 5- to 20-fold more potent than full-length bevacizumab in the same bioassays, in spite of the fact that it had only one antigen binding site and therefore lacked the potential for avidity effects (authors' unpublished observations). Similar to the parent antibody, ranibizumab

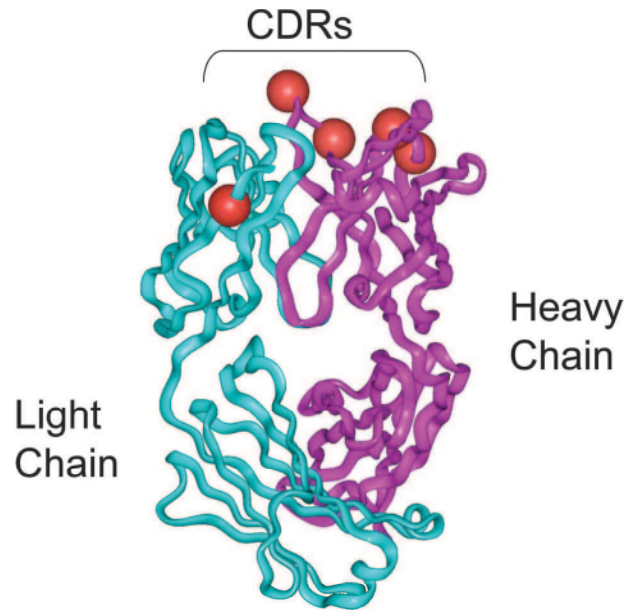


Fig. 2. Ribbon diagram of the structure of ranibizumab. The five red spheres designate the position of amino acid changes in the structure of ranibizumab⁹⁶ compared with bevacizumab Fab. A sixth amino acid change, introduced for cloning purposes in the constant domain of the heavy chain, is not indicated. See text for details. CDRs, complementarity-determining regions (loops in antibody light- and heavy-chain variable regions involved in antigen binding).

neutralized the biologic activities of all human alternatively spliced VEGF-A isoforms, as well as the proteolytic cleavage product VEGF₁₁₀.^{24,96,98} Ranibizumab has a very low-affinity interaction with murine VEGF-A⁹⁹; therefore, it is not suitable to assess the role of VEGF-A in rodent models of intraocular neovascularization.

Thus, in addition to the aforementioned desirable properties of a Fab, ranibizumab possessed a remarkably high level of biologic activity, including the ability to neutralize all bioactive forms of VEGF-A. Pharmacokinetic studies of primates showed that, after a single intravitreal administration, biologically effective retinal concentrations of ranibizumab were maintained for ≈ 1 month, while serum levels were 1,000- to 2,000-fold lower than the ocular levels.¹⁰⁰

Ranibizumab Clinical Trials

Genentech filed an Investigational New Drug for ranibizumab in October 1999 and initiated the first clinical trial including patients with neovascular AMD in February 2000. After encouraging data from phase 1 and phase 2 studies,^{101,102} Genentech proceeded to study ranibizumab rigorously in pivotal phase 3 trials. In an initial phase 1 study, 0.5 mg was identified as the maximum tolerated single intravitreal dose, with intraocular inflammation being the dose-limiting toxic-

ity.¹⁰¹ However, in a multiple escalating dose protocol, up to 2-mg doses of ranibizumab could be injected as frequently as every 2 weeks without clinically significant inflammation.¹⁰³ The reasons for the inflammation in the single-dose study and its attenuation after multiple doses remain to be fully elucidated. A third study demonstrated that intravitreal injections of 0.3 mg or 0.5 mg of ranibizumab administered every 4 weeks for up to eight injections were well tolerated and associated with a mean improvement in visual acuity from baseline compared with the usual care control group, who had a mean decline in vision during the controlled first half of the study.¹⁰²

The safety and efficacy of ranibizumab in the treatment of neovascular AMD have been evaluated in two large phase 3, multicenter, randomized, double-masked, controlled pivotal trials including different neovascular AMD patient populations. Results of one of these 2-year studies—MARINA trial—are currently in press,¹⁵ and data from the primary analysis at 1 year for the second study—ANCHOR trial—have been presented.

The MARINA trial¹⁵ randomized 716 subjects in the United States who had minimally classic or occult without classic subfoveal CNV to one of three treatment arms: monthly sham injections, monthly intravitreal injections of 0.3 mg of ranibizumab, or monthly intravitreal injections of 0.5 mg of ranibizumab. In the primary analysis at 1 year, the study met its primary end point, with 95% and 96% of subjects in the 0.3-mg and 0.5-mg ranibizumab groups, respectively, losing <15 letters compared with 62% in the sham injection group ($P < 0.0001$ vs sham). On average at 1 year, subjects treated with 0.3 mg and 0.5 mg of ranibizumab gained 6.5 letters and 7.2 letters, respectively, while subjects in the sham injection group lost an average of 10.4 letters ($P < 0.0001$ vs sham). A significantly larger percentage of subjects treated with ranibizumab gained ≥ 15 letters at 1 year (25% and 34% in the 0.3-mg and 0.5-mg ranibizumab groups, respectively) than did the sham injection recipients (5%; $P < 0.0001$ vs sham). These visual acuity benefits persisted through the second year of the study. Key serious ocular adverse events of uveitis and presumed endophthalmitis over the 2-year study period were uncommon, with each occurring in $\leq 1.7\%$ of ranibizumab-treated subjects. The rates of key nonocular serious adverse events (i.e., arterial thrombotic events as defined by the Antiplatelet Trialists' Collaboration¹⁰⁴ [nonfatal myocardial infarctions, nonfatal strokes, and vascular deaths including deaths of unknown cause]) over the 2-year study period were similar in the three treatment groups: 4.6% in each of

the ranibizumab groups and 3.8% in the sham injection group.

The ANCHOR trial randomized 423 subjects in the United States, Europe, and Australia who had predominantly classic CNV to one of three treatment arms: verteporfin photodynamic therapy with monthly sham ocular injections, monthly intravitreal injections of 0.3 mg of ranibizumab with a sham photodynamic therapy procedure, and monthly intravitreal injections of 0.5 mg of ranibizumab with a sham photodynamic therapy procedure. Results of the primary analysis at 1 year have been presented (P.K. Kaiser, presentation at "Macula 2006," January 14, 2006; New York, NY [A Phase III Study of Ranibizumab (Lucentis™) versus Verteporfin (Visudyne®) PDT in Predominantly Classic Subfoveal Neovascular AMD—Year 1 Results]). The study met its primary and secondary visual acuity end points. The treatment effect of ranibizumab at 1 year on the proportion of subjects losing <15 letters at 1 year (the primary end point), the mean change in visual acuity from baseline, and the proportion of subjects gaining ≥ 15 letters was similar to that observed in the MARINA trial, despite the presence of an active control in the ANCHOR study and a more aggressive target lesion type. The ocular and nonocular safety profile observed in the ANCHOR trial was similar to that observed in the MARINA trial.

Conclusions and Perspectives

Analyses of findings from two phase 3 studies (ANCHOR and MARINA trials) indicate that ranibizumab results not only in a slowing down of vision loss but also in a significant proportion of patients with clinically meaningful vision gain at the primary 12-month assessment. In the case of the MARINA study,¹⁵ these benefits were also observed through the final 24-month assessment. The visual acuity benefit over control was observed regardless of CNV lesion type. Furthermore, the benefit was associated with a low rate of serious adverse events. On June 30, 2006, ranibizumab was approved by the US Food and Drug Administration for the treatment of wet AMD.

It had been previously argued that a strategy aimed at targeting a single isoform, VEGF₁₆₅, is sufficient to suppress pathologic intraocular angiogenesis, while normal vessels would be "maintained" by VEGF₁₂₁ and other isoforms.^{105,106} However, as already discussed, various proteases known to be activated during inflammation and angiogenesis may cleave VEGF₁₆₅ and the longer VEGF-A isoforms to generate non-heparin-binding fragments.^{24,25,27} We suggest that the ability to block these molecular forms, in addition to intact VEGF₁₆₅, is particularly important

to account for the therapeutic effectiveness of ranibizumab. In this context, it is noteworthy that tumor studies have shown that even modest amounts of VEGF-A, produced by stromal cells, may be sufficient to drive tumor angiogenesis, emphasizing the importance of a complete VEGF-A blockade for maximal tumor growth inhibition.^{107,108}

The expression of VEGF receptors in different cell types in the normal retina⁴¹ and the recent emphasis on the potential role of VEGF-A as a neurotrophic factor¹⁰⁹ raised the concern that chronic, complete blockade of VEGF-A might result in detrimental effects on the neuroretina. However, the clinical benefit, including increases in visual acuity, observed in ranibizumab-treated AMD patients argues against this possibility, at least within a 2-year treatment period.

Given the complexity of VEGF-A biologic activities, an important question concerns the mechanism of the benefit brought about by anti-VEGF therapy in AMD patients. It appears that vision improvements correlate with reductions of the levels of intraretinal and subretinal fluid, as assessed by optical coherence tomography.¹³ What is the biologic basis for such effects? VEGF is known to be able to promote microvascular permeability.¹¹⁰ However, VEGF-induced changes in vascular permeability of preexisting vessels are acute and of relatively short duration.¹¹¹ On the other hand, there is much evidence from studies in other contexts, especially tumors, that VEGF blockade induces long-term changes in the functional properties of the microvasculature—including permeability—that cannot be dissociated from extensive remodeling of the vessel wall, such as regression of some endothelial cells and an increased percentage of capillaries being covered by pericytes.^{36,37,40} Therefore, it is possible that the initial reduction of intraretinal and subretinal fluid elicited by anti-VEGF therapy is due to blockade of the direct permeability-enhancing activities of the molecule. However, long-term benefits are likely to be secondary not only to inhibition of further CNV growth but also to a switch into a more stable and “normal” microvascular network within CNV lesions, thus reflecting the role of VEGF as a mitogen and survival factor for blood vessels. Furthermore, VEGF-A is able to promote recruitment of inflammatory cells, which may in turn release additional mediators of vascular leakage and angiogenesis.^{56,57} Thus, VEGF-A blockade is expected to result in a series of effects on the vascular endothelium—acute and long term and direct and indirect—that may contribute to the clinical benefit in AMD patients. Additional studies are required to elucidate and dissect the specific contribution of these various mechanisms.

Encouraging results of phase 1 and phase 2/3 trials

of ranibizumab followed by favorable results of preliminary phase 3 clinical trials, combined with the lack of general availability of ranibizumab due to its investigational status, have led to growing interest in the possibility of off-label use of bevacizumab as a therapeutic option for the treatment of neovascular AMD. Systemic bevacizumab was the subject of an uncontrolled, open-label, single-center study of nine subjects with neovascular AMD and 12-week follow-up that reported encouraging signs of activity as assessed by visual acuity and anatomical optical coherence tomography and fundus fluorescein angiography evaluations¹¹²; however, seven of the nine subjects required new or revised antihypertensive medication during the study. The same investigators subsequently reported a case of a patient with neovascular AMD who received an intravitreal injection of bevacizumab and, over the next 4 weeks, was observed to have subsequent resolution of subretinal fluid with no associated inflammation.¹¹³ This case report attracted much interest and prompted a more recent larger, uncontrolled, open-label series of 79 subjects with neovascular AMD and 4- to 15-week follow-ups during which improvements in nonstandardized visual acuity measurements and in retinal anatomy as assessed by optical coherence tomography and fundus fluorescein angiography were observed without associated inflammation or other significant safety issues.¹¹⁴ Although intriguing, these early findings are difficult to compare with data from rigorous, double-masked, controlled phase 3 trials of verteporfin photodynamic therapy, pegaptanib, and, more recently, ranibizumab. In addition, it is noteworthy that initial, uncontrolled phase 1 or 2 studies with pegaptanib¹¹⁵ or verteporfin photodynamic therapy¹¹⁶ suggested a considerably greater benefit in AMD patients than that eventually demonstrated in randomized phase 3 studies,^{13,117} further emphasizing the difficulty of interpreting early clinical results.

In conclusion, >1 decade of preclinical and clinical research has yielded a novel therapeutic that, for the first time, appears to have the potential to enable many AMD patients to obtain meaningful and sustained gain of vision. Further research is needed to determine whether the vision gain conferred by ranibizumab extends beyond 24 months and whether additional intraocular neovascular syndromes may benefit from this treatment.

Key words: vascular endothelial growth factor, monoclonal antibody, endothelium, angiogenesis, age-related macular degeneration, retinopathy.

References

1. Yancopoulos GD, Davis S, Gale NW, et al. Vascular-specific growth factors and blood vessel formation. *Nature* 2000;407:242–248.
2. Carmeliet P. Angiogenesis in health and disease. *Nat Med* 2003;9:653–660.
3. Jain RK. Molecular regulation of vessel maturation. *Nat Med* 2003;9:685–693.
4. Nyberg P, Xie L, Kalluri R. Endogenous inhibitors of angiogenesis. *Cancer Res* 2005;65:3967–3979.
5. Ferrara N. VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer* 2002;2:795–803.
6. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004;25:581–611.
7. Patz A. Studies on retinal neovascularization. *Invest Ophthalmol Vis Sci* 1980;19:1133–1138.
8. Garner A. Vascular diseases. In: Garner A, Klintworth GK, eds. *Pathobiology of Ocular Disease*. 2nd ed. New York: Marcel Dekker; 1994:1625–1710.
9. Michaelson IC. The mode of development of the vascular system of the retina with some observations on its significance for certain retinal disorders. *Trans Ophthalmol Soc UK* 1948;68:137–180.
10. Ferrara N. Vascular endothelial growth factor. The trigger for neovascularization in the eye. *Lab Invest* 1995;72:615–618.
11. Adamis AP, Aiello LP, D'Amato RA. Angiogenesis and ophthalmic disease. *Angiogenesis* 1999;3:9–14.
12. Adamis AP, Shima DT. The role of vascular endothelial growth factor in ocular health and disease. *Retina* 2005;25:111–118.
13. Gragoudas ES, Adamis AP, Cunningham ET Jr, et al. Pegaptanib for neovascular age-related macular degeneration. *N Engl J Med* 2004;351:2805–2816.
14. Ng EW, Sima DT, Calias P, et al. Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. *Nat Rev Drug Discov* 2006;5:123–132.
15. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration: 2-year results of the MARINA study. *N Engl J Med* 2006 (in press).
16. Senger DR, Galli SJ, Dvorak AM, et al. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983;219:983–985.
17. Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 1989;161:851–858.
18. Connolly DT, Olander JV, Heuvelman D, et al. Human vascular permeability factor. Isolation from U937 cells. *J Biol Chem* 1989;264:20017–20024.
19. Leung DW, Cachianes G, Kuang WJ, et al. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 1989;246:1306–1309.
20. Keck PJ, Hauser SD, Krivi G, et al. Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science* 1989;246:1309–1312.
21. Houck KA, Ferrara N, Winer J, et al. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* 1991;5:1806–1814.
22. Tischer E, Mitchell R, Hartman T, et al. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* 1991;266:11947–11954.
23. Plouet J, Schilling J, Gospodarowicz D. Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT20 cells. *EMBO J* 1989;8:3801–3808.
24. Houck KA, Leung DW, Rowland AM, et al. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *J Biol Chem* 1992;267:26031–26037.
25. Park JE, Keller G-A, Ferrara N. The vascular endothelial growth factor isoforms (VEGF): differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol Biol Cell* 1993;4:1317–1326.
26. Keyt BA, Berleau LT, Nguyen HV, et al. The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency. *J Biol Chem* 1996;271:7788–7795.
27. Lee S, Jilani SM, Nikolova GV, et al. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *J Cell Biol* 2005;169:681–691.
28. Pepper MS. Extracellular proteolysis and angiogenesis. *Thromb Haemostasis* 2001;86:346–355.
29. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med* 2003;9:669–676.
30. Connolly DT, Heuvelman DM, Nelson R, et al. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest* 1989;84:1470–1478.
31. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995;146:1029–1039.
32. Ferrara N, Carver Moore K, Chen H, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 1996;380:439–442.
33. Marnieros AG, Fan J, Yokoyama Y, et al. Vascular endothelial growth factor expression in the retinal pigment epithelium is essential for choriocapillaris development and visual function. *Am J Pathol* 2005;167:1451–1459.
34. Alon T, Hemo I, Itin A, et al. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat Med* 1995;1:1024–1028.
35. Gerber HP, McMurtrey A, Kowalski J, et al. VEGF regulates endothelial cell survival by the PI3-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 1998;273:30366–30373.
36. Benjamin LE, Golijanin D, Itin A, et al. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J Clin Invest* 1999;103:159–165.
37. Yuan F, Chen Y, Dellian M, et al. 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 USA* 1996;93:14765–14770.
38. Kamba T, Tam BY, Hashizume H, et al. VEGF-dependent plasticity of fenestrated capillaries in the normal adult microvasculature. *Am J Physiol Heart Circ Physiol* 2006;290:H560–H576.
39. Inai T, Mancuso M, Hashizume H, et al. Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of

- tumor vessels, and appearance of basement membrane ghosts. *Am J Pathol* 2004;165:35–52.
40. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 2005;307:58–62.
 41. Robinson GS, Ju M, Shih SC, et al. Nonvascular role for VEGF: VEGFR-1,2 activity is critical for neural retinal development. *FASEB J* 2001;15:1215–1217.
 42. Kim KJ, Li B, Winer J, et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth in vivo. *Nature* 1993;362:841–844.
 43. Manley PW, Martiny-Baron G, Schlaeppli JM, Wood JM. Therapies directed at vascular endothelial growth factor. *Expert Opin Investig Drugs* 2002;11:1715–1736.
 44. Presta LG, Chen H, O'Connor SJ, et al. Humanization of an anti-VEGF monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res* 1997;57:4593–4599.
 45. Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discov* 2004;3:391–400.
 46. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004;350:2335–2342.
 47. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. *Nature* 2005;438:967–974.
 48. Shibuya M, Yamaguchi S, Yamane A, et al. Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase (flt) closely related to the fms family. *Oncogene* 1990;8:519–527.
 49. Quinn TP, Peters KG, De Vries C, et al. Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc Natl Acad Sci USA* 1993;90:7533–7537.
 50. Terman BI, Carrion ME, Kovacs E, et al. Identification of a new endothelial cell growth factor receptor tyrosine kinase. *Oncogene* 1991;6:1677–1683.
 51. de Vries C, Escobedo JA, Ueno H, et al. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 1992;255:989–991.
 52. Park JE, Chen HH, Winer J, et al. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J Biol Chem* 1994;269:25646–25654.
 53. Olofsson B, Korpelainen E, Pepper MS, et al. Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. *Proc Natl Acad Sci USA* 1998;95:11709–11714.
 54. LeCouter J, Moritz DR, Li B, et al. Angiogenesis-independent endothelial protection of liver: role of VEGFR-1. *Science* 2003;299:890–893.
 55. Hiratsuka S, Nakamura K, Iwai S, et al. MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. *Cancer Cell* 2002;2:289–300.
 56. Barleon B, Sozzani S, Zhou D, et al. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. *Blood* 1996;87:3336–3343.
 57. Autiero M, Luttun A, Tjwa M, Carmeliet P. Placental growth factor and its receptor, vascular endothelial growth factor receptor-1: novel targets for stimulation of ischemic tissue revascularization and inhibition of angiogenic and inflammatory disorders. *J Thromb Haemost* 2003;1:1356–1370.
 58. Matsumoto T, Claesson-Welsh L. VEGF receptor signal transduction. *Science's STKE* 2001;112(RE21):1–17.
 59. Shalaby F, Rossant J, Yamaguchi TP, et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 1995;376:62–66.
 60. Carmeliet P, Tessier-Lavigne M. Common mechanisms of nerve and blood vessel wiring. *Nature* 2005;436:193–200.
 61. Klagsbrun M, Eichmann A. A role for axon guidance receptors and ligands in blood vessel development and tumor angiogenesis. *Cytokine Growth Factor Rev* 2005;16:535–548.
 62. Soker S, Takashima S, Miao HQ, et al. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* 1998;92:735–745.
 63. Miller JW, Adamis AP, Shima DT, et al. Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. *Am J Pathol* 1994;145:574–584.
 64. Pierce EA, Foley ED, Smith LE. Regulation of vascular endothelial growth factor by oxygen in a model of retinopathy of prematurity. *Arch Ophthalmol* 1996;114:1219–1228.
 65. Safran M, Kaelin WJ Jr. HIF hydroxylation and the mammalian oxygen-sensing pathway. *J Clin Invest* 2003;111:779–783.
 66. Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 1994;331:1480–1487.
 67. Malecaze F, Clemens S, Simorer-Pinotel V, et al. Detection of vascular endothelial growth factor mRNA and vascular endothelial growth factor-like activity in proliferative diabetic retinopathy. *Arch Ophthalmol* 1994;112:1476–1482.
 68. Aiello LP, Pierce EA, Foley ED, et al. Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. *Proc Natl Acad Sci USA* 1995;92:10457–10461.
 69. Adamis AP, Shima DT, Tolentino MJ, et al. Inhibition of vascular endothelial growth factor prevents retinal ischemia-associated iris neovascularization in a nonhuman primate. *Arch Ophthalmol* 1996;114:66–71.
 70. Robinson GS, Pierce EA, Rook SL, et al. Oligodeoxynucleotides inhibit retinal neovascularization in a murine model of proliferative retinopathy. *Proc Natl Acad Sci USA* 1996;93:4851–4856.
 71. Ozaki H, Seo MS, Ozaki K, et al. Blockade of vascular endothelial cell growth factor receptor signaling is sufficient to completely prevent retinal neovascularization. *Am J Pathol* 2000;156:697–707.
 72. Congdon N, O'Colmain B, Klaver CC, et al. Causes and prevalence of visual impairment among adults in the United States. *Arch Ophthalmol* 2004;122:477–485.
 73. Ferris FL 3rd, Fine SL, Hyman L. Age-related macular degeneration and blindness due to neovascular maculopathy. *Arch Ophthalmol* 1984;102:1640–1642.
 74. Lopez PF, Sippy BD, Lambert HM, et al. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. *Invest Ophthalmol Vis Sci* 1996;37:855–868.
 75. Kvant A, Alverre PV, Berglin L, Seregard S. Subfoveal fibrovascular membranes in age-related macular degenera-

- tion express vascular endothelial growth factor. *Invest Ophthalmol Vis Sci* 1996;37:1929–1934.
76. Okamoto N, Tobe T, Hackett SF, et al. Transgenic mice with increased expression of vascular endothelial growth factor in the retina: a new model of intraretinal and subretinal neovascularization. *Am J Pathol* 1997;151:281–291.
77. Campochiaro PA, Hackett SF. Ocular neovascularization: a valuable model system. *Oncogene* 2003;22:6537–6548.
78. Donoso LA, Kim D, Frost A, et al. The role of inflammation in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol* 2006;51:137–152.
79. Krzystolik MG, Afshari MA, Adamis AP, et al. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. *Arch Ophthalmol* 2002;120:338–346.
80. Saishin Y, Takahashi K, Lima e Silva R, et al. VEGF-TRAP(R1R2) suppresses choroidal neovascularization and VEGF-induced breakdown of the blood-retinal barrier. *J Cell Physiol* 2003;195:241–248.
81. Holash J, Davis S, Papadopoulos N, et al. VEGF-Trap: a VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci USA* 2002;99:11393–11398.
82. Jackson TL, Antcliff RJ, Hillenkamp J, Marshall J. Human retinal molecular weight exclusion limit and estimate of species variation. *Invest Ophthalmol Vis Sci* 2003;44:2141–2146.
83. Kuwabara T. The eye. In: Weiss L, ed. *Cell and Tissue Biology. A Textbook of Histology*. Baltimore: Urban & Schwarzenberger; 1988:1069–1105.
84. Tolentino MJ, Husain D, Theodosiadis P, et al. Angiography of fluoresceinated anti-vascular endothelial growth factor antibody and dextrans in experimental choroidal neovascularization. *Arch Ophthalmol* 2000;118:78–84.
85. Ryan AM, Eppler DB, Hagler KE, et al. Preclinical safety evaluation of rhuMABVEGF, an antiangiogenic humanized monoclonal antibody. *Toxicol Pathol* 1999;27:78–86.
86. Gordon MS, Margolin K, Talpaz M, et al. Phase I safety and pharmacokinetic study of recombinant human anti-vascular endothelial growth factor in patients with advanced cancer. *J Clin Oncol* 2001;19:843–850.
87. Smelser G, Ishikawa T, Pei Y. Electron microscopic studies of intra-retinal s: diffusion of particulate material. In: Rohen J, ed. *Eye Structure*. Stuttgart: Schattauer-Verlag; 1965.
88. Mordenti J, Cuthbertson RA, Ferrara N, et al. 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* 1999;27:536–544.
89. Goldenberg DM. Targeted therapy of cancer with radiolabeled antibodies. *J Nucl Med* 2002;43:693–713.
90. Shahar J, Avery RL, Heilweil G, et al. Electrophysiologic and retinal penetration studies following intravitreal injection of bevacizumab (Avastin). *Retina* 2006;26:262–269.
91. Raghavan M, Bjorkman PJ. Fc receptors and their interactions with immunoglobulins. *Annu Rev Cell Dev Biol* 1996;12:181–220.
92. Reff ME, Hariharan K, Braslawsky G. Future of monoclonal antibodies in the treatment of hematologic malignancies. *Cancer Control* 2002;9:152–166.
93. Muller YA, Chen Y, Christinger HW, et al. 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* 1998;6:1153–1167.
94. Baca M, Presta LG, O'Connor SJ, Wells JA. Antibody humanization using monovalent phage display. *J Biol Chem* 1997;272:10678–10684.
95. Kabat EA, Wu TT, Reid-Miller M, Perry H, Gottesman K. Sequences of proteins of immunological interest. 4th ed. Bethesda, MD: National Institutes of Health; 1987.
96. Chen Y, Wiesmann C, Fuh G, et al. Selection and analysis of an optimized anti-VEGF antibody: crystal structure of an affinity-matured Fab in complex with antigen. *J Mol Biol* 1999;293:865–881.
97. Morea V, Lesk AM, Tramontano A. Antibody modeling: implications for engineering and design. *Methods* 2000;20:267–279.
98. Kim KJ, Li B, Houck K, et al. The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies. *Growth Factors* 1992;7:53–64.
99. Liang WC, Wu X, Peale FV, et al. Cross-species VEGF-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. *J Biol Chem* 2006;281:951–961.
100. Gaudreault J, Fei D, Rusit J, et al. Preclinical pharmacokinetics of ranibizumab (rhuFabV2) after a single intravitreal administration. *Invest Ophthalmol Vis Sci* 2005;46:726–733.
101. Rosenfeld PJ, Schwartz SD, Blumenkranz MS, et al. Maximum tolerated dose of a humanized anti-vascular endothelial growth factor antibody fragment for treating neovascular age-related macular degeneration. *Ophthalmology* 2005;112:1048–1053.
102. Heier JS, Antoszyk AN, Pavan PR, et al. Ranibizumab for treatment of neovascular AMD. A phase I/II multicenter, controlled, multidose study. *Ophthalmology* 2006 (in press).
103. Rosenfeld PJ, Heier JS, Hantsbarger G, Shams N. Tolerability and efficacy of multiple escalating doses of ranibizumab (Lucentis) for neovascular age-related macular degeneration. *Ophthalmology* 2006;113:631–632.
104. Antiplatelet Trialists' Collaboration. Collaborative overview of randomised trials of antiplatelet therapy—I. Prevention of death, myocardial infarction, and stroke by prolonged antiplatelet therapy in various categories of patients. *BMJ* 1994;308:81–106.
105. Usui T, Ishida S, Yamashiro K, et al. VEGF164(165) as the pathological isoform: differential leukocyte and endothelial responses through VEGFR1 and VEGFR2. *Invest Ophthalmol Vis Sci* 2004;45:368–374.
106. Ishida S, Usui T, Yamashiro K, et al. VEGF164-mediated inflammation is required for pathological, but not physiological, ischemia-induced retinal neovascularization. *J Exp Med* 2003;198:483–489.
107. Gerber HP, Kowalski J, Sherman D, et al. Complete inhibition of rhabdomyosarcoma xenograft growth and neovascularization requires blockade of both tumor and host vascular endothelial growth factor. *Cancer Res* 2000;60:6253–6258.
108. Dong J, Grunstein J, Tejada M, et al. VEGF-null cells require PDGFR alpha signaling-mediated stromal fibroblast recruitment for tumorigenesis. *EMBO J* 2004;23:2800–2810.
109. Lambrechts D, Storkebaum E, Morimoto M, et al. VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. *Nat Genet* 2003;34:383–394.
110. Dvorak HF, Detmar M, Claffey KP, et al. Vascular permeability factor/vascular endothelial growth factor: an impor-

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- tant mediator of angiogenesis in malignancy and inflammation. *Int Arch Allergy Immunol* 1995;107:233–235.
111. Collins PD, Connolly DT, Williams TJ. Characterization of the increase in vascular permeability induced by vascular permeability factor in vivo. *Br J Pharmacol* 1993;109:195–199.
 112. Michels S, Rosenfeld PJ, Puliafito CA, et al. Systemic bevacizumab (Avastin) therapy for neovascular age-related macular degeneration: twelve-week results of an uncontrolled open-label clinical study. *Ophthalmology* 2005;112:1035–1047.
 113. Rosenfeld PJ, Moshfeghi AA, Puliafito CA. Optical coherence tomography findings after an intravitreal injection of bevacizumab (Avastin) for neovascular age-related macular degeneration. *Ophthalmic Surg Lasers Imaging* 2005;36:331–335.
 114. Avery RL, Pieramici DJ, Rabena MD, et al. Intravitreal bevacizumab (Avastin) for neovascular age-related macular degeneration. *Ophthalmology* 2006;113:363–372.
 115. Eyetech Study Group. Anti-vascular endothelial growth factor therapy for subfoveal choroidal neovascularization secondary to age-related macular degeneration. Phase II study results. *Am J Ophthalmol* 2003;110:979–986.
 116. Schmidt-Erfurth U, Miller JW, Sickenberg M, et al. Photodynamic therapy with verteporfin for choroidal neovascularization caused by age-related macular degeneration: results of retreatments in a phase 1 and 2 study. *Arch Ophthalmol* 1999;117:1177–1187.
 117. TAP Study Group. Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin. One-year results of 2 randomized clinical trials—TAP report 1. *Arch Ophthalmol* 1999;117:1329–1345.