Ocular Anti-VEGF Therapy for Diabetic Retinopathy: The Role of VEGF in the Pathogenesis of Diabetic Retinopathy

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Diabetic retinopathy is the leading cause of visual impairment and preventable blindness, and represents a significant socioeconomic cost for health care systems worldwide. Therefore, new approaches beyond current standards of diabetes care are needed. Based on the crucial pathogenic role of vascular endothelial growth factor (VEGF) in the development of diabetic macular edema (DME), intravitreal anti-VEGF agents have emerged as new treatments. To provide an understanding of the rationale for use and clinical efficacy of anti-VEGF treatment, we examine this topic in a two-part Bench to Clinic narrative. In the Bench narrative, we provide an overview of the role of VEGF in the pathogenesis of diabetic retinopathy, the molecular characteristics of anti-VEGF agents currently used, and future perspectives and challenges in this area. In the Clinic narrative that follows our contribution, Cheung et al. provide an overview of the current evidence from clinical trials on anti-VEGF therapy for diabetic retinopathy.

Diabetic retinopathy is the most common complication of diabetes and one of the leading causes of preventable blindness (1). The tight control of blood glucose levels and blood pressure are essential in preventing or arresting diabetic retinopathy development. However, these therapeutic objectives are difficult to achieve and diabetic retinopathy develops in a high proportion of patients. In fact, population-based studies suggest that one-third of the diabetic patients have signs of diabetic retinopathy and one-tenth have vision-threatening states of retinopathy, such diabetic macular edema (DME) and proliferative diabetic retinopathy (PDR) (2). The main pathogenic element in DME is the disruption of the blood-retinal barrier (BRB), whereas neovascularization is the hallmark of PDR. However, vascular endothelial growth factor (VEGF) plays a central role in both DME and PDR development (3).

When diabetic retinopathy appears, the prior standard of care relied on laser photocoagulation, which is inherently destructive, associated with unavoidable side effects (i.e., visual field loss and impairment of either dark adaptation or color vision), and not universally effective in reversing or preventing visual loss. Intravitreal corticosteroids have been successfully used in eyes with persistent DME and loss of vision following the failure of conventional treatment. However, reinjections are commonly needed, and there are substantial adverse effects, such as infection, glaucoma, and cataract formation. Vitreoretinal surgery is an expensive treatment that should be carried out only by vitreoretinal specialists experienced in this procedure and it is normally reserved for the blinding complications of PDR, such as severe vitreous hemorrhage and secondary retinal detachment (4).

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In recent years, intravitreal anti-VEGF agents have emerged as new treatments for DME. However, this is an invasive procedure that can have complications, such as endophthalmitis and retinal detachment, and may have deleterious effects for the remaining healthy retina. This is especially important in diabetic patients in whom long-term administration is to be expected. Apart from local side effects, anti-VEGF agents might also produce systemic complications (i.e., hypertension, proteinuria, ischemic cardiovascular disease) due to their capacity to pass into systemic circulation. Current evidence indicates that this is very unlikely, but further studies in diabetic patients on the long-term effectiveness and safety of intravitreal anti-VEGF agents are still needed (5). In this article, an overview of the role of VEGF in the pathogenesis of diabetic retinopathy will be given. In addition, the molecular characteristics of anti-VEGF agents currently used, their differences, and future perspectives and challenges in this area will be provided.

VEGF: GENERAL ACTIONS, ISOFORMS, AND RECEPTORS

The VEGF family is composed of five structurally related ligands: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF). These ligands bind in an overlapping pattern to three tyrosine kinase receptors (VEGFR-1, VEGFR-2, and VEGFR-3) (6).

VEGF-A, which we refer to as VEGF, is a potent mitogen for micro- and macrovascular endothelial cells derived from arteries, veins, and lymphatics. VEGF is required for normal vascular development and even deletion of one VEGF allele is embryonic lethal (6). VEGF is also a survival factor and plays an essential role in maintaining the integrity of endothelial cells via antiapoptotic signaling (7). In addition, there is evidence to suggest that pericyte support of vessel survival is closely related to VEGF-induced expression (8).

The human VEGF gene is organized in eight exons, separated by seven introns, and is localized in chromosome 6p21.3. Alternative exon splicing results in the generation of four isoforms, having 121, 165, 189, and 206 amino acids, respectively, after signal sequence cleavage ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$). The predominant molecular variant is $VEGF_{165}$, a basic, heparin-binding homodimeric glycoprotein of 45 kDa. $VEGF_{165}$ specifically binds to neuropilin-1, thus enhancing VEGFR2 signaling (8). Less frequent splice variants have been reported, including $VEGF_{145}$, $VEGF_{183}$, $VEGF_{162}$, and $VEGF_{165b}$, paradoxically a variant reported to have an inhibitory effect on VEGF-induced angiogenesis (9). This isoform inhibits neovascularization but not revascularization, and is cytoprotective for endothelial and epithelial cells in vivo and in vitro.

The affinity for heparin may profoundly affect the bioavailability of VEGF. $VEGF_{121}$ fails to bind to heparin and, therefore, is a freely soluble protein. In contrast, $VEGF_{189}$ and $VEGF_{206}$ bind to heparin with high affinity and consequently, they are almost completely sequestered in the extracellular matrix. $VEGF_{165}$ has intermediate properties, as it is secreted and circulates as diffusible protein, but a significant fraction remains bound to the cell surface and the extracellular matrix. However, the isoforms quenched in the extracellular matrix may be released in a diffusible form by heparin, heparinase, or plasmin. Therefore, $VEGF_{165}$ may act both as a diffusible factor and as a heparin-binding form that is released upon heparin cleavage (6).

VEGF-A and -B exert their actions through two high-affinity tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR, human; Flk-1, mouse), which are mainly expressed on the cell surface of vascular endothelium. VEGFR-3 is a member of the same family, but binds VEGF-C and -D (VEGF-related molecules) rather than VEGF-A. PlGF and VEGF-B are other VEGF-related members that bind VEGFR-1 but not VEGFR-2 or VEGFR-3. VEGFR-2 signal transduction has been examined in great detail, and early signaling events that control survival, proliferation, and permeability have been identified while final downstream effectors are less well characterized. A schematic highlighting some of the important signaling mechanisms of VEGFR-2 activation is provided in Fig. 1, while a comprehensive review on VEGF

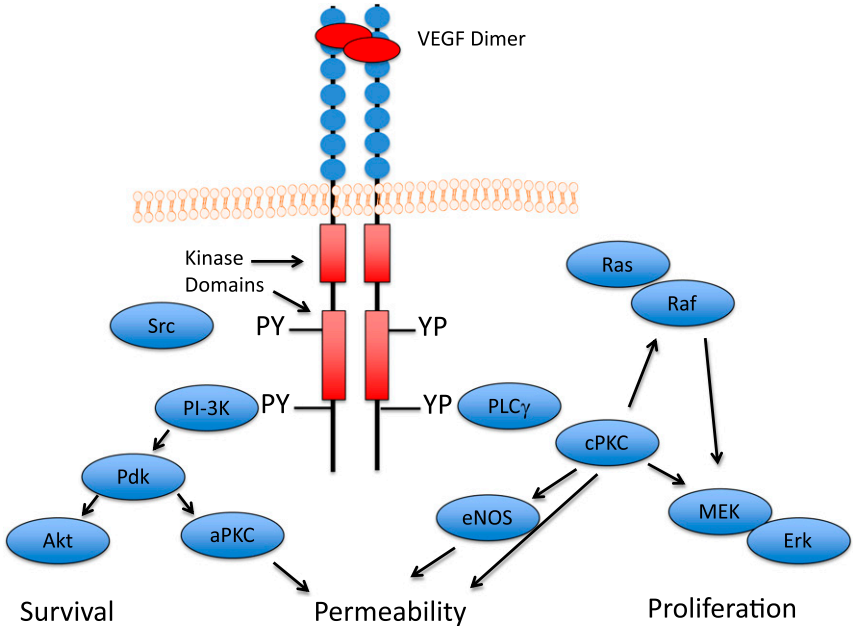

Figure 1—Schematic of VEGF signal transduction. VEGF-A forms a dimer and binds to VEGFR-2 in a typical receptor tyrosine kinase activation pathway, inducing kinase domain activation and cross-receptor phosphorylation. The phosphorylation of the receptor (and adaptors, not shown) allows binding of signaling molecules possessing phosphotyrosine-binding motifs, such as SH2 domains. Signal transduction pathways include activation of the p42/p44 extracellular regulated kinase (Erk) pathway that contributes to control of proliferation. PKC pathways, including conventional (cPKC) and atypical (aPKC), are both activated and, along with endothelial nitric oxide synthase (eNOS), contribute to vascular permeability and macular edema. The phosphatidylinositol 3-kinase (PI-3K) pathway and activation of Akt contributes to endothelial cell survival. Targeting VEGF-A with antibodies effectively prevents both VEGF-dependent angiogenesis and vascular permeability.

signal transduction has been previously reported (10).

There is much evidence to suggest that VEGFR-2 is the major mediator of endothelial cell mitogenesis, survival, and microvascular permeability. Indeed, tyrosine kinase activity of VEGFR-2 is one order of magnitude higher than that of VEGFR-1 in experimental conditions (6). In contrast, VEGFR-1 does not mediate an effective mitogenic signal in endothelial cells, and, at least in early development, it may have inhibiting properties by sequestering VEGF and rendering this factor less available to VEGFR-2 (6). Such a "decoy" role could be also performed by the alternative spliced soluble VEGFR-1 (sFlt-1). However, it has been proposed that activation of VEGFR-1 by PlGF produces a transphosphorylation of VEGFR-2, thus amplifying VEGF-driven angiogenesis through VEGFR-2 (11). In fact, in vivo studies have demonstrated that an antibody against VEGFR-1 is capable of inhibiting tumor and retinal angiogenesis (12). Therefore, cross talk between VEGF receptors likely exists, and, depending on circumstances, a dual activity of VEGFR-1 can be exerted.

VEGFR-1 seems the predominant receptor in microvessels of the normal human retina, whereas VEGFR-2 expression becomes increased during diabetic retinopathy development (13). In addition, it should be noted that apart from the eventual negative regulation of VEGFR-2 signaling, VEGFR-1 activation induces vascular permeability, monocyte and macrophage migration, and the recruitment of hematopoietic progenitor cells (6,14). Finally, VEGFR-3 is a critical regulator of developmental and adult vasculogenesis and lymphangiogenesis (6,14).

MECHANISMS REGULATING VEGF EXPRESSION

Several mechanisms have been shown to participate in the regulation of VEGF gene expression (Fig. 2). Hypoxia is one of the most well-characterized factors that induce VEGF gene expression. Transcriptional activation leading to VEGF upregulation in response to hypoxia requires the stabilization of hypoxia-inducible factor 1 (HIF-1) (6).

Changes in glucose levels are another major factor involved in VEGF expression. In this regard, both long-term high glucose concentration and acute glucose deprivation can upregulate VEGF in cultured bovine retinal pigment epithelium (RPE) cells (15). Production of advanced glycation end products (AGEs) could be one of the mechanisms by which chronic hyperglycemia stimulates VEGF mRNA expression, and conversely this could also explain the angiogenic properties of AGEs. In this regard, it has been reported that intravitreal injection of AGEs in rats and rabbits increased VEGF mRNA levels in the ganglion, inner nuclear, and RPE cell layers of the retina (16). This effect was dose- and time-dependent, additive with hypoxia, and could be blocked by using anti-VEGF antibodies (16). In addition, HIF-1α activation by AGEs through the extracellular regulated kinase pathway could be also involved in VEGF expression mediated by AGEs (17).

Apart from hypoxia, changes in blood glucose levels, and AGEs, a variety of growth factors, including insulin, IGF-I, fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF), as well as proinflammatory cytokines (i.e., interleukin [IL]-1 and IL-6), several hormones (i.e., TSH, ACTH, and gonadotropin), and oncogenes (i.e., K-ras, Wnt) could upregulate VEGF mRNA expression (6). It is important to note the relationship observed between insulin and VEGF expression. Lu et al. (18) reported that insulin increased VEGF mRNA and secreted protein levels in RPE cells through enhanced transcription of the VEGF gene. Yamagishi et al. (19) obtained evidence that in human skin microvascular endothelial cells, VEGF is the main factor responsible for the angiogenic activity of insulin. Poulaki et al. (20) demonstrated that acute intensive insulin therapy produced a transient worsening of diabetic BRB breakdown via an HIF-1α–mediated increase in retinal VEGF expression. Finally, Hernández et al. (21) provided evidence that diabetic patients with blurred vision after starting insulin therapy present a significant transient increase of macular biometrics (volume and thickness) that is associated with a

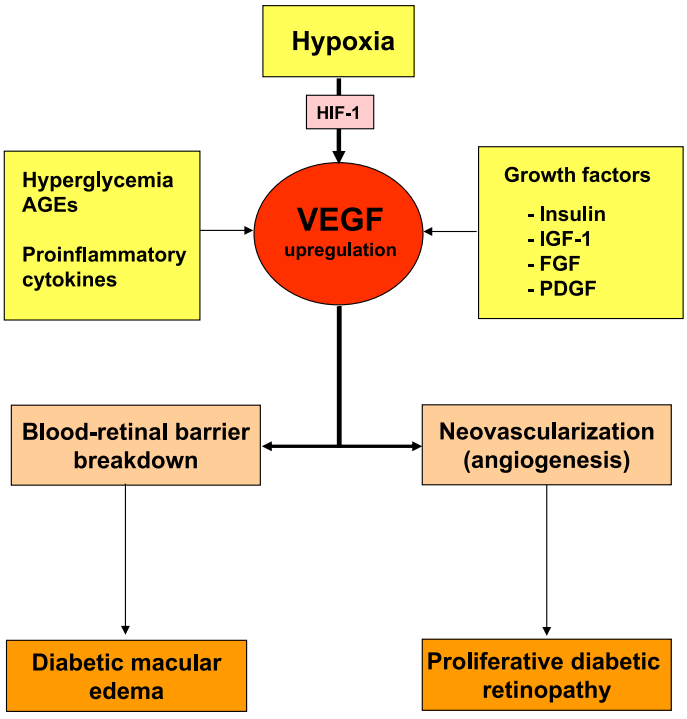

Figure 2—Main mechanisms involved in VEGF upregulation and its consequences in the pathogenesis of diabetic retinopathy. Hypoxia is the most important trigger for VEGF upregulation mainly through HIF-1. Hyperglycemia, AGEs, and proinflammatory cytokines are among the other stimulating factors that could increase VEGF production by the diabetic retina. The main deleterious consequences of VEGF overexpression are the disruption of the blood-retinal barrier (the pathogenic hallmark of diabetic macular edema) and neovascularization (the hallmark of proliferative diabetic retinopathy). FGF, fibroblast growth factor; PDGF, platelet-derived growth factor.

decrease in circulating sFlt-1. Taken together, these findings could contribute to understanding of the transient worsening of diabetic retinopathy that might occur after starting intensive insulin therapy.

VEGF-MEDIATED ACTIONS IN DIABETIC RETINOPATHY

VEGF is a well-known pathogenic factor for the disruption of the BRB and neovascularization, which are the primary pathogenic events of DME and PDR, respectively (Fig. 2). In addition, there is emerging evidence that VEGF has also significant neurotrophic and neuroprotective properties (22–24).

Numerous retinal cells synthesized VEGF, including RPE cells, pericytes, endothelial cells, glial cells, Müller cells, and ganglion cells (25). The specific site in which VEGF is produced is important in accounting for the local actions. For instance, the major function of ganglion cell–derived VEGF is not pathogenic and is important as an autocrine-paracrine neurotrophic factor for neuron cell survival under stress conditions (23). By contrast, conditional gene deletion studies demonstrate that Müller cell–derived VEGF seems to be primarily responsible for BRB breakdown and neovascularization in rodent disease models (26,27). VEGF is also an important link between the neurodegenerative process that occurs in early stages of diabetic retinopathy and the BRB breakdown (28). The most important VEGF-mediated actions in the pathogenesis of diabetic retinopathy are the breakdown of the BRB and angiogenesis.

Breakdown of BRB

The BRB consists of the outer BRB formed by the RPE and the inner BRB involving the endothelial cells of the vasculature network within the inner retina. Tight junctions between neighboring RPE cells and neighboring endothelial cells are essential in the strict control of the fluid and solutes that cross the BRB. Vascular leakage as a consequence of the breakdown of the BRB, in particular the inner BRB, contributes to the pathogenesis of DME. For many patients, this change in vascular permeability is mediated by increased VEGF, also known as vascular permeability factor (VPF) due to its ability to induce vascular leakage. VEGF may induce permeability by transport through cells by inducing fenestrae and vesicles by breakdown of the junctional complex (10). VEGF-induced breakdown of the BRB requires conventional protein kinase C (PKC)β activation, which phosphorylates the tight junction protein occludin, thus inducing the disassembly of the tight junctions and contributing to vascular permeability (29,30). In addition, activation of atypical PKC also contributes to VEGF-induced permeability in a separate but required pathway with, as yet, unidentified downstream targets (31). Further, phosphorylation and internalization of the adherens junction protein, VE-cadherin, also contributes to VEGF-induced permeability (32). In addition, the critical role of nitric oxide in VEGF-induced vascular permeability and angiogenesis has been reported (33). These downstream signaling pathways may provide opportunities for development of next-generation inhibitors to prevent VEGF action on BRB permeability. In this regard, a multipoint model based on well-established drug development principles, with the goal of improving the success of clinical drug development focused on anti-VEGF therapy, has been recently proposed (34).

Angiogenesis

It has been extensively reported that both VEGF and its receptors are increased in the retina of animal models with either ischemic retinopathy or diabetes, as well as in diabetic patients (25). VEGF concentration has been found strikingly higher in the vitreous fluid of PDR patients than in nondiabetic control subjects and expression is related to active retinopathy (35). It should be emphasized that the vitreal concentration of VEGF detected in PDR patients can induce proliferation using in vitro models, and studies of both gain and loss of function for VEGF demonstrate this factor is capable of inducing retinal angiogenesis.

The mechanisms by which VEGF induces neovascularization in PDR are multifactorial; however, critical factors have been identified and are highlighted here. Activation of PKC-β2 isoform has been reported to be important for VEGF-dependent retinal neovascularization (36), likely involving activation of extracellular regulated kinase pathway. Apart from the mitogenic effect, VEGF has other properties that contribute to neovascularization. VEGF induces the expression of serine proteases, tissue-type plasminogen activators, and metalloproteinases, as well as significantly decreasing the tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2 (37,38). These effects are crucial in favoring the proteolysis of the basement membrane, which is the first step in the angiogenic process that leads to PDR. Another mechanism by which VEGF could promote angiogenesis is through the enhancement of intercellular adhesion molecule 1 (ICAM-1) (27) and vascular adhesion molecule 1 (VCAM-1) (39). In this regard, a direct correlation between VCAM-1 and VEGF in the vitreous fluid of diabetic patients with PDR has been reported (40).

CURRENT ANTI-VEGF THERAPIES

Clinicians now have a number of options for reducing VEGF signaling in the retina through intravitreal injection of anti-VEGF biologics.

Pegaptanib is a conjugated to polyethylene glycol neutralizing RNA aptamer with an extremely high affinity for human $VEGF_{165}$ (5). An important feature of aptamers is that, unlike a number of recombinant human proteins, they are essentially nonimmunogenic. Pegaptanib was the first anti-VEGF approved by the U.S. Food and Drug Administration for treatment of exudative age-related macular degeneration (December 2004). However, the lack of robust clinical trials has prevented its approval for the treatment of DME or PDR.

Current evidence indicates that there are three anti-VEGF agents that could be useful for diabetic retinopathy: bevacizumab, ranibizumab, and aflibercept (34) (Table 1). Bevacizumab is a full-length murine monoclonal, anti-VEGF antibody that has been humanized, which has the distinct advantage of the lowest cost of the available therapies, but has no large-scale clinical trial data to support selecting this therapy for DME or PDR. In addition, it should be noted that intravitreous injections of bevacizumab is an "off-label" therapy. Ranibizumab is an affinity-enhanced antibody fragment developed from bevacizumab (34). The Fab fragment lacks the Fc portion and is smaller with monovalent binding to VEGF, as opposed to the bivalent binding of the bevacizumab

Table 1—Main characteristics of current anti-VEGF agents used for treatment of diabetic retinopathy

Anti-VEGF therapy	Class	Molecular weight	Targets	K_D for $VEGF_{165}$	
Bevacizumab	Monoclonal Ab	149 kDa	All VEGF isoforms	58 pM	
Ranibizumab	Fab	48 kDa	All VEGF isoforms	46 pM	
Aflibercept	VEGF-Trap	115 kDa	All VEGF isoforms VEGF-B PlGF	0.49 pM	

Biochemical traits of current anti-VEGF therapies include bevacizumab, an antibody to VEGF; ranibizumab, a fragment of an antibody to VEGF; and aflibercept, an in-line fusion protein of VEGFR-1 (red) and VEGFR-2 (green) extracellular ligand–binding domains linked to the Fc portion of human IgG1. Ab: antibody; Fab: antibody fragment.

antibody but with a higher affinity (Table 1). Ranibizumab is the only approved drug for intravitreal use in DME in the U.S. and Europe. Finally, the VEGF-Trap, aflibercept, was created as an in-line fusion protein of VEGFR-1 and VEGFR-2 extracellular ligand–binding domains linked to the Fc portion of human IgG1 (41).

Recent clinical trials with anti-VEGF therapies have demonstrated significant improved visual acuity for patients suffering from DME, ranibizumab being the most thoroughly tested (42).

Comparison of binding of aflibercept with ranibizumab and bevacizumab reveals a markedly higher binding affinity of aflibercept for $VEGF\text{-}A_{165}$, approximately 100-fold, (Table 1) (43). Modeling studies suggest that this difference in binding affinity may allow aflibercept to provide effective VEGF-A binding for a significantly longer time after injection, potentially reducing the time between repeat injections (44).

The presence of the Fc portion on bevacizumab and aflibercept, but not ranibizumab, may explain several differences in pharmacokinetics. Production of the smaller ranibizumab may allow better diffusion, but the presence of an Fc-binding receptor in the BRB may affect transport dynamics across the retina or into circulation. Indeed, recent measures of serum VEGF concentration in a small cohort after bevacizumab or ranibizumab intravitreal injection reveals that bevacizumab, but not ranibizumab, reduces plasma VEGF for up to 1 month after injection (45). However, the half-lives of all three molecules are very similar, ranging from 3.2 days (ranibizumab) to 4.8 days (aflibercept) to 5.6 days (bevacizumab) (44), thus suggesting the Fc portion does not have a dramatic affect on transport out of the retina.

Clearly, additional studies on the differences in kinetics of bevacizumab versus ranibizumab and aflibercept are needed to ensure minimal systemic effects of intravitreal anti-VEGF therapy. Most notably, aflibercept binds VEGF-B and PlGF, which may contribute to vascular eye disease, and thus, coupled with higher affinity for binding VEGF-A, may have distinct advantage in effectiveness with reduced time of dosing. It will be critical to also determine any effects on neurodegeneration as the higher affinity and broader binding profile could affect a potential role of VEGF signaling in the neuroretina.

A study directly comparing the change in visual acuity after 1 year for ranibizumab, bevacizumab, and aflibercept was initiated in 2012 and will be completed in 2016 by the Diabetic Retinopathy Clinical Research Network (ClinicalTrials.gov, http://clinicaltrials.gov/ct2/show/NCT01627249).

ADVERSE EVENTS RELATED TO ANTI-VEGF TREATMENT

A recent meta-analysis was conducted to determine the effectiveness of anti-VEGF for the treatment of DME to determine the rate of ocular and nonocular side effects. Overall, these events are rare and have been studied imprecisely to date. Across all studies included in the analysis, infectious endophthalmitis occurred in 9 of 2,287 total patients (3.9 patients per 1,000). Nonocular side effects, as defined by the Antiplatelet Trialists' Collaboration (ATC) events and death, were not different in anti-VEGF–treated arm versus the assumed risk group (46). However, it should be noted that most data were obtained at 1 year and, therefore, long-term confirmation is needed.

There is some concern that consistent exposure to anti-VEGF may result in geographic atrophy. A recent study demonstrated in mice that VEGF produced by the RPE contributes to maintenance of the choriocapillaris (the vascular network that underlies the retina) (47). While genetic ablation of soluble VEGF from the RPE did not lead to complete choriocapillary loss, there was a reduction in vascular area covered and the ERG A-wave and B-wave amplitude. Anti-VEGF therapy related to geographic atrophy has been noted as occurring slightly more frequently in patients treated with monthly ranibizumab versus bevacizumab for wet age-related macular degeneration. However, extrapolation of these data to patients with diabetic retinopathy should be done cautiously given the disparate underlying pathophysiology. The potential for anti-VEGF treatment to cause geographic atrophy in diabetic retinopathy will require further study.

Finally, the potential inhibition of the neuroprotective effects of VEGF should also be taken into account in long-term administration of anti-VEGF agents. The neuroprotective and neurotrophic effects of VEGF in the brain are well established (48), and there is growing

evidence that this is also true in the retina (22–24). In this regard, a dose-dependent decrease in ganglion cells has been reported following the injection of an antibody that blocks all VEGF isoforms in rats (23). These findings could have clinical implications as to date clinical trials using anti-VEGF treatment have focused only on studying the systemic side effects, such as cardiovascular, hypertension, proteinuria, or bleeding, but not the incidence of retinal neurodegeneration, such as retinal atrophy or RPE cell degeneration (6). Therefore, further research on this issue is urgently needed.

CONCLUSIONS

Diabetic retinopathy remains the most common cause of visual impairment in working-aged individuals. Therefore, new therapeutic strategies are needed to reduce the significant burden associated with this common complication of diabetes. As retinal overexpression of VEGF plays an essential role in the development of both DME and PDR, intravitreal anti-VEGF agents have emerged as new treatments for these debilitating complications of diabetes. At present, anti-VEGF agents have been shown to be effective and safe treatments for DME, and ongoing and future studies will reveal their potential value for treating PDR. However, as diabetic retinopathy is a chronic condition, long-term data to evaluate both the efficacy as well as local and systemic adverse effects are needed. In addition, head-to-head studies of these drugs would provide useful information. Finally, new therapeutic approaches to selectively block VEGF angiogenic and permeabilizing actions, while sparing VEGF neuroprotective and choroidal trophic actions seem warranted.

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