Review

VEGF-A and its inhibitors in age-related macular degeneration – pharmacokinetic differences and their retinal and systemic implications

Alexa Klettner

Abstract: Age-related macular degeneration (AMD) is the main cause for legal blindness in the industrialized countries. In its exudative form, vessels grow from the choroid into the subretinal and retinal space, resulting in a rapid loss of vision. A major factor in the development of neovascularization is Vascular Endothelial Growth Factor (VEGF) A, which is therapeutically inhibited to treat exudative (wet) AMD. In addition to its pathological effects, VEGF-A has important physiological functions both systemically and in the retina. Current VEGF-antagonists are the Fab-Fragment ranibizumab, the fusion protein aflibercept and the off-label used antibody bevacizumab. In addition, the aptamer pegaptanib is approved for the treatment of AMD. These molecules differ on a molecular level, which impacts on their pharmacological behavior such as their effects on retinal cells, ocular and systemic clearance, or systemic VEGF-A inhibition. These differences may result in different systemic and retinal effects of the respective therapeutic molecule, which may cause different long term effects after prolonged usage.

Keywords: VEGF-A, ranibizumab, bevacizumab, aflibercept, pegaptanib

Age-related macular degeneration

Age-related macular degeneration (AMD) is considered the main cause for vision loss in the elderly and the main reason for legal blindness in the industrialized countries [1]. Early AMD is mainly asymptomatic for the patient with the presence of drusen in the retina, but in its late forms, vision can be severely impaired. The late forms of AMD present themselves as different entities, a so called atrophic, or “dry form”, in which the retinal pigment epithelial (RPE) layer degenerates and a secondary atrophy of the photoreceptors results in vision loss, and an exudative, or “wet” form, in which vessels grow from the choroid into the subretinal or retinal layers. These vessels are immature and leak fluid in the surrounding areas, resulting in edema, scar formation and severe vision loss [2]. A major factor in neovascularization is the Vascular Endothelial Growth Factor (VEGF) A [3].

Vascular endothelial growth factor

VEGF-A is indispensable for the angiogenesis of a developing organism. A fine balance has to be kept in order to ensure developmental vascularization, as both heterozygous knock-out as well as overexpression is lethal for an organism [4-6]. In the development of the retina, a time-dependent VEGF-A expression is needed to ensure the vascularization of the retina, which is perturbed for example in retinopathy of prematurity [7]. In addition, VEGF-A is important for the development and maturation of the brain,
VEGF-A is produced in several cells of the retina, e.g. Müller cells, retinal ganglion cells, astrocytes and pericytes [27]. Most notably, VEGF-A is constantly secreted by the retinal pigment epithelium (RPE) [28]. VEGF-A derived by the RPE is mainly of the isoform VEGF165, but VEGF121 and VEGF189 have also been described. The proportion of each isoform has been shown to change with age [29]. VEGF-A is mainly secreted basally from the RPE in order to uphold the fenestration of the retina and to protect the endothelial cells [28, 30]. To a lesser extent, VEGF-A is also secreted apically, where it exerts neuroprotective functions on the neuroretina and on the RPE itself [31-35]. In addition, VEGF-A is involved in its own regulation in the RPE/choroid complex [36, 37]. Early studies indicated VEGF165 to be mainly responsible for pathological angiogenesis, while VEGF121 was considered to be mainly physiological [38]. However, this differentiation could not be upheld. Possibly, a differentiation between beneficial and pathophysiological effects can be made with VEGFxxxb and VEGF, but this needs to be further investigated.

VEGF receptors

VEGF-A has two main receptors, which are receptor tyrosine kinases, designated VEGFR-1 and VEGFR-2. In order to activate the down-stream signaling pathway, VEGF-A binds as a dimer in an antiparallel fashion and dimerizes the bound receptor which, in turn, autophosphorylates several tyrosine amino acid residues. Here, different tyrosine residues activate different pathways [23, 39]. In addition to the tyrosine receptors VEGFR-1 and VEGFR-2, VEGF-A may also bind to neuropilins (NPR) and heparan-sulfate proteoglycans, depending on the isoform of VEGF-A [23, 27].

The receptor with the highest affinity for VEGF-A is VEGFR-1, however, its signal transduction activity is considered to be weaker than VEGFR-2 [40]. VEGFR-1 exists in a membrane-bound and in a soluble fashion. The soluble receptor is considered to be a decoy receptor, which exerts inhibiting functions [41]. For the membrane-bound receptor, its role is not clear so far. It is considered a negative regulator of angiogenesis, however, pro-angiogenic properties have also been described [42, 43]. In addition, synergistic effects between VEGFR-1 and VEGFR-2 have been shown [44].

VEGFR-2 is considered the main angiogenic receptor for VEGF-A on endothelial cells [45]. In addition, protective functions are also mediated by VEGFR-2, as are autoregulatory functions of VEGF-A expression [31, 34, 37, 46].

In contrast to the tyrosine kinase receptors, neuropilins (NRP1, NRP2) are transmembrane glycoproteins which lack signal transduction capacity. They are considered coreceptors which enhance the probability of VEGF binding to its receptor and, in addition, modulate receptor activity [47, 48].
Heparan sulfate proteoglycans are negatively charged linear polysaccharides, expressed on the cellular surface and in the extracellular matrix. VEGF-A isoforms of 165 amino acids and longer can bind to HSP via their heparin binding domain, enhancing the half-life of VEGF-A. The heparin-binding ability is indispensable for concentrations gradients, which in turn are needed for vascular development [49, 50].

VEGF-A in the retina and choroid

VEGF-A is indispensable for the development of the retinal and choroidal vasculature [7]. In the development of the retina vasculature, a hypoxia-induced vascularization takes place. Hypoxia induces the expression of VEGF-A via the transcription factor hypoxia-induced factor (HIF-1). The developing vessels follow a VEGF-A gradient, which is isoform dependent [51, 52]. The development of the choroid also depends on VEGF-A; here the expression and secretion of VEGF-A by the RPE is vital [53]. In contrast to retinal development, the expression of VEGF-A by the RPE during choroidal development is not hypoxia-dependent and does not require HIF-1 [53, 54]. Independent of its role in the vascularization of the retina and the choroid, VEGF-A is important for the proper development of the neuronal retina [55, 56]. In the adult eye, VEGF-A is strongly involved in the maintenance of the choriocapillaris, where it upholds the fenestration of the endothelial cells and protects them from apoptosis [15, 57, 58]. In addition, VEGF-A exerts protective effects on the retina, shown for retinal ganglion cells [34, 35], photoreceptor cells [33], Müller cells [32], and the RPE [31] (Figure 1).

VEGF-A regulation

The expression and secretion of VEGF-A is regulated on the transcriptional, translational and posttranslational level [27]. On the transcriptional level, several transcription factors are involved, most notably SP1, AP-1, Stat3, NFKB and HIF-1 [22, 59, 60]. For the constitutive expression of VEGF-A in the RPE, NFKB and SP-1 have been shown to be of importance [37]. VEGF-A is strongly regulated on the translational level, as its mRNA has a short half-life, which can be enhanced by mRNA binding proteins [61]. On a posttranslational level, VEGF-A secretion is regulated by chaperones that can control the processing through the endoplasmatic reticulum and the Golgi apparatus [62, 63]. Several factors may induce VEGF-A expression, the most prominent being hypoxia, oxidative stress, hyperglycemia or several cytokines [27] (Figure 2).

VEGF-A in exudative age-related macular degeneration

In exudative AMD, subretinal neovascularization (NV) may exert from the retina (retina angiomatous proliferation, RAP) or from the choroid [64]. RAP, in which vessels grow from the retinal vasculature through the retina into the subretinal space, can be induced by VEGF-A overexpression in photoreceptors [65]. The most common form of exudative NV in AMD, however, is choroidal neovascularization (CNV), in which vessels grow from the choroid into the subretinal space or into the retina. The developing vessels

![Fig. 1: Physiological functions of VEGF-A in the retina. In the developing organism, VEGF-A is strongly involved in the development of the vasculature of the choroid and the retina, and in the neurogenesis of the retina. In the adult organism, VEGF-A protects the choroid and upholds the fenestration of the choriocapillaris and exerts neuroprotective effects.](image1)

![Fig. 2: Regulation of VEGF-A expression. VEGF-A is controlled on 1) the transcriptional level by several transcription factors (e.g. SP-1, NFkB and HIF-1), on the 2) translational level, as VEGF mRNA needs to be protected from degradation by nucleases and 3) on the posttranslational level by chaperones, such as alphaB-crystallin.](image2)
Fig. 3: Model of VEGF-A in choroidal neovascularization. In diseased tissue, RPE is secreting exceeded amounts of VEGF-A towards the choroid. Vessels originating from the choroid grow through lesion in the Bruch’s membrane and enter the subretinal space by violating the RPE-barrier.
mRNA, thus preventing it from being translated. Double-stranded RNA of a particular sequence is applied, which is cleaved in siRNA by an enzyme named DICER and incorporated in a RISC complex. This complex recognizes its specific target, which then is cleaved [89]. While this approach is intriguing, the siRNA developed so far did not reach clinical approval for the treatment of AMD [90].

A second approach is the inhibition of the signal transduction induced by the VEGFR, which can be achieved by small molecular inhibitors. Such inhibitors have been developed, e.g. pazopanib, however, none of the inhibitors is specific for VEGFR-2, but inhibit a plethora of receptor tyrosine kinases (RTK) [91]. So far, none of the RTK-inhibitors has been approved for the treatment of AMD, but clinical phase I and phase II studies have been conducted [92].

A third approach is the extracellular binding of VEGF-A, thus preventing its association with the VEGFR. Here, several therapeutic molecules have been developed and approved to treat AMD. In chronological order, these are pegaptanib, ranibizumab and aflibercept. In addition, bevacizumab, which is neither created nor approved for the treatment of AMD, has been extensively used to treat wet AMD. These molecules are described and discussed below. An overview is given in table 1.

**Pegaptanib**

Pegaptanib, sold as Macugen, differs from the other VEGF-A antagonists (see below), as it is not a protein based therapeutic substance, but an RNA-based aptamer. It has been developed utilizing a “Systematic Evolution of Ligands by Exponential Enrichment (SELEX)” in vitro evolution technique and is derived from a modified 2’ fluoro pyrimidin RNA inhibitor to VEGF-A. These aptamers show a high specificity towards their target. Pegaptanib selectively binds to the heparin-binding domain of VEGF165 and does not target the smaller, soluble VEGF121, which does not carry this domain [93]. The rationale of this approach was the notion that VEGF165 was responsible for the pathological changes in the retina, while VEGF121 mediated the physiological functions [38]. Structurally, pegaptanib consists of 27 bases, stabilized by 2’-O-methyl and 2’-O-fluoro modification to protect the molecule against ribonuclease attacks. To avoid renal clearing, a 40 kDa 5’-polyethyleneglycol moiety was added. In addition, a 3’cap has been introduced to protect pegaptanib from exonucleases. The molecule presents itself as a stable hairpin at ambient temperatures. The binding of pegaptanib the heparin binding domain displays a high affinity (12 nM) and requires Ca²⁺ [94]. The binding of pegaptanib to VEGF165 is supposed to prevent an interaction of the receptor binding domain with the VEGFR in a matter of steric interference. Alternatively, the prevention of the interaction with the heparan sulfates and the co-receptor NRP1 may contribute to its inhibitory properties. NRP1 enhances the receptor signaling upon binding, thus an inhibition of the binding to NRP1 may restrict the receptor signaling rather than abolishing the VEGFR triggered response [36].

Intravitreally injected pegaptanib is cleared from the eye to the circulation with an estimated half-life of 94 hours, but can be still be found in the aqueous humor 28 days after a single injection [95]. Its polyethylene moiety is designed to prolong the vitreal half-life; it also protects pegaptanib from degradation in the serum [96]. It can be found in serum for up to 1 week after intravitreal injection of lower doses (1 mg), while at higher doses (3 mg), pegaptanib can be found in the plasma up to 3-4 weeks [96]. No accumulation could be found after multiple injections [96]. Pegatanib is cleared from the serum by renal elimination with a systemic half-life of 9.3 hours [97, 98]. It exhibits an excellent safety profile, with very few ocular adverse effects and no systemic activity [99]. In addition, it has no immunogenic capabilities [96]. Pegaptanib use for the treatment of AMD has been tested in the VISION clinical trials, where it was shown to slow down the loss of visual acuity compared to the control group by approximately 50% in the first year and stabilized vision in the second year [93]. However, its efficacy is less compared to the results obtained in the clinical studies assessing ranibizumab, where an improvement of visual acuity of more than 15 letters could be achieved for approximately 30% of the patients [100] (see below). The lesser efficacy of pegaptanib may be attributed to the specificity of binding, as only VEGF165 but not VEGF121 is inhibited; also, its ability to prevent VEGFR binding may be less efficient compared to ranibizumab [36]. Because of the lesser efficacy, the current use of pegaptanib in AMD is marginal. However, some studies do see improvement of visual acuity

<table>
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<th>Molecule</th>
<th>Ocular clearance</th>
<th>Systemic clearance</th>
<th>Immune complexes</th>
<th>Approved</th>
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<tr>
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<td>~10 hours</td>
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<td></td>
<td>Fab-fragment</td>
<td>7-9 days</td>
<td>~2 hours</td>
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<td>2 days</td>
<td>No</td>
</tr>
<tr>
<td>Afibercept</td>
<td>IgG</td>
<td>5-8 days</td>
<td>20 days</td>
<td>Yes</td>
</tr>
<tr>
<td>Bevacizumab</td>
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Table 1: Overview of properties of VEGF-inhibitors used in the treatment of AMD. n.d.= not determined
with pegaptanib [101], and the use of pegaptanib in maintenance therapy has been suggested [102]. In addition, there have studies showing good results in treating choroidal neovascularizations of different etiology with pegaptanib, such as myopic CNV [103].

Ranibizumab

Ranibizumab, sold as Lucentis, is a Fab-fragment, which is based on a murine VEGF-A antibody, developed for enhanced VEGF-A-binding in a series of recombinant DNA and phage-display selection steps [104]. The recombinant protein is produced in E.coli. Ranibizumab binds to VEGF at the receptor binding domain, preventing activation of the VEGFR by its ligand through steric inhibition [23]. Ranibizumab is a pan-VEGF inhibitor, binding to all active VEGF-A isoforms [105], but not to other members of the VEGF family. Intravitreally injected ranibizumab is cleared from the eye into the circulation with a half-life of 7-9 days in humans [106, 107] and approximately 3 days in rabbits and monkeys [108, 109]. Ranibizumab can be found in the serum, but the concentrations detected are considered below the necessary threshold for a sufficient VEGF inhibition [110]. Only very limited effects on the serum concentration of VEGF were seen [70, 72, 111]. Ranibizumab is not protected from serum elimination by an Fc-fragment and rapidly cleared from the circulation, possibly via renal elimination with a serum half-life of approximately 2 hours; however, due to the slow release from the eye, the serum half-life after intravitreal injection is 9 days, reflecting the vitreal half-life [107]. A beneficial effect of ranibizumab on the contralateral eye has not been described for AMD patients [110], and may only be connected to an impaired blood-retinal barrier [112]. In accordance, ranibizumab displayed a good safety profile and a high tolerability in patients. However, some concerns have been raised as some studies show an elevated risk of non-ocular hemorrhages and a potential risk for thromboembolic events. Ranibizumab may exert some immunogenic effects as low titers of anti-ranibizumab antibodies have been detected in a small subset of patients [113].

When used in a monthly regime, ranibizumab has been shown to increase visual acuity by a mean of 6-10 letters [114], and the gain of visual acuity may exceed 15 letters in approximately 30% of the patients [100].

Bevacizumab

Bevacizumab is a humanized anti-VEGF-A antibody, developed form the murine anti-VEGF-A antibody A4.6.1 [23]. In contrast to ranibizumab, bevacizumab is a full length antibody, and as such carries the Fc-fragment. Bevacizumab binds Fcγ receptors, but does not induce Fc mediated cell lysis [115]. It is produced in mammalian CHO cells. Bevacizumab, like ranibizumab, binds to all active isoforms of VEGF-A, but not to other members of the VEGF-family and inhibits VEGFR activation through steric inhibition [23]. Of note, bevacizumab can form multimeric immune complexes with VEGF, which has not been described for any of the other VEGF-A inhibitors [69]. Bevacizumab injected into the vitreous clears the eye with a half-life of 4-6 days in rabbits and 5–8 days in humans [116-120]. It readily penetrates the retina and is cleared through the circulation, possibly by active transportation pathways [121]. In contrast to ranibizumab, it is found in biologically active concentration in the serum [115, 119, 120]. Accordingly, intravitreal bevacizumab has been shown to reduce the serum level of VEGF-A [72, 122]. Serum half-life of bevacizumab has been described to be 20 days after intravenous injection in humans [123]. In rabbits treated with intravitreal bevacizumab, a serum half-life of approximately 7 days was described [116]. The extended half-life of bevacizumab, especially when compared to ranibizumab, can most likely be attributed to the Fc-moiety present in bevacizumab. IgGs are protected and kept in circulation via their Fc-moiety by the binding and re-shuttling of the neonatal Fc receptor (FcRn) [124]. In addition, some bevacizumab is found in the fellow eye, but whether the concentration found is high enough to exert any effects in the fellow eye is under debate [116, 120, 125]. The high serum concentration of bevacizumab after intravitreal application, the prolonged systemic half-life and the inhibition of VEGF-A in the circulation has raised concerns about the systemic safety of intravitreal bevacizumab treatment [126, 127]. In addition, bevacizumab is taken up by platelets [128] and may induce platelet activation and aggregation, which in turn may induce thromboembolic events [129]. Moreover, the high molecular immune complexes of bevacizumab and VEGF may accumulate in the renal glomeruli [69]. These properties of bevacizumab may be of particular importance, as bevacizumab is not approved for the treatment of AMD and used off-label due to considerable lower costs compared to other treatment options [130]. The data regarding systemic safety of intravitreal bevacizumab is not conclusive. While there has been indication that bevacizumab has a higher rate of serious systemic adverse effect than ranibizumab [131, 132], other studies do not find any differences between the two drugs. As these serious adverse events are rare, conducted studies generally do not have the power to reliably detect these differences [133]. In addition to possible systemic adverse effect, distinct effects on retinal cells have been shown. Bevacizumab, in contrast to ranibizumab, has been shown to be taken up and stored in cultured RPE and endothelial cells [134-136]. Intracellular bevacizumab reduces the phagocytic ability of RPE cells [135].

The clinical efficacy of bevacizumab has been investigated in two major clinical trials, conducted in the US (CATT) and in Great Britain (IVAN). In both studies, bevacizumab was compared with ranibizumab. Generally, bevacizumab was found to be non-inferior to ranibizumab [122, 131].
Aflibercept

Aflibercept, sold as Eylea for the treatment of AMD, is a fusion protein which consists of the second Ig domain of VEGFR-1 and the third Ig domain of the VEGFR-2 fused to the Fc fragment of human immunoglobulin G [137]. Aflibercept displays a high affinity for all VEGF-A isoforms. In contrast to the other VEGF antagonists described above, aflibercept also binds to VEGF-B and PIGF [136, 137]. In contrast to bevacizumab, aflibercept binds VEGF-A in a 1:1 stoichiometry which remains stable in the circulation [69]. Because of this stoichiometry and the inert nature of the complexes, aflibercept is not expected to activate platelets or accumulate in renal glomeruli as it has been found for bevacizumab [69].

There was very little immunogenicity associated with aflibercept [138]. Aflibercept can be detected in the systemic circulation after intravitreal application of 2 mg. The concentration of free aflibercept was described to be 0.02 μg/ml which could be found 1-3 days after intravitreal application but was not detectable two weeks after application. An accumulation after repeated application was not seen [138]. Tissue diffusion of systemic aflibercept has been reported to be low [139]. Plasma concentration of aflibercept as applied for cancer treatment has been described to have a half-life of two days because of clearance by renal filtration [140].

In clinical trials investigating the treatment of AMD, the efficacy of aflibercept at a 2-month regimen was found to be clinically equivalent of ranibizumab, administered at a monthly rate [138].

VEGF inhibitors and systemic effects

VEGF-A is a major factor in the development of exudative AMD and the therapeutic approach of VEGF-A inhibition has been proven to be a valuable and effective treatment. VEGF-A, however, is highly expressed in healthy tissue [69] where it exerts many physiological functions. A systemic inhibition of VEGF as practiced in cancer patients may lead to several severe adverse effects, including hypertension, thromboembolic events or gastrointestinal perforation [141]. The question therefore remains whether intravitreally applied anti-VEGF molecules may exert systemic effects. A systemic exposure after intravitreal application with a consequent VEGF-A inhibition is seen for bevacizumab, but not or ranibizumab, indicating possible differences in systemic effects of these drugs. In addition, the systemic half-life of bevacizumab is very high, especially compared to ranibizumab, which has a very short systemic half-life and is readily cleared from the circulation, but also compared to aflibercept [107, 123, 140]. In addition, bevacizumab may actively be involved in thromboembolic events, as it is described to be taken up into and activate platelets [128, 129]. Furthermore, bevacizumab, in contrast to the other anti-VEGF molecules described, forms high molecular weight immunocomplexes which may aggregate in tissues such as the renal glomeruli [69]. Indeed, bevacizumab displayed a significantly higher rate of overall adverse effects than ranibizumab in the CATT study and in a meta-analysis [131, 142]. Further research is needed to investigate this issue.

According to the molecular structure and pharmacokinetic properties of ranibizumab, as described above, the risk of systemic effects should be low. It is rapidly cleared from the circulation and does not form immunocomplexes. However, a potential risk for thromboembolic events or hemorrhages has been postulated [113]. Again, further research needs to be conducted.

Little data on aflibercept concerning systemic effects after intravitreal application is available. A clearance from the eye into the systemic circulation has been shown [138], however, aflibercept does not form high molecular immunocomplexes but inert 1:1 complexes with VEGF [69], indicating that systemic effects may be neglectable. Further research is needed to investigate this issue.

VEGF inhibitors and retinal effects

The retinal functions of VEGF-A have raised concerns about possible long term side effects of retinal VEGF inhibition [7]. On a cellular level, no toxicity of VEGF-antagonists on retinal could be found [134, 143, 144]. However, VEGF-inhibitors did suppress VEGF-A-mediated protection against oxidative stress [35]. Bevacizumab applied in animal eyes did not induce functional changes [145, 146], nor did bevacizumab or ranibizumab change functional parameters in isolated vertebrate retina [147, 148]. Of note, aflibercept did alter functional parameters in this system [149]. Long term inhibition of VEGF-A in mice expressing transgenic soluble VEGFR-1 in the retina did not display functional or anatomical alterations [150, 151]. However, VEGF-A inhibition results in the reduction of choioicapillary fenestration, which may impede nutrient supply in the retina [15]. Indeed, a dramatic effect of the absence of VEGF-A on photoreceptors has been described [33] and there is some clinical evidence studied that prolonged VEGF-A inhibition may induce atrophy [152]. Especially considering new, more effective VEGF inhibitors, the safety of prolonged VEGF-A inhibition in the retina should be monitored carefully. Also, side effects accountable to the molecular structure should be considered, as bevacizumab but not ranibizumab reduces the phagocytic function of RPE cells in vitro [135], and immunocomplexes formed by bevacizumab may have detrimental effects on the choroidal circulation [153].

Conclusion

VEGF is the main factor for angiogenesis and has important functions both systemically and in the retina. Its
inhibition in order to treat the exudative form of AMD is an effective therapy, however, due to possible retinal and systemic long term effects, the impact of the used inhibitors has to be more thoroughly investigated.

**Conflict of interest**

AK has been a consultant for and received lecture fees and research grants from Novartis Pharma.

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