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Perspective

VEGF and Intraocular Neovascularization: From Discovery to Therapy

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I am honored and humbled to be one of the awardees of the 2014 A. Champalimaud Vision Award. I offer my heartfelt thanks to the Champalimaud Foundation President, Leonor Beleza, and to the Award Committee Members for this wonderful recognition.

I feel especially fortunate to have had the opportunity to witness my scientific discoveries move from the bench to the clinic. Scientific discovery is hugely exciting, but the ability to translate that work into potentially helping someone lead a better life is even more fulfilling. This Award is dedicated to the patients.

Introduction

The existence of factors capable of inducing growth of cells and tissues was hypothesized already at the beginning of the last century. In 1913, Carrel¹ described the ability of tissues extracts to stimulate cell proliferation in cultured tissue explants. Early investigators also speculated that biochemical mediators are responsible for the growth of blood vessels associated with tumorigenesis and other pathological conditions (reviewed previously²). In 1939, the observation that tumors transplanted in transparent chambers inserted in the rabbit ear induce rapid and extensive neovascular growth, led Ide et al.³ to postulate the existence of a tumor-derived “blood vessel growth stimulating factor.” In 1945, Algire et al.⁴ announced the seminal hypothesis that “the rapid growth of tumor transplants is dependent upon the development of a rich vascular supply.” In the late 1940s and in the 1950s, other investigators postulated the existence of a diffusible angiogenic factor (“Factor X”), produced in the ischemic retina.^{5–7} This hypothetical molecule was thought to be responsible for neovascularization associated with diabetic retinopathy and other retinal disorders.^{5–7} Notwithstanding these seminal studies, very little progress was possible

at that time, given the daunting challenge of isolating growth factors, which typically are active at very low concentrations. Beginning in the 1970s, the availability of powerful protein purification techniques, combined with the development of cDNA cloning methodologies, enabled major advances. The greatest challenge at that time was purifying the proteins to homogeneity to obtain a partial amino acid sequence, which could be used to design probes suitable for cDNA cloning, thus, dramatically expanding the possibilities of investigating the molecules of interest.²

In 1971, Folkman⁸ published an elegant synthesis of the aforementioned early studies and hypotheses, and also proposed that antiangiogenesis could be a novel strategy to inhibit tumor growth. This key hypothesis stimulated the search for regulators of angiogenesis. By the mid 1980s, several proangiogenic molecules had been identified and characterized, including epidermal growth factor (EGF), tumor growth factor (TGF)- α , TGF- β , a-fibroblast growth factor (aFGF), bFGF, and angiogenin (reviewed previously^{2,9}). However, while these factors were able to promote angiogenesis in various bioassays, their role as endogenous mediators of angiogenesis remained uncertain, suggesting that in all likelihood some key molecules remained to be discovered (reviewed previously^{2,10}).

The Discovery of Vascular Endothelial Growth Factor (VEGF)

Independent efforts contributed to the discovery of VEGF. In 1983, Senger et al.¹¹ at Beth Israel Hospital (Boston, MA) reported an initial biochemical characterization of vascular permeability factor (VPF), a permeability-enhancing protein identified in the conditioned media of a guinea pig tumor cell line. However, the lack of amino acid sequence data precluded molecular cloning and establishing whether VPF was distinct from the known mediators of vascular permeability or from other proteins. Therefore, it is not surprising that limited progress in elucidating the significance and function of VPF took place during the next several years.¹² Senger et al. reported the full purification of guinea pig VPF in 1990.¹³

In 1989, we reported the isolation and cloning of a heparin-binding endothelial cell mitogen.^{14,15} This project began while I was a postdoctoral fellow at the University of California, San Francisco (UCSF) in the mid 1980s. At that time, I was able to isolate and culture a population of nonhormone-secreting cells from bovine pituitary, termed “follicular” or “folliculo-stellate” cells.¹⁶ Earlier investigators noted that they establish intimate contacts with the pituitary perivascular spaces, suggesting a role in the development or maintenance of the pituitary vasculature.¹⁷ In the course of these studies, I discovered that follicular cells release in their culture supernatants an endothelial cell mitogen. In 1988, I joined Genentech, where I had the opportunity to pursue the isolation of this mitogen. By early 1989, we were able to determine the amino terminal amino acid sequence of the purified protein. We found that this sequence was unique, since it had no match with known sequences in available databases.^{14,15} Because this molecule appeared to have growth-promoting activity selectively for vascular endothelial cells, we proposed the name “vascular endothelial growth factor” (VEGF). We then isolated bovine and human clones encoding multiple molecular species (isoforms) of VEGF, due to alternative mRNA splicing.¹⁵ In this early study, we identified three VEGF isoforms: VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉. Subsequent studies revealed the existence of additional VEGF isoforms (reviewed previously¹⁸).

After our cloning paper was accepted for publication,¹⁵ we learned that a group at the Monsanto Company had submitted at approximately the same

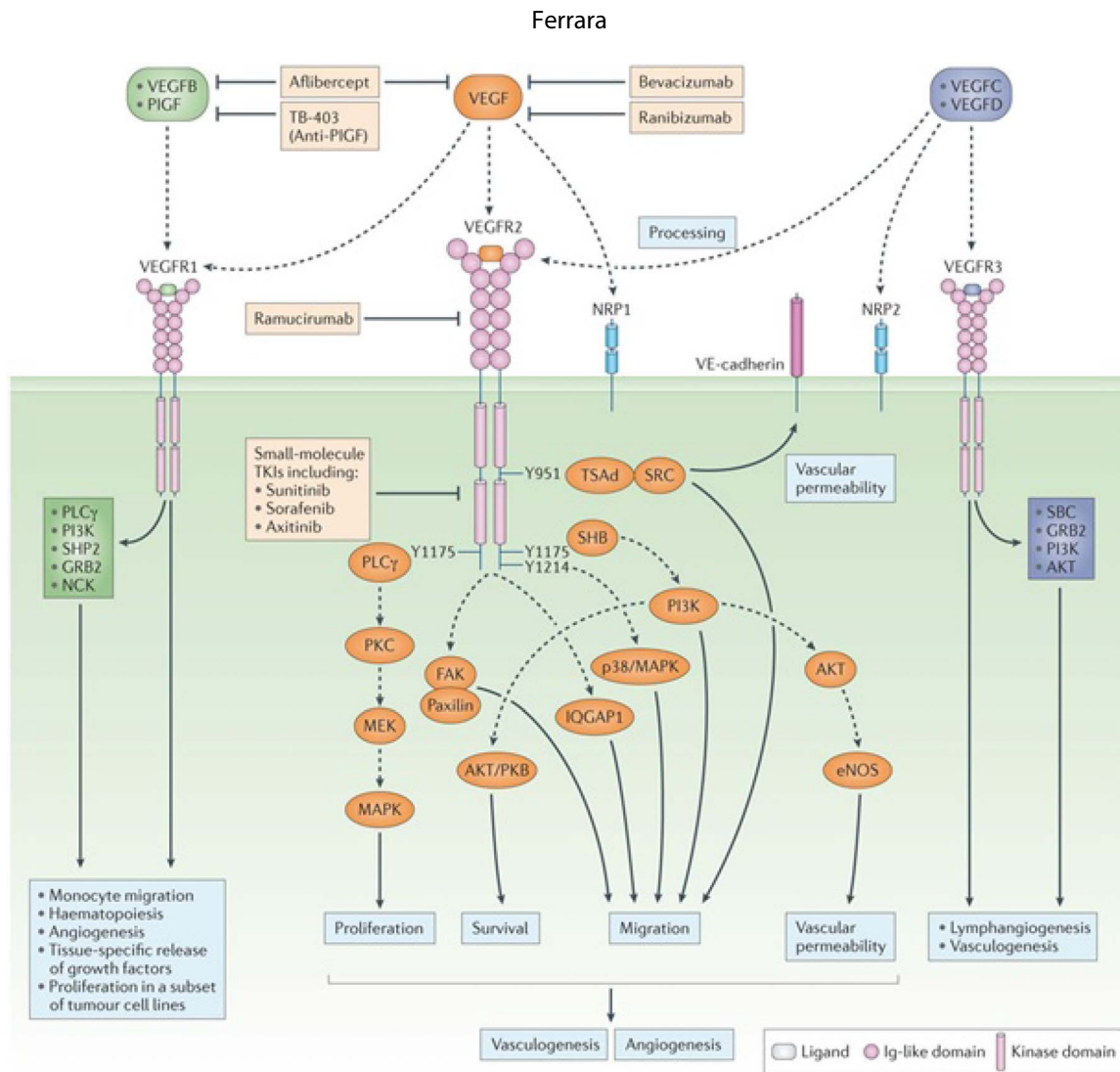
time a manuscript reporting on the cloning of VPF.¹⁹ These investigators described a human clone that encoded a protein identical to VEGF₁₈₉.¹⁹ This group followed up on the earlier work by Senger et al.¹¹ and was able to isolate and sequence VPF. Therefore, it appeared that the same molecule possesses mitogenic and permeability-enhancing activities.

VEGF as a Key Regulator of Normal and Tumor Angiogenesis

The cloning of VEGF (today also known as VEGF-A following the discovery of several related molecules, VEGF-B, VEGF-C, VEGF-D, and PlGF) generated significant interest in the angiogenesis field,² but it took several years before we could establish that VEGF was truly a pathophysiologically relevant mediator. It became clear that the VEGF isoforms are well suited to generate biochemical gradients, a requirement for angiogenesis in vivo, due to their differential diffusibility, which depends on their affinity for heparan-sulfate proteoglycans.^{20,21} A key question was whether VEGF has a role as an angiogenic factor in vivo. The earliest evidence that VEGF expression is temporally and spatially correlated with neovascularization was from a study published in 1990, where we examined the expression of VEGF mRNA in the rat ovary by in situ hybridization.²² We reported that the expression was low in the avascular granulosa cells, but was strongly upregulated in the highly vascularized corpus luteum.²² Furthermore, in 1992 we reported that the high affinity binding sites for VEGF are selectively expressed in endothelial cells in vivo.²³

The identification of the VEGF tyrosine kinase receptors represented a major milestone in the quest to understand VEGF action. In 1992, in collaboration with Lewis (Rusty) Williams at UCSF, we identified the *fms*-like tyrosine kinase²⁴ (presently known as VEGFR-1) as a high-affinity VEGF receptor.²⁵ In the same year, Terman et al.²⁶ identified a highly homologous tyrosine kinase receptor, known as KDR or VEGFR-2. It now is well established that VEGFR-2 is the main signaling VEGF receptor.²⁷ Figure 1 illustrates the current view of the roles of the VEGF receptors and signaling pathways.

To elucidate the role of VEGF in vivo, we employed multiple strategies to inhibit its function. In 1993, we reported that administration of an anti-VEGF monoclonal antibody substantially reduced



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Figure 1. The VEGF signaling pathways and inhibitors. The two VEGF tyrosine kinase receptors are expressed primarily in endothelial cells. The VEGF-related molecules PIGF and VEGFB bind selectively to VEGFR-1, while VEGF binds VEGFR-1 and VEGFR-2. VEGFC and VEGFD bind to VEGFR3, a key regulator of lymphangiogenesis, but following proteolytic processing also have the ability to bind and activate VEGFR-2.⁵⁶ Heparin binding VEGF isoforms and PIGF also bind the coreceptor neuropilin (NRP)1.⁶⁷ NRP1 increase the binding affinity to VEGFR-2.⁶⁸ Direct effects of VEGF or PIGF on NRP1, independent of VEGF receptor activation, also have been reported.⁶⁸ In some circumstances, VEGFR-1 may function as a “decoy” receptor, preventing VEGF from binding to VEGFR-2. However, VEGFR-1 is able to regulate the expression of a variety of genes in the endothelium, including MMP9 and certain growth factors, such as hepatocyte growth factor and connective tissue growth factor, which are known to have an important role in tissue homeostasis and regeneration.⁶⁹ Also, VEGFR-1 is expressed in monocytes and macrophages and, in some cases, also in tumor cells where in it can mediate tumor proliferation in response to VEGF or PIGF.⁷⁰ VEGFR-2 is the main signaling receptor that mediates endothelial cell mitogenesis and vascular permeability.⁵⁶ Multiple inhibitors can block VEGF-induced signaling. Bevacizumab and ranibizumab bind VEGF. The soluble chimeric receptor aflibercept binds VEGF, PIGF, and VEGFB. The anti-VEGFR-2 monoclonal antibody ramucirumab prevents VEGFR-2 dependent signaling. Also, numerous small molecule kinase inhibitors (e.g., sunitinib, sorafenib, and axitinib) inhibit VEGFR signaling (reproduced from the study of Ferrara and Adamis⁵⁶).

the growth of several human tumor cell lines implanted in immunodeficient mice.²⁸ These findings were unexpected at that time, as it was widely believed that tumor angiogenesis is multifactorial and, there-

fore, reflects the contribution of numerous mediators. They paved the way for subsequent clinical development of VEGF inhibitors as cancer therapeutics, including a humanized variant of this anti-VEGF

antibody²⁹ (bevacizumab), which has been approved for therapy of multiple tumor types.³⁰ The impact of these findings, however, went beyond the tumor angiogenesis field. They rapidly stimulated studies aimed at directly probing the role of VEGF in other biological contexts, including intraocular neovascularization.

Inactivation of the *vegf* gene in mice provided evidence for the crucial role of this molecule in the early development of the vasculature. These findings, reported in 1996 by our group³¹ and by Carmeliet et al.,³² were nothing short of striking. Loss of even a single *vegf* allele resulted in defective vascular development and early embryonic lethality. This phenotype was even more dramatic than that of VEGFR2 knockout, which requires inactivation of both alleles to elicit a lethal phenotype.³³ A few years later, we developed VEGF *loxP* mice.³⁴ Crossing these mice with various *Cre*-transgenic lines enables conditional VEGF inactivation in specific cell types or tissues.³⁴ These studies reinforced the notion that VEGF is required for angiogenesis in many tissues and organs (reviewed previously²⁷). In parallel with the genetic reagents, we developed soluble chimeric VEGF receptors (or “VEGF-traps”), which, unlike many monoclonal antibodies, can block VEGF across species.³⁵ Also, structure–function studies of VEGFR-1 led to the discovery that of the seven extracellular Ig-like domains, domain two is the critical element for high-affinity VEGF binding,³⁶ enabling the design of smaller and more stable soluble receptors. The availability of these tools allowed us to establish the role of VEGF in neovascularization associated not only with such essential physiological processes as organ and skeletal growth^{34,37} or cyclical growth of the ovarian corpus luteum,³⁸ but also with pathological retinal neovascularization.³⁹

Looking back at that period, it is almost impossible not to recall a sense of excitement permeating through the angiogenesis field. After decades of largely descriptive work, it finally was possible to unravel some of the secrets of this process and provide a molecular explanation for a variety of fundamental pathophysiological processes. A commentary by Klagsbrun and Soker,⁴⁰ published in 1993, reflects this excitement. According to the authors, “. . . VEGF/VPF may be the best candidate for the principle regulator of normal and tumor angiogenesis.”⁴⁰ I feel extremely fortunate that my lab was at the forefront of this revolution.

VEGF as a Mediator of Intraocular Neovascularization

As pointed out above, by the early 1990s it was apparent that VEGF was implicated in normal as well as in pathologic angiogenesis. Vascular endothelial growth factor also had several features consistent with “Factor X,”⁵ being diffusible and selective for vascular endothelial cells. Also, in 1992 two studies reported that VEGF mRNA expression is induced by hypoxia.^{41,42} Therefore, it is not surprising that VEGF became the top candidate as a mediator of retinal ischemia-related neovascularization. In 1994, in a collaborative study with Lloyd Aiello and George King at the Joslin Diabetes Center in Boston, we tested this hypothesis. Taking advantage of sensitive assays newly developed in our group, we measured the VEGF levels in the eye fluids from 164 patients.⁴³ We found a striking correlation between VEGF concentrations and active proliferative retinopathy associated with diabetes, occlusion of central retinal vein, or prematurity.⁴³ Adamis et al.⁴⁴ at the Massachusetts Eye & Ear Infirmary in Boston also reported elevated VEGF levels in the vitreous of patients with diabetic retinopathy.⁴⁴ At approximately the same time, a French group also reported similar findings.⁴⁵

Subsequent studies revealed that VEGF upregulation in the eye is not limited to ischemic retinal disorders. In 1996, two groups reported the immunohistochemical localization of VEGF in choroidal neovascular membranes from patients with wet age-related macular degeneration (AMD), the leading cause of irreversible severe vision loss in the adult population.^{46,47}

Proof-of-concept studies supported the hypothesis that VEGF is, indeed, a major mediator of intraocular neovascularization. As already mentioned, administration of chimeric soluble VEGF receptors resulted in a marked reduction of retinal neovascularization in a mouse model of retinopathy of prematurity.³⁹ Also, in collaboration with Tony Adamis and Joan Miller, we tested the effects of the anti-VEGF monoclonal antibody used in the cancer studies²⁸ in a primate model of iris neovascularization induced by central retinal vein occlusion.⁴⁸ Similar to the tumor models, we observed a substantial inhibition of blood vessel growth following administration of the antibody.⁴⁸ These effects were not limited to models of retinal ischemia. As described in the next section, wet (neovascular)

AMD became the primary clinical target of our anti-VEGF efforts in the eye.⁴⁹ To this end, we engineered an affinity-matured antibody fragment (Fab) derived from the murine antibody parent of bevacizumab.^{50,51} Krzystolik et al.⁵² kindly agreed to support these efforts by testing this Fab, subsequently known as ranibizumab, in a primate model of choroidal neovascularization.⁵² These studies showed a dramatic inhibition of neovascularization and leakage following intravitreal administration of ranibizumab.⁵²

An Anti-VEGF Therapy for the Eye

The development path of anti-VEGF agents for wet AMD and other intraocular neovascular disorders has been described previously.⁵¹ Briefly, developing an anti-VEGF therapy for wet AMD presented at that time a number of significant challenges.⁵¹ We initially considered testing the intravenous administration of bevacizumab, but the possibility of cardiovascular adverse events in elderly patients led us to discard this possibility in favor of the intraocular route of administration. However, one could not rule out that long-term injection of full-length antibodies in human eyes might result in complement-mediated or cell-dependent cytotoxicity that might be triggered by interaction of the antibody Fc portion with receptors in inflammatory or immune cells.⁵³ We felt that removing the Fc would be prudent. As already noted, we created an affinity-matured Fab variant of bevacizumab to further enhance its binding affinity.⁵⁰ Genentech initiated the first clinical trial in subjects with wet AMD in February 2000. After encouraging data from phase I and phase II studies,^{54,55} ranibizumab was tested in pivotal phase III trials. Examining in detail the phase III studies with ranibizumab and other VEGF inhibitors (bevacizumab and aflibercept) is beyond the scope of this article, which mainly focuses on the discovery and science of VEGF. A very recent review summarizes such clinical trials and discusses a decade of clinical experience with VEGF inhibitors.⁵⁶ Suffice to say here that these agents have had a dramatic impact in ophthalmology. Patients with different variants of wet AMD receiving monthly intravitreal injections of ranibizumab experienced significantly improved visual acuity compared to sham-injected⁵⁷ or verteporfin-treated⁵⁸ patients. In addition, near vision, reading speed, and overall quality of life, were improved.⁵⁹ Subsequent large

randomized clinical trials have demonstrated the efficacy of ranibizumab and other VEGF inhibitors in several other vision-threatening diseases, including diabetic macular edema and retinal vein occlusion.⁵⁶

A few years ago, Bressler et al.⁶⁰ modeled visual acuity outcomes in patients with wet AMD in the United States population based on data from the ranibizumab phase III trials. Their analysis indicated that ranibizumab has the potential to reduce the rate of legal blindness from neovascular AMD over two years by 72%.⁶⁰ In good agreement with these predictions, recent studies have documented a marked reduction in the incidence rate of legal blindness due to AMD in some countries following the introduction of intravitreal VEGF inhibitors in 2006.^{61,62} However, not all patients receive adequate treatment to experience maximal visual improvement. A recent multicountry, retrospective study of wet AMD patients treated with ranibizumab indicated that, especially in some countries, patients receive fewer injections and have poorer outcomes than those reported in clinical trials.⁶³ Therefore, the cost and burden of chronic therapy in some cases limits benefit of anti-VEGF treatment. It is hoped that long-acting delivery technologies will address the gap in visual outcomes between clinical trials and “real life” clinical practice.⁵⁶

Conclusions

I am gratified and humbled that work that I initiated almost 30 years ago during my years as a postdoctoral fellow eventually resulted in a therapy for wet AMD and other intraocular neovascular disorders. The magnitude of the benefit, particularly the visual acuity gains, vastly exceeded my expectations, considering that previous treatments only slowed down the rate of vision loss.^{64,65}

Numerous trials currently are exploring a variety of novel therapeutic agents.⁶⁶ These efforts give hope that combining VEGF inhibitors with agents that target additional pathways may go beyond the benefits achieved so far from targeting VEGF alone.

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This manuscript is based in part on review articles cited in the text.^{2,12,49,56}

Disclosure: **N. Ferrara**, None

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