Review

Mechanisms of Acquired Resistance to Anti-VEGF Therapy for Neovascular Eye Diseases

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Purpose. The purpose of this study was to evaluate clinical reports of response-loss in patients with neovascular eye diseases, such as neovascular age-related macular degeneration (AMD) and diabetic macular edema (DME), after repeated anti-vascular endothelial growth factor (VEGF) therapy. To assess experimental evidence of associations of other angiogenic growth factors and endothelial glycolytic pathways with the diseases and to propose the underlying mechanisms.

Methods. Review of published clinical studies and experimental investigations.

Results. Intravitreal injection of anti-VEGF biologic drugs (e.g. bevacizumab, ranibizumab, and aflibercept) is the front-line treatment for neovascular AMD and DME, and acts by halting the progression of excess blood vessel growth and leakage. Despite favorable clinical results, exudation returns in a number of patients after repeated administrations over time. Patients suffering from disease recurrence may have developed an acquired resistance to anti-VEGF therapy. We have analyzed clinical and preclinical findings on changes to angiogenic signaling pathways following VEGF-targeted treatment and hypothesize that switching to alternative pathways could potentially bypass VEGF blockade, accounting for development of resistance to anti-VEGF therapy. We have also discussed potential reprogramming of ocular endothelial glycolysis in response to VEGF antagonism and proposed that metabolic adaptations could impair blood-retinal barrier function, counteracting the clinical efficacy of VEGF-targeted therapies and contributing to a decline of response to them.

Conclusions. Future studies of the mechanisms proposed in this review may shed some light on how these adaptations result in the development of acquired resistance to anti-VEGF therapy, which should help discover new therapeutic strategies for overcoming anti-VEGF resistance and improving clinical efficacy.

Keywords: angiogenesis, anti-vascular endothelial growth factor (VEGF), age-related macular degeneration (AMD), proliferative diabetic retinopathy (PDR), diabetic macular edema (DME)

Age-related macular degeneration (AMD) is a leading cause of blindness within the elderly population and diabetic retinopathy (DR) is a primary cause of blindness in the working-age population.[1,2] The growth of elderly, obese, and diabetic populations has resulted in increased prevalence of AMD and DR, both of which are predicted to continue to increase, representing significant challenges for global public health.[3] AMD is recognized as a multifactorial disease caused by combined multiple factors of aging, genetic variants, and environment, as well as lifestyle (including ethnicity, dietary habits, and cigarette smoking), whereas diabetes-related hyperglycemia is the key promoter for the development and progression of DR. Despite the differences in their pathophysiological manifestations, both disease states are primarily driven by the aberrant expression of vascular endothelial growth factor (VEGF) upregulated mainly through hypoxia-inducible factor-1 in response to retinal ischemic hypoxia, activating angiogenesis, and increasing vascular permeability.[4,5] In the case of AMD, drusen deposits develop between the retinal pigment epithelium (RPE) and the choroid, causing damage to RPE cells through ischemia and hypoxia. This progresses to the angiogenic late-stage of the disease, termed neovascular (or wet) AMD, when hypoxia triggers overexpression and release of VEGF causing aberrant neovascularization of the choroidal blood vessels and increased vascular permeability.[6,7] Untreated neovascular AMD can lead to loss of central visual acuity and can rapidly progress over weeks or months.[8]

DR is the most severe ocular complication of diabetes mellitus. Diabetic macular edema (DME) is the most common cause of visual impairment in patients with DR and primarily manifests when hyperpermeable retinal blood vessels leak into the macula area.[9,10] Diabetes and the

resulting hyperglycemia progressively affect retinal endothelial cell (EC) metabolism to induce detrimental oxidative stress and cause EC dysfunction with diminished inner retinal microcirculation, resulting in retinal ischemic hypoxia (see Dysregulation of EC Glucose Metabolism in DR). The hyperglycemia-induced ischemia present in DR is considered a major contributing factor to the upregulation of VEGF, which in turn plays a central role in the development of DME and proliferative diabetic retinopathy (PDR). VEGF elicits aberrant angiogenesis of the retinal vasculature (the hallmark of PDR) and disrupts the blood-retinal barrier (the hallmark of DME) to cause vascular leakage, resulting in loss of vision. Thus, VEGF-targeted therapies are a primary treatment option for such neovascular eye diseases (NVEDs). Anti-VEGF biological therapies, such as ranibizumab and bevacizumab (discussed below), directly target ocular VEGF-A in patients with neovascular AMD and DME.

Alongside VEGF-A, other members of the VEGF family include VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). VEGF-A (referred to hereafter as VEGF), plays a pivotal role in angiogenesis. Multiple VEGF isoforms, including $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, result from alternative splicing of mRNA from a single VEGF gene. $VEGF_{165}$ is the most widely expressed VEGF isoform in tissues, with a crucial role in pathological angiogenesis.[11] Despite the wide-scale use of anti-VEGF biologics, long-term clinical data suggests that VEGF-targeted therapy can be limited by the return of visual decline after repeated administrations over time, indicating a loss of efficacy following an initial response.[12,13] Patients suffering from disease recurrence may have acquired resistance to anti-VEGF therapy, the mechanisms of which are poorly understood. In addition, up to 30% of sufferers of NVEDs do not respond at all to anti-VEGF therapy.[10,14]

This review aims to provide an overview of clinical observations and experimental evidence on the role of alternative angiogenic pathways which could account for development of resistance to VEGF-targeted treatment, with particular focus on VEGF-independent pathways mediated through neuropilin-1 (NRP-1). The review also will discuss potential perturbation of endothelial glucose metabolism after anti-VEGF therapy and propose how these adaptation mechanisms potentially bypass VEGF blockade and promote resistance to the VEGF inhibitors responsible for recurrence of NVEDs.

Anti-VEGF Therapy in NVEDs

Numerous clinical trials have been conducted to determine the clinical effectiveness of anti-VEGF therapies in neovascular AMD and DME. In particular, landmark studies over recent years have demonstrated improvements in visual outcomes after treatment (Table 1).

VEGF-targeted biological medicines include monoclonal antibodies against VEGF and decoy receptors comprising modified VEGF receptor extracellular domains. Ranibizumab (sold as Lucentis, developed by Genentech/Novartis) is a recombinant humanized monoclonal antibody Fab fragment targeted against VEGF created from the same parental mouse antibody as bevacizumab (discussed below). Approved in 2006 for the treatment of neovascular AMD and in 2012 for the treatment of DME (see Table 1),[15,16] ranibizumab inhibits angiogenesis through binding with high affinity to all VEGF isoforms to prevent activation of VEGF receptor-1 (VEGFR1) and VEGF receptor-2 (VEGFR2), located on the surface of endothelial cells.[17] Bevacizumab (Avastin, developed by Genentech/Roche) is a recombinant humanized full-length monoclonal antibody which, similarly to ranibizumab, bind all isoforms of VEGF with high affinity, preventing VEGF receptor binding. Approved by the US Food and Drug Administration (FDA) in 2004 for the first-line treatment of metastatic colorectal cancer, current therapeutic indications include a wide range of cancer types.[18] Despite the absence of formal approval for the use of bevacizumab in ocular diseases, it is still widely used as an off-label treatment for neovascular AMD due to its cost-effectiveness in comparison with ranibizumab.[19,20]

Aflibercept (Eyelea/VEGF-TRAP, developed by Regeneron) is a recombinant fusion protein, which combines the extracellular immunoglobulin-like (Ig) domain 2 of VEGFR1 and the extracellular Ig domain 3 of VEGFR2 fused to the Fc portion of human IgG1. It acts as a soluble decoy VEGF receptor and was approved in 2011 for the treatment of AMD and in 2014 for the treatment of DME.[21,22] Similar to bevacizumab and ranibizumab, aflibercept binds to multiple VEGF isoforms but with comparatively higher affinity. Having the second Ig domain of human VEGFR1, aflibercept also binds the VEGFR1 ligands VEGF-B and PlGF.[23] Brolucizumab (Beovu, developed by Novartis) is recently developed anti-VEGF biologic treatment, which was approved for wet AMD treatment in 2019 after showing promising results in the HAWK and HARRIER clinical trials.[24] It is a humanized single-chain antibody fragment, which neutralizes all isoforms of VEGF. As the emerging Angiopoietin-2/Tie-2 pathway has been identified to play an important and complementary role alongside VEGF in NVEDs, faricimab (Vabysmo, developed by Roche), the first bispecific monoclonal antibody to target both VEGF and angiopoietin-2, has very recently demonstrated vision benefits at an extended treatment interval (every 16 weeks) comparable with VEGF pathway inhibition alone with aflibercept given at 8-week intervals for neovascular AMD and DME, thereby reducing treatment burden in patients.[27,28] A summary of ocular anti-VEGF treatments is presented in Table 2.

Combined clinical trial data has suggested that around 30% of patients with DME are nonresponsive to intravitreal anti-VEGF treatment.[10,14] Similarly, in patients with AMD, the CATT study revealed that even after 2 years of treatment, 67.4% of patients treated with bevacizumab and 51.5% of patients treated with ranibizumab showed persistent retinal fluid accumulation.[19] Although there is currently no consensus on the categorization of response status to anti-VEGF therapy, patients manifesting persistent or increased retinal exudation and no improvement in visual acuity despite four or six monthly consecutive injections have been described as nonresponsive patients or nonresponders,[5,10] suggesting that other pathological mechanisms are largely involved in the multifactorial disease. Collectively, these findings draw attention to the presence of significant innate resistance to anti-VEGF therapy, highlighting the need for further research elucidating the underlying mechanisms of disease in nonresponsive patients.

Furthermore, these studies show that intravitreal administration of bevacizumab to patients with neovascular AMD results in a progressive decrease in therapeutic and biological responses to treatment over time, a phenomenon that is not counteracted by increased treatment dosage.[29–31] Similarly, 17% to 56% of patients with PDR saw recurrence of vitreous hemorrhage after initial intravitreal bevacizumab treatment[32,33]; however, the repeated treatment

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Table 1. Summary of Landmark Clinical Trials Which Pertained to Approval of Anti-VEGF Biologic Therapies

Author(s)	Year	Trial Name	Treatment	Against	Test Period	Major Findings
Rosenfeld et al.[15]	2006	MARINA	Ranibizumab (0.3 mg or 0.5 mg)	Sham	24 mo	Ranibizumab increased VA with low rates of ocular adverse events in patients with AMD compared to sham injections
Brown et al.[16]	2006	ANCHOR	Ranibizumab (0.3 mg or 0.5 mg)	Verteporfin	24 mo	Ranibizumab increased VA with low rates of ocular adverse events in patients with AMD compared to verteporfin
Nguyen et al.[25]	2010	READ-2	Ranibizumab (0.5 mg)	Laser PCG	24 mo	Ranibizumab alone improved BCVA to a greater extent than laser alone or combination therapy in DME
Martin et al.[19]	2012	CATT	Bevacizumab (1.25 mg)	Ranibizumab (0.5 mg)	24 mo	Bevacizumab and ranibizumab elicited similar effects on visual acuity in patients with AMD over 2 y
Heier et al.[21]	2012	VIEW 1 and VIEW 2	Aflibercept (0.5 mg)	Ranibizumab (0.5 mg)	12 mo	Intravitreal aflibercept elicited similar efficacy and safety outcomes as ranibizumab in patients with AMD
Brown et al.[26]	2013	RISE and RIDE	Ranibizumab (0.3 mg)	Ranibizumab (0.5 mg)	36 mo	0.3 mg and 0.5 mg doses of ranibizumab in DME had similar efficacies and improve VA over a 36-mo period
Korobelnik et al.[22]	2014	VIVID and VISTA	Aflibercept	Laser PCG	12 mo	Intravitreal aflibercept demonstrated superior visual outcomes compared to laser therapy in DME
Dugel et al.[24]	2020	HAWK and HARRIER	Brolucizumab (3 mg or 6 mg)	Aflibercept (2 mg)	48 wk	Brolucizumab showed similar efficacy to aflibercept in patients with AMD in a 12-mo period
Heier et al.[27]	2022	TENAYA and LUCERNE	Faricimab (6 mg)	Aflibercept (2 mg)	48 wk	Faricimab every 16 weeks was non-inferior to aflibercept every 8 weeks in patients with AMD
Wykoff et al.[28]	2022	YOSEMITE and RHINE	Faricimab (6 mg)	Aflibercept (2 mg)	12 mo	Faricimab every 8 weeks or up to every 16 weeks was non-inferior to aflibercept every 8 weeks in patients with DME

VA, visual acuity; PCG, photocoagulation; BCVA, best corrected visual acuity.

Table 2. Summary of Anti-VEGF Biologic Therapies

INN	Trade Name	Molecular Weight	K_D for VEGF165	Indication
Bevacizumab	Avastin	149 kDa	58 pM	Choroidal neovascularization (in AMD and other - off-label); diabetic macular edema (off-label); central retinal vein occlusion (off-label)
Ranibizumab	Lucentis	48 kDa	46 pM	Choroidal neovascularization (in AMD and other); diabetic macular edema; macular edema secondary to retinal vein occlusion
Aflibercept	Eyelea/VEGF-TRAP	115 kDa	0.49 pM	Neovascular AMD; diabetic macular edema; macular edema secondary to retinal vein occlusion; myopic choroidal neovascularization
Brolucizumab	Beovu	26 kDa	28.4 pM	Neovascular AMD
Faricimab	Vabysmo	150 kDa	3.5 nM (22 nM for Ang-2)	Neovascular AMD; diabetic macular edema

INN, international non-proprietary name; Ang-2, angiopoietin-2.

effects of bevacizumab in patients with PDR remains unclear. A number of clinical studies have also described the recurrence of DME after intravitreal bevacizumab injection.[34,35]

The response-loss (known as tachyphylaxis) refers to the phenomenon whereby some patients who had a good initial response with the resolution of exudation after injections of

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anti-VEGF drug, then developed new fluid after repeated administration over time and became resistant to further treatment.[5,13]

Retrospective studies of intravitreal ranibizumab treatment in patients with neovascular AMD showed recurrence in 66% to 76% of patients after 12 months of repeated treatment and in 74.8% of patients after 24 months of treatment.[36,37] Gasperini et al.[38] reported that patients with AMD with choroidal neovascularization following repeated administration of either ranibizumab or bevacizumab over time developed a diminished therapeutic response; both ranibizumab and bevacizumab showed attenuation of efficacy after an average of five and seven injections, respectively. Interestingly, however, switching from one treatment to the other after resistance occurrence largely elicited restoration of therapeutic effect in the majority of eyes. Eleven percent to 31% of patients with PDR had pathophysiological recurrence after initial intravitreal ranibizumab treatment.[39,40] However, the long-term effects of repeated ranibizumab treatments in PDR recurrence requires further investigation. Recent studies have also reported disease recurrence in 9% to 55% of patients with neovascular AMD treated with repeated intravitreal aflibercept injections,[13,41] demonstrating an acquired resistance to aflibercept-based anti-VEGF therapy. It was noted by Hara et al.[13] that occult with no classic type and polypoidal choroidal vasculopathy (lesions beneath the RPE and no intraretinal edema) were the only AMD subtypes that developed the resistance (tachyphylaxis) to aflibercept. The reason for this is unclear, and the intravitreal aflibercept achieved the initial resolution in these two subtypes, suggesting the access of aflibercept into lesions of choroidal neovascularization beneath the RPE. They also found similar percentages of the RPE detachment, a common manifestation of AMD, in eyes without and with tachyphylaxis (30% vs. 32%),[13] whereas the lack of intraretinal edema was only observed in eyes with tachyphylaxis. Perhaps, the absence of intraretinal edema indicates less disruption of the RPE-mediated outer blood-retinal barrier, which could limit penetration of aflibercept to lesions beneath the RPE, contributing to the loss of response and the development of resistance.

Overall, although many patients with NVEDs benefit from anti-VEGF treatment, a significant proportion see no effect of treatment, and most responding patients experience recurrence of disease over time. Although studies have endeavored to characterize patients who either lack an anti-VEGF response or experience decline in response over time,[5] the mechanisms behind both the failure to respond and decreased responsiveness remains unclear. Whereas there is a potential genetic component to innate anti-VEGF resistance,[42,43] the mechanisms of acquired resistance are largely unknown.

Despite this, some pathways have been hypothesized to participate in disease recurrence after anti-VEGF treatment. Neuropilin-1 (NRP-1), a transmembrane glycoprotein implicated in neuronal development, angiogenesis, and immune regulation, is a co-receptor for VEGF and several other cytokines. A number of NRP-1 ligands have been highlighted as potential candidates through which recurrence of NVEDs may occur after anti-VEGF treatment (see Role of NRP-1 and NRP-1-binding Cytokines in NVEDs). Further to this, alterations in the glycolytic pathway also has the potential to elicit some of the pathological recurrences of NVEDs after anti-VEGF treatment (see Endothelial Glycolysis and its Potential Role in NVED Pathology and Recurrence).

Role of NRP-1 and NRP-1-Binding Cytokines in NVEDs

NRP-1

NRP-1 is a receptor for class 3 semaphorins, such as semaphorin 3A (SEMA3A), as well as a co-receptor for a variety of ligands including VEGF, PlGF, transforming growth factor-βs (TGF-βs), and platelet-derived growth factor (PDGF),[44,45] as illustrated in Figure 1. By forming a co-receptor complex with VEGFR2, NRP-1 facilitates optimal VEGF signaling via VEGFR2, alongside optimal downstream biological function.[46,47] NRP-1 signaling has been widely investigated for its role in angiogenesis and vascular permeability pathways.[48,49]

Studies have associated endothelial NRP-1 with tip cell function during angiogenesis, alongside mediation of tumor angiogenesis, vascular permeability, and vascular remodeling mechanisms.[50–52] A role of NRP-1 in NVEDs has recently emerged. Fernández-Robredo et al.[53] observed that endothelial specific NRP-1 knock-out mice had reduced choroidal and retinal neovascularization in models of laser-induced choroidal neovascularization and oxygen-induced retinopathy compared to wild-type mice, indicating a potential role of NRP-1 in the pathological progression of NVEDs, such as AMD and DR. By using the vaso-permeability Miles assay, Roth et al.[52] showed that stimulation of NRP-1 with VEGF or a CendR peptide increased vascular leakage in vivo in both VEGFR2-dependent and independent manners, suggesting a role of NRP-1 signaling in vascular permeability and EC barrier function.

Although these findings indicate a relationship between NRP-1 and pathological ocular neovascularization, there remains a lack of clinical evidence of its role in the development of acquired resistance to anti-VEGF therapy. In vivo studies, such as that of Roth et al.,[52] raise the possibility of VEGF-independent pathological activity of endothelial NRP-1. Therefore, further study into the mechanisms behind this is required, investigating the various NRP-1 ligands and the pathways through which they may elicit acquired anti-VEGF resistance (see Fig. 1).

Semaphorin 3A

Semaphorin 3A (SEMA3A) is a class 3 semaphorin which primarily cues axonal guidance through interaction with NRP-1 and members of the plexin family.[54,55] Through this pathway, SEMA3A has also been associated with ocular pathophysiology, with Kwon et al.[56] identifying SEMA3A as a potential biomarker for DR.

To assess ocular SEMA3A/NRP-1 signaling, Cerani et al.[44] evaluated the presence of SEMA3A in the vitreous of patients with DME and found elevated quantities of SEMA3A in patients with DME compared to control patients. Further evaluation of the role of ocular SEMA3A in vivo showed that increased SEMA3A levels contributed to increased ocular vascular leakage resulting from compromised blood-retinal barrier function. Interestingly, these effects were prevented by either using a recombinant mouse soluble NRP-1 in a mouse model of streptozotocin-induced diabetes or knock-out of vascular NRP-1 in mice, demonstrating the induction of retinal vascular permeability through NRP-1/SEMA3A interactions. Furthermore, higher levels of SEMA3A were observed in the vitreous of patients suffering from late-stage PDR, and in a murine model of oxygen-induced retinopathy

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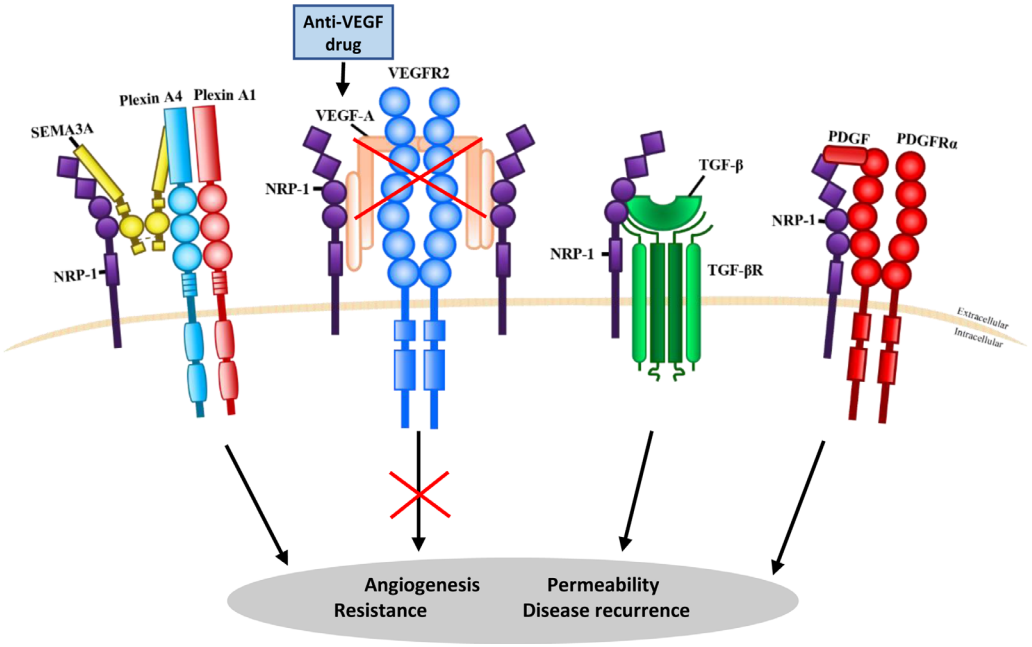

Figure 1. Neuropilin-1 (NRP-1) binding to multiple ligands and their potential involvement in anti-VEGF resistance. NRP-1 can function as a receptor for semaphorin 3A (SEMA3A) and a co-receptor for a variety of ligands, including vascular endothelial growth factor A (VEGF-A) through vascular endothelial growth factor receptor 2 (VEGFR2); transforming growth factor-βs (TGF-βs) through transforming growth factor-β receptors (TGF-βR); and platelet-derived growth factor (PDGF) through the platelet-derived growth factor receptor a (PDGFRα). Following anti-VEGF therapy, NRP-1-mediated VEGF-independent pathways may be switched on to bypass VEGF blockade for pathological angiogenesis and vascular permeability, accounting for disease recurrence due to the development of acquired resistance.

SEMA3A recruited NRP-1-positive mononuclear phagocytes to sites of pathological neovascularization in the retina, which was essential for disease progression.[57]

In addition, Guo et al.[58] investigated the aqueous level of SEMA3A in patients with retinal vein occlusion with macular edema, which often occurs following DR, and observed increased levels of SEMA3A in the aqueous humor of patients. This positively correlated with central retinal thickness as well as negatively correlated with the ganglion cell-inner plexiform layer, suggesting a pathological role of ocular SEMA3A in macular edema and ganglion cell degeneration. Andriessen et al.[59] also recently reported elevated SEMA3A and VEGF-A in the vitreous of patients with AMD at times of active choroidal neovascularization and provided evidence that NRP1-expressing myeloid cells promote and maintain choroidal neovascularization in a mouse model.

Placental Growth Factor

The PlGF, originally isolated from human placenta, is a member of the VEGF family,[60] binding VEGFR1 and NRP1 but not VEGFR2. Despite this, PlGF is capable of enhancing VEGF activation of VEGFR2 through synergistic crosstalk.[61] This occurs through PlGF displacement of VEGF from VEGFR1, increasing availability of VEGF to VEGFR2, enhancing VEGF-induced angiogenic activity.[62] In addition to forming homodimers, PlGF and VEGF can also form heterodimers, which exhibit only weak biological activity.[63] Unlike VEGF, PlGF is dispensable for developmental angiogenesis, but its expression is associated with pathological angiogenesis and inflammation.[61]

Although the role of PlGF in tumor growth and metastasis has been extensively studied,[64] several studies suggest that PlGF could also be implicated in retinal vascular disease.[65,66] Clinical studies revealed the elevation of aqueous and vitreous levels of PlGF in patients with PDR, with neovascular glaucoma due to DR or DME compared to non-diabetic patients or non-DR control patients, suggesting that increased PlGF levels were correlated with progressive ischemic retinopathies.[67–70] Furthermore, in an in vivo model of PDR, PlGF overexpression in rat ocular media was associated with abnormal vascular properties, including microaneurysm formation, junction ruptures, and aberrant vascular sprouting.[71] Using an electrical cell-impedance sensing system to measure trans-endothelial electrical resistance, Huang et al.[72] showed that PlGF negatively regulated retinal endothelial cell barrier function in an in vitro model of the blood-retinal barrier.

Furthermore, Zehetner et al.[73] explored the effects of intravitreal administration of ranibizumab, bevacizumab, or aflibercept on systemic PlGF levels in patients with neovascular AMD and reported a significant upregulation of plasma PlGF levels after treatment with aflibercept but not with bevacizumab or ranibizumab, suggesting a counter-regulatory response to aflibercept injection. However, little is known about any changes in ocular PlGF levels after anti-VEGF therapy, and this requires further investigation.

Transforming Growth Factor-β

The TGF-β subfamily of proteins, consisting of TGF-β1 to β3, regulates cellular growth, development, and homeostasis in a cell type and condition specific manner.[74] As a co-receptor for TGF-β, NRP-1 is capable of modulating TGF-β-dependent EC development to control sprouting angiogenesis and immune responses through TGF-β receptors.[75,76] To further

investigate this mechanism, Aspalter et al.[77] examined the effect of reduced NRP-1 expression on TGF-β signaling, finding that NRP-1 downregulation increases phosphorylation of vascular SMAD2/3 and drives endothelial stalk cell behavior. These findings may suggest that in the presence of NRP-1, TGF-β/SMAD2/3 signaling-inhibited endothelial tip cell capacity for sprouting angiogenesis may be attenuated. Conversely, inhibition of TGF-β has been associated with decreased phosphorylated SMAD2/3 and impaired vascular development and function in ocular vasculature, suggesting an important role of the TGF-β/SMAD2/3 signaling in ocular neovascularization.[78,79]

Tosi et al.[80] investigated TGF-β1 levels in the aqueous of patients with neovascular AMD before and after repeated doses of ranibizumab. The study found that baseline TGF-β1, prior to ranibizumab injection, was higher in patients with neovascular AMD than control patients and aqueous TGF-β1 levels were persistently elevated with a tendency for further increase after treatment, presenting an upregulating effect of VEGF blockade on ocular TGF-β1. Although TGF-β1 is largely associated with induction of angiogenic mechanisms, other studies are consistent with a therapeutic effect of TGF-β1 secreted from mesenchymal stem cells, through suppression of retinal neovascularization in vivo.[81]

The vascular effects of TGF-β1 in the presence of NRP-1, or the pathogenic or homeostatic effects of NRP-1/TGF-β interaction on ocular angiogenesis remain poorly understood. Additionally, the ability of TGF-β to both positively and negatively regulate angiogenic pathways in a context-dependent manner warrants much further investigation into the function of upregulated TGF-β1 in NVEDs.

Other Growth Factors

The PDGF family and their receptors play a role during vascular development mainly through their essential functions in pericyte and vascular smooth muscle cell recruitment to developing vessels.[82] Several studies indicate a role of NRP-1 activation in the modification of PDGF signalling.[82–86] In vivo studies on corneal neovascular rabbits and subretinal neovascular mice have associated combined anti-VEGF and anti-PDGF treatment with reduced AMD pathophysiology, such as reduced choroidal neovascularization, retinal detachment, and subretinal neovascularisation.[87–89] This stimulated interest in clinical investigation into dual anti-PDGF and anti-VEGF therapy for AMD, although lack of improvement to visual acuity compared to anti-VEGF alone has been reported recently.[90,91] Furthermore, Muhl et al.[86] found that in vitro PDGF-D treatment was associated with translocation of NRP-1 to EC junctions and that ex vivo PDGF-D/NRP-1 binding retains pericyte coverage and interaction with ECs during angiogenic sprouting, potentially mediating vascular permeability. Therefore, alternative pathways of PDGF signaling, such as NRP-1 co-activation pathways, may be worth further investigation to examine whether PDGF signaling participates in acquired anti-VEGF resistance.

The hepatocyte growth factor (HGF)/c-MET signaling pathway plays a prominent role in developmental and homeostatic angiogenesis.[92,93] There are a number of clinical investigations into the HGF level in association with eye disease progression in PDR and non-PDR, indicating a higher expression level in the disease state.[94,95] Further to this, increased aqueous levels of HGF have been observed after anti-VEGF treatment in patients with AMD and DME.[96,97] In vivo study of HGF activity in mice with ischemic retinopathy indicated a role of HGF/c-MET activation in retinal neovascularization, with HGF acting as a pro-inflammatory, pro-permeability, and pro-angiogenic factor.[98] As a co-receptor for HGF, NRP-1 is capable of enhancing HGF binding and activity.[99] Various studies have sought to investigate NRP-1/HGF interactions in cancer.[100,101] Although these studies further clarify the role of HGF in angiogenic pathophysiology and the effect of HGF/NRP-1 in disease states, the role of HGF in acquired resistance to anti-VEGF therapy in NVEDs remains unclear.

A recent study found that expression of angiopoietin-like 4 (ANGPTL4), is increased in the eyes of diabetic mice and patients with DME.[102] These authors also showed that ANGPTL4 binds to endothelial NRP-1, resulting in activation of RhoA/ROCK signaling and subsequent loss of endothelial cell-cell junction barrier function. Furthermore, a soluble extracellular fragment of NRP-1 (sNRP-1) inhibited ANGPTL4-induced endothelial permeability in diabetic mice, and the permeability-increasing activity of aqueous fluid from patients with DME.[102]

Endothelial Glycolysis and Its Potential Role in NVED Pathology and Recurrence

Endothelial Glycolysis in Ocular Neovascularization

Increased endothelial glycolysis has recently been recognized as a driving force of angiogenesis alongside the well-established angiogenic growth factor VEGF. Activated ECs rely on glycolysis as opposed to oxidative metabolism for adenosine triphosphate (ATP) production to fuel the migrating tip ECs as well as proliferating stalk ECs during neovascular development.[103] The consequence is that less than 1% of glycolytic pyruvate enters the mitochondria to undergo oxidative metabolism for ATP production in physiological conditions.[104] In healthy ECs, a common glycolysis pathway is followed in the production of ATP in order to produce the energy required for EC proliferation and migration during angiogenic processes (Fig. 2). During healthy adulthood, quiescent ECs sustain basal levels of glycolysis and other metabolic pathways to serve energy production, biomass synthesis, and redox homeostasis required for their multiple functions, including vascular protection against oxidative stress and maintenance of vascular tone and blood-tissue barrier integrity and stability.[105] Healthy quiescent ECs produce up to 85% of their ATP from glycolysis and they are more glycolytic than other healthy cell types.

Although EC metabolism provides the driving force for proliferation during angiogenesis, some studies relate alterations in this mechanism to the progression of pathological angiogenesis in certain disease states.[106] Alongside the common glycolysis pathway, glycolysis side pathways exist to allow the generation of macromolecules required for EC proliferation and migration.[107] One such pathway, the pentose phosphate pathway (PPP), has an oxidative branch PPP (oxPPP) which produces reduced nicotinamide adenine dinucleotide phosphate (NADPH). This NADPH is utilized in the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH), a potent reactive oxygen species (ROS) scavenger (see Fig. 2), presenting a major function of the PPP as an antioxidative defense pathway.[108]

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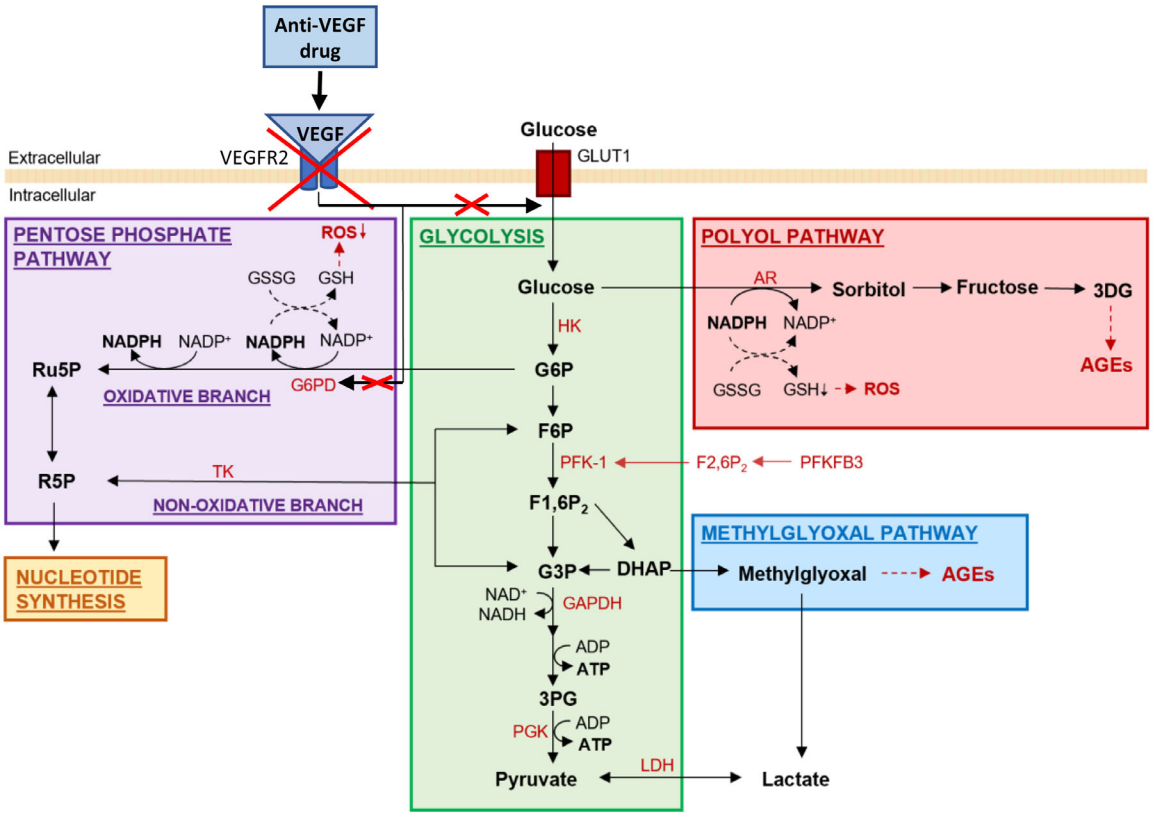

Figure 2. Endothelial cell glycolysis and three glycolytic side pathways potentially influenced by anti-VEGF therapy. The glycolytic main pathway along with the pentose phosphate side pathway physiologically exist in ECs to allow energy production, biomass synthesis and redox homeostasis. The polyol side pathway and the methylglyoxal side pathway become pathologically significant during hyperglycemia. VEGF antagonism may cause EC oxidative stress and cellular damage through abolishment of VEGF-stimulated activity of glucose-6-phosphate dehydrogenase (G6PD) and depletion of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the pentose phosphate pathway, resulting in decreased reduced glutathione (GSH) and accumulated reactive oxygen species (ROS). Please note for simplicity, not all enzymes, metabolites and glycolysis side pathways are illustrated in this diagram. Abbreviations: 3DG, 3-deoxyglucosone; 3PG, 3-phosphoglycerate; ADP, adenosine diphosphate; AGE, advanced glycation end product; AR, aldose reductase; ATP, adenosine triphosphate; DHAP, dihydroxyacetone phosphate; F1,6P2, fructose 1,6-bisphosphate; F2,6P2, fructose-2,6-bisphosphate; F6P, fructose 6-phosphate; G3P, glyceraldehyde 3-phosphate; G6P, glucose 6-phosphate; GLUT1, glucose transporter 1; GSSG, oxidized glutathione; HK, hexokinase glucokinase; LDH, lactate dehydrogenase; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; PFK-1, 6-phosphofructo-1-kinase; PFKFB3, phosphofructokinase-2/fructose-2,6-bisphosphatase 3; PGK, phosphoglycerate kinase; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; TK, transketolase.

Moreover, the presence and activity of certain growth factors, including some discussed as NRP-1 ligands above, can regulate EC glycolysis. PlGF inhibition has been found to upregulate glucose-6-phosphate dehydrogenase (G6PD), a key regulator of the oxPPP, in human retinal cells in vitro.[109] The role of PlGF in downregulation of G6PD has been associated with oxidative cellular damage as well as negative regulation of retinal EC barrier function resulting in vascular hyperpermeability.[72] Furthermore, VEGF and TGF-β1 individually have been associated with increased glycolysis in cancer,[110,111] and thus could be worth investigating for their effects on retinal EC glycolysis.

Dysregulation of EC Glucose Metabolism in DR

Hyperglycemia is the hallmark of diabetes and perturbs EC metabolism in a number of ways which can cause EC dysfunction, contributing to the pathogenesis of PDR and DME. One such mechanism is the inhibition of the PPP flux through downregulation of G6PD, the rate-limiting enzyme of the pathway that maintains the level of NADPH. This in turn decreases the generation of NADPH, subsequently impairing maintenance of the GSH level, so that ROS is progressively accumulated after GSH depletion (see Fig. 2).[104,112] Consequently, hyperglycemia-induced ROS accumulation inhibits GAPDH to stall glycolysis and concomitantly generate upstream glycolytic intermediates, which are subsequently diverted into several pathological glycolytic side pathways including the polyol pathway and the methylglyoxal pathway (see Fig. 2), whereas high levels of glucose continuously overwhelm glycolysis and excess glucose also floods to the polyol pathway.[104] These pathways give rise to a further increase in ROS levels and production of advanced glycation end products (AGEs), which can result in cellular inflammation and degradation.[104] The degradation of retinal ECs through such mechanisms can give rise to the microaneurysm formation and vascular hyperpermeability present in DR and DME.

Using metabolomic analysis of the vitreous of patients with PDR, Barba et al.[113] found lactate to be the most abundant metabolite present compared to non-diabetic patients, evidencing increased glycolytic metabolism in the

eyes of patients with PDR. Furthermore, recent studies by Haines et al.[114] and Wang et al.[115] reported significantly altered metabolites in the vitreous and aqueous humor samples of patients with DR. Of note, increased *sn*-glycerol-3-phosphate, a product of dihydroxyacetone phosphate (DHAP) reduction, and fructose 6-phosphate (F6P) were observed. As both DHAP and F6P are upstream intermediates in glycolysis (see Fig. 2), it highlights the implications for their diversions from glycolysis to the pathological polyol pathway and the methylglyoxal pathway to generate ROS and AGEs in DR. In addition, Schoors et al.[116] determined that in vivo inhibition of phosphofructokinase-2/fructose-2,6-bisphosphatase 3 (PFKFB3), a glycolytic activator which mediates blood vessel formation,[107] impairs retinal vessel sprouting and reduces vascular hyperbranching and pathological angiogenesis; this could present another way in which dysregulation of EC glucose metabolism pathways allows the progression of pathological angiogenesis.

Potential Influence of Anti-VEGF Drugs on EC Glucose Metabolism

Whereas clinical investigations have demonstrated the impact of high glucose levels on the progression of DR, accumulating evidence also indicates the importance of glycemic control on the treatment response to VEGF antagonists. Studies by Ozturk et al.[117] and Matsuda et al.[4] noted that clinical responsiveness to anti-VEGF treatment (ranibizumab and bevacizumab) for NVEDs was influenced by serum glycated hemoglobin A1c (HbA1c) levels, which were negatively correlated with anti-VEGF-mediated improvements in central subfield macular thickness and best-corrected visual acuity. Similar findings from patients with DME were reported by Sharma et al.,[118] wherein better treatment outcome of intravitreal bevacizumab was associated with good glycemic control (low HbA1c). These studies also suggest that HbA1c can serve as a predictor for response to anti-VEGF therapy in DME.

Interestingly, it was demonstrated that bevacizumab treatment induced metabolic adaptation in glioblastomas with reduced oxidative metabolism and increased glucose metabolism, alongside upregulated glycolytic enzymes including pyruvate dehydrogenase kinase and lactate dehydrogenase.[119] They also observed that bevacizumab led to a depletion in GSH levels, indicating that the treatment caused oxidative stress in the tumors. Retinal ECs are basically glycolytic, and both the oxPPP and non-oxPPP branches are used for production of GSH to provide antioxidative defense and the synthesis of nucleotides to enable cellular growth. The oxPPP flux is regulated by G6PD, which itself is partially regulated by VEGF.[103,120] Thus, long-term anti-VEGF treatment in NVEDs may be associated with EC damage leading to macular edema through attenuation of the antioxidative oxPPP. However, whether anti-VEGF therapy reprograms glycolytic metabolism in DR has not been investigated. Given the ability of VEGF to upregulate EC glycolysis[121] as well as antioxidative mechanisms,[120] it is plausible that long-term anti-VEGF treatment can influence EC metabolism and impair EC function undesirably through the enhancement of hyperglycemia-instigated ROS accumulation and the resulting production of AGEs to worsen oxidative stress and cellular inflammation (see Fig. 2), leading to the disruption of blood-retinal barrier integrity to cause vessel leakage, which counteracts the clinical efficacy of anti-VEGF therapy.

Conclusions and Future Perspectives

Anti-VEGF biologic treatment is a frontline therapy for NVEDs and although many patients benefit from its therapy, a significant proportion either do not respond to treatment, or experience a reduced response after repeated treatments. In this review, we have discussed alternative angiogenic pathways that may be mechanisms underlying acquired resistance to VEGF-targeted therapies. NRP-1 has an emerging role in the development of acquired resistance to anti-VEGF biologic therapy, with a number of its ligands upregulated after anti-VEGF treatment and associated with VEGF-independent angiogenic modulation.[73,80,96,97] In spite of these associations, the reason for alteration in expression of these ligands after anti-VEGF ocular therapy remains unclear, and their role in angiogenic modulation post-treatment requires elucidation. Studies into the alterations to ocular EC metabolism such as changes in glycolytic pathways after ocular anti-VEGF treatment are scarce. As glycolysis is a known driver of angiogenic growth and anti-VEGF treatment may mediate altered glucose metabolism, further investigation into alterations or mechanistic switches in these pathways after ocular anti-VEGF therapy is warranted. For the development of an effective therapy for NVEDs, such as wet AMD, PDR, and DME, it is imperative to determine the mechanisms by which anti-VEGF treatment response declines and acquired resistance develops. Closer study of the mechanisms considered in this review may shine a light upon novel targets and allow the development of more rational strategies to treat and prevent progression of neovascular ocular diseases.

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