Treating Age-Related Macular Degeneration – Interaction of VEGF-Antagonists with their Target

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Abstract: The neutralization of VEGF is the current treatment of choice for age-related macular degeneration. Current approaches include anti-VEGF-antibodies and –Fab Fragments, aptamers, soluble receptors (Traps) and siRNA. The molecular properties of VEGF and its antagonists are reviewed and the pathways of action of these substances are discussed.

Key Words: VEGF, ranibizumab, lucentis, AMD, pegaptanib, VEGF-trap.

INTRODUCTION

Age-related macular degeneration (AMD) is the main cause for legal blindness of the elderly in the industrialized world. A lot of effort has been made to understand the underlying pathways of this devastating disease, yet the pathogenesis remains unclear. In the exudative ("wet") form of AMD, choroidal neovascularization develops, consisting of immature, leaking vessels and leading to the degeneration of photoreceptors, vision deterioration and eventually the complete loss of central vision [1]. Even though the etiology of AMD is not fully understood, current therapies concentrate on angiogenesis. Angiogenesis is the formation of new capillary blood vessels which physiologically occurs in the adult in the female reproductive cycle and in wound healing. Angiogenesis plays a profound role in pathological events, including, but not limited to, AMD, cancer or proliferative diabetic retinopathy. It is triggered by a wide variety of physiological stimuli and is controlled by pro- and anti-angiogenic growth factors, which are released by a variety of cells in response to hypoxia, hypoglycaemia, inflammatory proteins, oxidative stress or genetic alterations [2]. The most important pro-angiogenic factor is the Vascular Endothelial Growth Factor (VEGF). Even though an inhibition of angiogenesis cannot prevent or cure AMD, the introduction of VEGF-antagonists to fight angiogenesis in order to fight vision loss has revolutionized AMD therapy.

VEGF

Judah Folkman hypothesized an angiogenic factor as early as 1971 [3]. It was later discovered and described as Vascular Permeability Factor [4] or Vascular Endothelial Growth Factor [5]. The VEGF family is a member of the cystine-knot growth factor family [6], which is characterized by the presence of six conserved cysteine residues forming the typical cystine-knot structure [7]. The VEGF family consists of a variety of members (VEGF-A, -B, -C, -D, -E, -F and placental growth factor) [8], of which VEGF-A is most important for angiogenesis and will be focused on in this article. VEGF-A is indispensable in embryogenesis, as even a knock-out of a single allel of the gene is lethal [9]. The human VEGF-A (subsequently called VEGF) gene is localized on the chromosome 6q21 and organized in 8 exons, separated by 7 introns [10]. Different isoforms are expressed due to alternative splicing and are called according to the number of amino acids (AS) they consist of, $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$. All transcripts contain exons 1-5 and 8, but are alternatively spliced in exon 6 and 7. A hydrophobic signal sequence essential for secretion of VEGF is encoded within exon 1 and a small region of exon 2 [11]. The isoforms differ in their molecular mass and their biological properties, most prominently in their ability to bind to heparin or heparan sulphate proteoglycans, resulting in different solubilities. The smallest of these isoforms, $VEGF_{121}$, lacks exon 6 and 7, does not bind to heparin and is freely soluble, while $VEGF_{165}$, which lacks exon 6, has intermediate properties. $VEGF_{189}$ and $VEGF_{206}$ are highly basic proteins which are completely sequestered by the extracellular matrix, from which they can be released by heparinase or plasmin protein lysis at the carboxy terminus [12]. The isoforms also differ in their binding affinity to the Neuropilin co-receptors. All of the isoforms have the same 110 N-terminal region, which contains the receptor-binding-domain and the dimerization functionality, and 6 C-terminal residues (CDKPRR) [2] (Fig (**1**)). Also, additional isoforms ($VEGF_{111}$, $VEGF_{145}$) are formed by proteolytic cleavage. Recently, inhibitory isoforms of VEGF have been described, which differ in their C-terminal amino acid residues (CDKPRR is replaced by SLTRKD) [13,14]. VEGF is glycosylated at Asn75. This glycosylation is necessary for heparan sulphate to induce a structural change in $VEGF_{165}$, stabilizing the active conformation of the growth factor [15].

While the receptor binding is conducted by the N-terminal 110 AS, called the receptor-binding-domain, the heparin binding function of $VEGF_{165}$ is completely mediated by the carboxyl-terminal domain (111-165), leaving $VEGF_{111}$ and $VEGF_{121}$ incompetent to bind heparin. This 55 amino acid carboxyl portion of $VEGF_{165}$ is very basic (pI estimated to be 11.6), contains another eight cysteine residues and represents a heparin binding structure unique to VEGF with no known protein homolog [16]. The basic heparin-binding-

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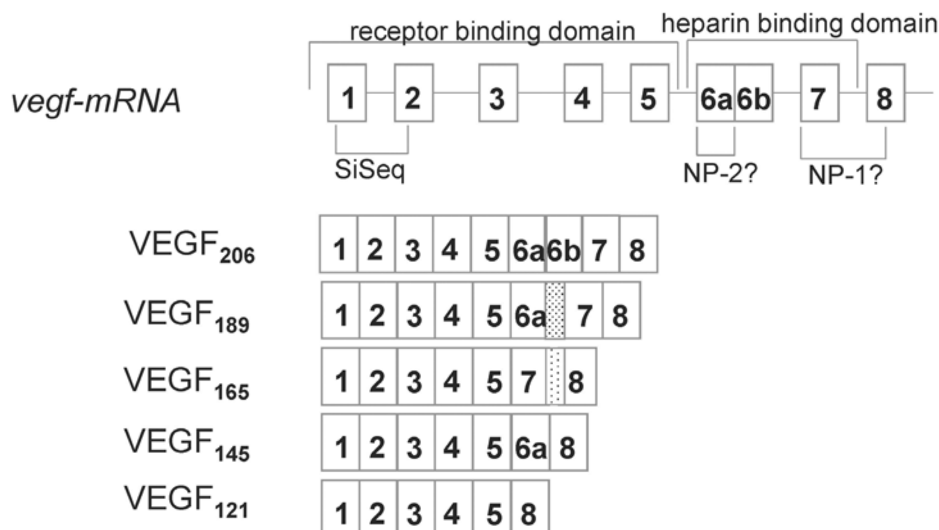

Fig. (1). Alternative splicing of the 8 exons of the VEGF gene results in different VEGF isoforms. SiSeq: Signaling sequence, NP-2?: putative Neuropilin-2 binding site, NP-1?: putative Neuropilin 1 binding site [2, 11, 17,18, 61].

domain is encoded by exon 6, while the NP-1/heparin-binding domain encoded by exons 7, Cys137 being functionally important, and exon 8 [17]. The later region is composed of two subdomains, including two disulphide bridges each, a short two-stranded antiparallel ß sheet, and a short alpha helix in the carboxy-terminal domain [11, 18].

The VEGF molecule dimerizes in an antiparallel, side-by-side fashion. Each monomer contains a core cystine-knot structure held together by three intrachain disulphide bonds. The monomers are covalently linked by two symmetrical disulfide bonds between Cys51 and Cys60 [6]. $VEGF_{121}$ carries a third disulfide bond, between Cys116 of each monomer [19]. VEGF interacts with the VEGF-receptors *via* binding sites at the end poles of each monomer. This arrangement indicates that VEGF dimerizes its receptor in order to induce signal transduction. The main receptors for VEGF are the tyrosine kinase receptors VEGFR-2 (KDR) and VEGFR-1 (Flt-1).

VEGF-RECEPTORS

The VEGFR-1 gene consists of 30 introns and encodes for two polypeptides, the soluble form of VEGFR-1 and the full length receptor form of VEGFR-1 [20]. The soluble form of VEGFR-1 consists of the 1st to 6th Ig-like domains with an additional 31 amino acid residues in the carboxyl terminal region [21]; the receptor form consists of 7 extracellular Ig domains and an intracellular tyrosine kinase domain with a long kinase insert [22]. The 1st, 2nd and 3rd Ig-like domains are essential for ligand binding. The 2nd domain is the direct binding region for VEGF, but to form the appropriate tertiary structure, several peptides from the 1st and 2nd domain are required. The 4th Ig-like domain is important for receptor dimerization [23]. VEGFR-1 is heavily glycosylated. The role of VEGFR-1 is not fully elucidated yet. It has a much higher affinity for VEGF than VEGFR-2 (Kd: 1-2 pM [24]), but its signal transduction activity is rather weak [25,26]. Commonly, it is regarded as a decoy receptor for VEGF [27], but its role is more complex. E.g., monocytes and macrophages express membrane bound VEGFR-1, which is probably involved in VEGF-induced migration of peripheral blood monocytes [28].

VEGFR-2 is considered to be the main VEGF receptor to mediate VEGF responses in endothelial cells [29]. VEGFR-2, like VEGFR-1, belongs to the receptor type tyrosine kinase superfamily. It consists of 7 extracellular Ig-like domains, has a short transmembrane domain and an intracellular region containing a tyrosine kinase domain, split by a 70 amino acid insert [30]. VEGF binds to the 2nd and 3rd extracellular Ig-like domains of VEGFR-2 with a Kd of 75-125 pM [31]. The binding of VEGF to the Ig-like domains 2 and 3 of one VEGFR-2 monomer increases the probability of a second receptor binding. Through the binding of two receptors to one VEGF dimer, homotypic interactions between the Ig-like domains 7 of both receptors are possible which further stabilize the receptor dimer [32]. This dimerization induces autophosphorylation of the receptors by their intracellular kinase domains.

In the receptor binding region of VEGF, crystal structure analysis revealed five residues to contribute to most of the binding energy of the receptor binding epitope for VEGF-R2, Phe17, Ile46, Glu64, Gln79 and Ile83, which are clustered in two different patches across the VEGF dimer interface. [6]. They are located on one face (“receptor binding face”) of the dimer. Another crystal structure analysis adds Pro85 and Ile43 to these interacting residues [33]. Alanine-scanning mutagenesis identified the residues Arg82, Lys84 and His86, which are located on a hairpin loop, to be critical for binding to VEGFR-2 [31].

Important for the binding to Flt-1 (VEGFR-1) are the residues Asp63, Glu64 and Glu67 [6,34]. Additionally, Pro106, Arg105, Cys104, Glu103, Glu64, Asp63, Cys60, Cys26, Tyr25, Arg23, Gln22 and Tyr21 are proposed to be aiding interactions between VEGF and VEGFR-1 [35,36]. All isoforms of VEGF bind to the VEGFR-1 and VEGFR-2, but differ in their binding properties to the co-receptors, neuropilin 1 (NP-1) and neuropilin 2 (NP-2) [37].

NEUROPILINS

NP-1 and NP-2 are transmembrane glycoproteins with a similar domain structure that are 44 % identical at the amino acid level [38]. They consist of large extracellular regions (two CUB domains, two b1/b2 domains, and a MAM domain), a single transmembrane domain and small cytoplasmic domains. The CUB domains are essential for the binding to semaphorine, proteins important for axon guiding and not related to angiogenesis. $VEGF_{165}$ bind to the b1/b2 domain [39].

The binding of VEGF to NP-1 is mediated by the basic heparin-binding-domain and the NP-1/heparin-binding domain of VEGF [17]. NP-2 binds to $VEGF_{165}$ and $VEGF_{145}$, but not $VEGF_{121}$. As $VEGF_{145}$ lacks exon 7 and does not bind NP-1, the NP-2 binding site is probably located in exon 6 [40]. The specificity of NP-1 for $VEGF_{165}$ has been challenged as a binding of NP-1 to $VEGF_{121}$ has been reported. This implies that not exon 7 but the C-terminus of the exon-8 encoded region is important for VEGF binding to NP-1 [41]. The importance of exon 8 for the NP-1 binding has also been shown by other authors [42,43].

The exact function of NP binding has not been completely elucidated yet. Both the binding to NP and the binding to heparan sulphate have profound effects on the function of $VEGF_{165}$. NP-1 is considered a co-receptor of VEGF, dependent on the heparin-binding-domain to increase the affinity of VEGF for its signaling receptor VEGFR-2. Co-transfecting of NP-1 to VEGFR-2 expressing cells enhances the binding of VEGF to VEGFR-2 and the VEGFR-2-mediated mitogenic and chemotactic activity of VEGF [17]. NP-1 enhances the interaction of $VEGF_{165}$ with VEGFR-2 but not with VEGFR-1, which binds to NP-1 without VEGF, suggesting that NP-1 shares a common surface for its interaction with VEGFR-1 and VEGF [44]. Due to its short C-terminal domain, NP-1 is not believed to be able to participate in signal transduction of its own [40]. The view of NP-1 as merely a co-receptor for VEGF, however, has recently been challenged, as NP-1 has been shown to have an influence on tumour growth independently of VEGFR-2 [45] and seems to be able to an independent signal transduction *via* its C-terminus [46].

HEPARIN/HEPARAN SULPHATE

Heparin and heparan sulphate are negatively charged linear polysaccharides. They consist of repeating uronic acid D-glucosamine disaccharide subunits. The uronic acid of the repeating disaccharide can be either L-iduronic acid or D-glucoronic acid and display a variable degree of N-sulphation, 2-O- and 6-O sulphation and N-acetylation [47,15]. Both heparan sulphate and heparin are synthesized as proteoglycans (HSPGs). After synthesis, heparin chains are randomly cleaved into smaller polysaccharides [48]. Heparan sulphate is biosynthesized and secreted by all mammalian cells, while heparin is specific for mast cells. HSPGs are expressed on cell surfaces and in the extracellular matrix in a tissue-specific manner. $VEGF_{165}$ binds to HSPGs *via* its heparin binding domain (residues 110-165), the most important residues for HSPG binding being Arg124, Arg145, Arg149 and Arg159 [49].

$VEGF_{165}$ binding to VEGFR-1 is dependent on cellular HSPG which can be partially restored by exogenous heparin [50]. The binding of $VEGF_{165}$ to VEGFR-2 is enhanced by cellular or exogenous heparin/HS [51], possibly by the formation of VEGF-VEGFR-2-HS ternary complexes [52]. Additionally, Heparin/HS facilitate $VEGF_{165}$ interaction with the neuropilin co-receptors [53]. The differences in HSPG affinity of the isoforms result in a concentration gradient which is important for the development of the vasculature [54]. Additionally, HS interaction prolongs the extracellular half life of VEGF by protecting it from degradatory pathways, additionally it may act as a chaperone [49, 55]. In the presence of heparin, the amount of VEGF bound to VEGFR-2 increased more than 3-fold; however, the apparent affinity of VEGF for VEGFR-2 was unchanged. The stability of VEGF-heparan sulphate-receptor complexes probably contributes to effective signal transduction and stimulation of endothelial cell proliferation [34].

VEGF ANTAGONISTS

In order to treat AMD, several VEGF antagonists have been developed. The following agents are either FDA approved for AMD (Ranibizumab, Pegaptanib), used off label (Avastin) or are currently assessed in clinical trials (VEGF-Trap, Sirna027, Bevasiranib).

VEGF ANTIBODIES

Ranibizumab and Bevacizumab

In 1992, a murine anti-VEGF antibody was developed, designated A.4.6.1, which recognized three species of VEGF, $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ [56]. It was reported to be a neutralizing VEGF antibody, which potently inhibited the growth of several tumour cell lines in nude mice, but not *in vitro* [57]. As murine antibodies may induce a significant immune response when used in humans, the murine MAb A4.6.1 was humanized, using a previously utilized consensus human framework [58]. The Fab consists of a complete human framework (variable light domain kappa subgroup I and variable heavy domain subgroup III), of which six complementary-determining regions (CDRs) were changed to the murine A.4.6.1 sequence.. Transferring these CDRs from the murine antibody to the human framework strongly reduced the antibodies binding to VEGF. Seven non-CDR framework residues in the variable heavy domain and one framework residue in the variable light domain were altered to achieve a better binding. This Fab fragment was designated Fab-12. The variable heavy and variable light domains of Fab-12 were combined with human IgG1κ constant domains to produce a full length antibody composed of two identical light chains (214 amino acid residues) and two heavy chains (453 amino acid residues), with a total molecular weight of 149 kDa. The heavy chains demonstrate C-terminal heterogeneity and contain one N-linked glycosylation site at Asn303. The oligosaccharides are of complex biantennary structure with core fucose and two branches, terminating mainly with zero, one or two galactose residues. Each light chain is covalently coupled through a disulfide bond at Cys214 to a heavy chain at Cys226 [59]. It was designated rhu Mab-VEGF [58], later Bevacizumab (Avastin©) [60], being 93% human and 7 % murine. Bevacizumab binds human VEGF with a dissocia-

tion constant of 1.1 nM, which is very similar to the murine Mab [61].

In order to reach maximum inhibition, a molar ratio of 2.6:1 of Bevacizumab to homodimeric $VEGF_{165}$ is needed. In monocytes, which express predominantly VEGFR-1, the ratio is 10.4:1 [46]. Bevacizumab can bind to Fcγ receptors, but it does not induce Fc mediated cell lysis, suggesting that the binding and neutralizing of VEGF does not occur on the cell surface [46]. This is consistent with the finding that VEGF is not found on the cell surface when overexpressed in CEN cells [62].

For Ranibizumab, a Fab-fragment similar to Fab-12, designated MB1.6, was adapted for improved VEGF-binding through a series of recombinant DNA and phage-display selection steps [63-65]. *Via* various steps to improve the affinity of the fragment to VEGF,

Y0317 was created, which contains five variable domain substitutions and one constant domain substitution (at the C-terminus of the heavy chain) (Fig. (**2**)). Affinity improvement of this new fragment, later called Ranibizumab (Lucentis©), was 100-fold and 5-20 fold more potent than Bevacizumab when tested in bioassays [65]. An independent publication found a 6-fold higher efficacy of Ranibizumab compared with Bevacizumab *in vitro* [66].

The residues of VEGF essential for Fab-12 binding have been analyzed. Twenty-five residues of the Fab fragment become buried in the antigen-antibody interface of which 8 are crucial for binding (light chain: Trp96; heavy chain:

Light chain

1 … 50
Fab12 DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYF
Y0317 DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPDKAPKVLIYF
CDR L1

100
Fab12 TSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQ
Y0317 TSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQ
CDR L2 CDR L3

150
Fab12 GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV
Y0317 GTKVEIDRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV

200
Fab12 DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
Y0317 DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG

Fab12 LSSPVTKSFNRGEC
Y0317 LSSPVTKSFNRGEC

Heavy chain

1 … 50
Fab12 EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGW
Y0317 EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVRQAPGKGLEWVGW
CDR H1

100
Fab12 INTQTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYP
Y0317 INTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYP
CDR H2

150
Fab12 HYYGSSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC
Y0317 YYYGTSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC
CDR H3

200
Fab12 LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG
Y0317 LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG

Fab12 TQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
Y0317 TQTYICNVNHKPSNTKVDKKVEPKSCDKTHL

Fig. (2). Amino acid sequence of Fab12 and Y0317. Complementarity binding regions are depicted in italic. Amino acids that differ between Fab12 and Y0137 are bold. Residues that contribute most to the binding to VEGF are depicted in boxes [6, 63, 64].

Asn31, Tyr32, Trp50, Asn52, Tyr95, Ser106 (100B according to [67]), Trp108 (100D according to [67])). Nineteen VEGF residues participate in the interface between Fab-12 and VEGF. The VEGF residues 86-94 form hydrogen bonds with the Fab fragment and are bound in a shallow cleft formed by its CDRs. A short parallel ß ladder is formed between VEGF residues 91-93 and the CDR H3 residues 98-104. VEGF residue Gly88 is buried in a deep pocked containing of residues of the CDRs L3, H2 and H3. Gly92 and His90 are also buried inside the CDRs. Gln87 and Gln89 are hydrogen bonded to residues of CDR H3 and L3, and H1 and H2, respectively. Of the 19 residues that participate in the interface, most important for the antigen binding are Met81, Arg82, Ile83, Gly88, Gln89 and Gly92. To a lesser extent, the residues 48, 79, 84, 93 and 94 also contribute significantly to the binding. Only a single epitope, Ile83, is an important binding determinant for both the antibody and the receptor. Thus, the neutralizing effect appears to be due to steric hindrance and not due to competition for the same critical binding determinants [64] (Fig. (**3**)). However, additional pathways to the steric inhibition of receptor binding might contribute to VEGF neutralization. In an experimental setting in a perfusion organ culture, the effect of Bevacizumab as well as Ranibizumab extended the persistence of VEGF-inhibitors in the culture and a significant alleviation of $VEGF_{165}$ expression in RPE cells by Bevacizumab and Ranibizumab, respectively, could be seen [66]. The exact mechanisms through which VEGF-antagonists influence VEGF expression need to be elucidated further, but a possible mechanism could be an interference with feedback mechanisms since VEGF has been described to influence its own expression. In endothelial cells, the addition of VEGF to the medium resulted in an activation of HIF-1 and an upregulation of VEGF mRNA [68].

PEGAPTANIB

The RNA-based aptamer Pegaptanib (NX1838, Macugen©) is derived from a modified 2'fluoro pyrimidine RNA inhibitor to VEGF. Aptamers are nucleic acids which are generated by systematic evolution of ligands by exponential enrichments ("Selex") *in vitro* evolution technology. They exhibit high specificity and can easily distinguish between closely related proteins. Pegaptanib was develop to distinguish between $VEGF_{165}$ and $VEGF_{121}$, binding to $VEGF_{165}$ only, as it has been suggested that $VEGF_{165}$, but not $VEGF_{121}$, is involved in pathological alteration in AMD [69]. Pegaptanib consist of 27 bases and is stabilized by 2'-O-methyl (purine, all but 2 of the 2'-OH-purine position are subsidized) and 2'-O-fluoro modifications (pyrimidine, all are subsidized). The substitution of the 2'-position of the ribonucleotides with these moieties confers resistance to ribonucleases which depend on the 2'-OH group for cleavage of the phosphodiester bond. The stability of the secondary structure of the aptamer is not affected by these substitutes [70]. To additionally stabilize the RNA in solution and to avoid rapid renal clearing, a 40 kDa 5'-polyethyleneglycol moiety was added. To protect the molecule from exonuclease attacks, a 3'-dt has been attached *via* a 3'-3' linkage (3'cap) [71]. The secondary structure of Pegaptanib is a stable hairpin at 25°, and a thermally induced hairpin to single strand transition is highly reversible [72]. Of the two energetically favourable structures, one was identified in NMR-studies [71] (Fig. (**4**)).

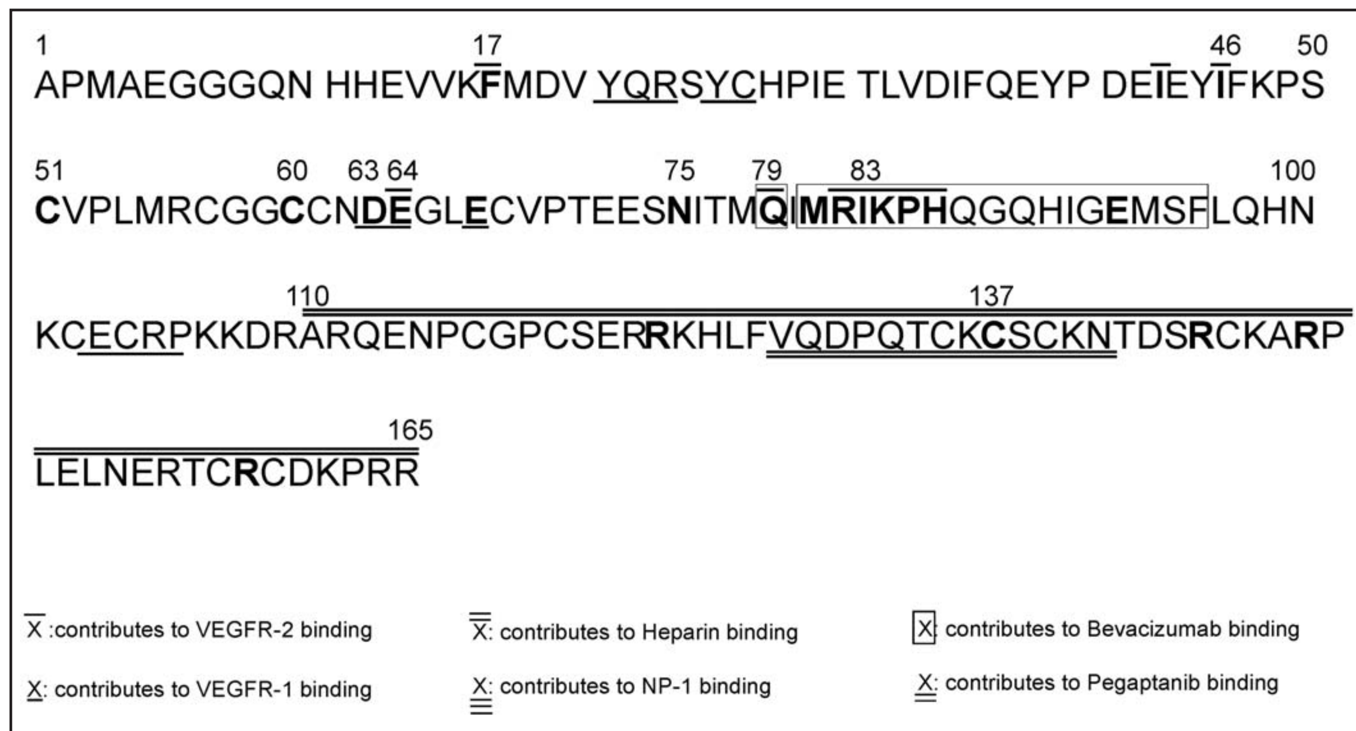

Fig. (3). Amino acid sequence of $VEGF_{-165}$. Single overlined residues contribute to VEGFR-2 binding, single underlined residues contribute to VEGFR-1 binding. Double overlined residues contribute to Heparin binding, triple underlined residues contribute to NP-1 binding, double underlined contribute to Pegaptanib binding and residues contributing to Bevacizumab binding are depicted in boxes. Residues that contribute strongly to their respective binding partner are depicted in bold [6, 16, 17, 33, 34, 49, 64].

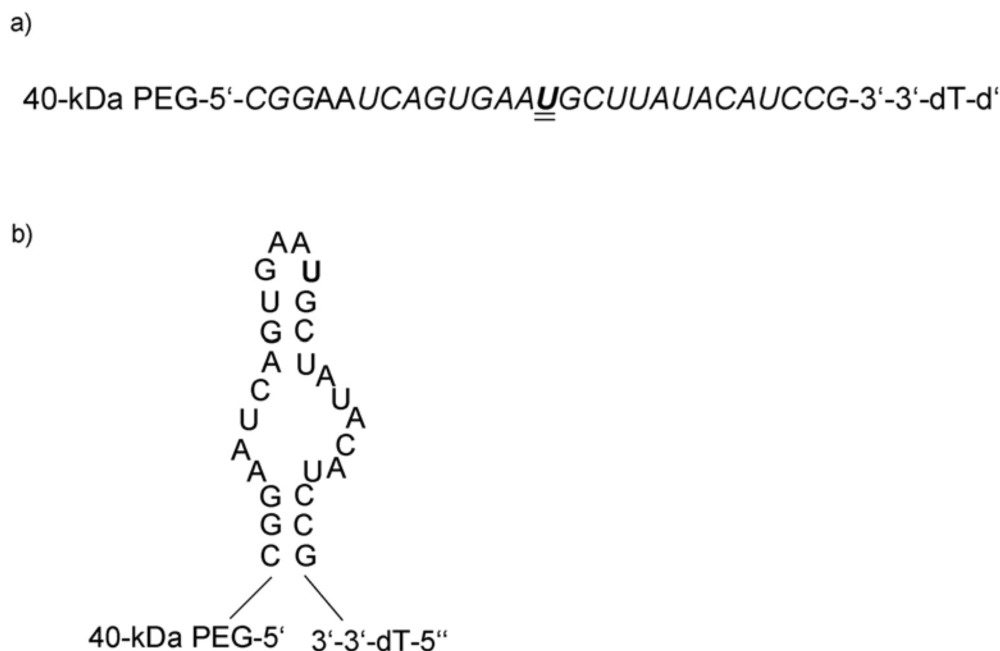

Fig. (4). Nucleotide sequence of Pegaptanib (a) and secondary structure (b). Underlined (a) and bold (b) is the uridine residue that forms a photo-inducible cross-link with Cys137 of VEGF [70, 71].

Pegaptanib binds to $VEGF_{165}$ with high affinity which requires Ca^{2+} ions for binding. It binds to the 55 amino acid heparin-binding-domain (HBD) *in vitro* with 12 nM affinity and the isolated HBD efficiently competes with full-length VEGF for Pegaptanib binding in cell culture. Pegaptanib binds in the area of VEGF residues 129-141, with the most important amino acid for the binding being Cys137 [70]. The individual base pairs and the overall secondary structure of Pegaptanib are greatly stabilized upon binding to the isolated HBD or full-length VEGF [73]. It is specific for $VEGF_{165}$, no binding to $VEGF_{121}$ was found. The backbone conformation of the aptamer and the interactions with the phosphates are conserved between the HBD-aptamer and the $VEGF_{165}$-aptamer complex. The receptor-binding-domain is either providing additional interactions with the aptamer or indirectly stabilizing new or existing interactions of the aptamer with the HBD. It appears that the Ca^{2+} binding site is formed only in the aptamer-protein complex, and this Ca^{2+} binding site may contribute to the isoform-specific recognition of the aptamer [71].

As pathways of action, two possibilities have been suggested. One possibility is that the aptamer inhibits $VEGF_{165}$ through a steric interference mechanism, where the bulky aptamer prevents interaction of the receptor-binding-domain with the cell-surface receptors, most notably VEGFR-2. Another possibility is the prevention of the interaction with heparan sulphates and NP-1. The heparin-binding-domain might increase the local concentration of $VEGF_{165}$ at the cell surface by interacting with heparan sulphate proteoglycans. $VEGF_{165}$ being bound to the cell surface greatly enhances the probability of receptor binding by restricting diffusion. Thus, the therapeutic activity of this aptamer may arise by capturing soluble $VEGF_{165}$, therefore preventing interaction with heparan on cell surface proteoglycans. Also, the heparin-binding-domain binds to NP-1 (though this has been challenged by [41]) so the VEGF aptamer may block the interaction between NP-1 and $VEGF_{165}$, especially since both NP-1 and Pegaptanib bind to Cys137, thereby diminishing $VEGF_{165}$-induced signal transduction [62]. A recent publication indicates that steric hindrance is not the mechanism of $VEGF_{165}$ inhibition. In an experimental setting, Pegaptanib was not able to prevent the binding of antibodies to the receptor binding domain, suggesting that Pegaptanib would not be able to prevent the binding of VEGF to the receptor, either [66]. This would offer a logical explanation as to why Pegaptanib is not as efficient as Ranibizumab when clinically used to treat CNV. While the VEGF-antibodies prevent VEGF-receptor binding, thereby preventing the induction of angiogenesis, Pegaptanib would inhibit the enhancement of receptor signaling by inhibiting the binding to heparan sulphates and NP-1, but not the induction of receptor signaling itself.

VEGF-TRAP

The highest-affinity VEGF blocker described to date is a soluble decoy receptor created by fusing the first three Ig domains of the VEGFR-1 to an Ig constant region. However, this fusion protein had very poor pharmacokinetic properties which might be due to the high positive charge of these proteins, resulting in non-specific adhesion to highly negatively charged proteoglycans in the extracellular matrix. To create VEGF-Trap, the first Ig domain of the decoy receptor was removed and the third domain was switched with the third domain of VEGFR-2 [74] resulting in a recombinant soluble VEGF-receptor protein combining the binding domains of VEGFR-1 and 2 with the Fc portion of immunoglobulin G. The receptor has a very high affinity for all VEGF-A isoforms (K_d<1pmol/l). Additionally, an affinity for placental

growth factor 1 and 2, VEGF-B, VEGF-C and VEGF-D has been described [75]. Unlike Avastin, which forms multimeric complexes with VEGF, VEGF-Trap forms a 1:1 complex which remains stable in the systemic circulation [76].

If feedback mechanisms contribute to VEGF expression, VEGF trap would probably have additional effects *via* this pathway.

siRNA

While Bevacizumab, Ranibizumab, Pegaptanib and VEGF-Trap all neutralize VEGF by extracellularly sequestering VEGF, the siRNA approach works intracellularly. The mechanism involved is called RNA interference. It is a sequence specific, posttranscriptional gene silencing method. In order to suppress certain genes, double stranded RNA homologues are introduced into the cell and processed by a cellular RNase III, called Dicer, to generate siRNA duplexes of ca. 21 nts with 3' overhangs. These siRNAs are incorporated into a multiprotein RNA-inducing silencing complex (RISC). The duplex RNA is unwound, leaving the antisense strand to guide RISC to its homologous target mRNA for endonucleolytic cleavage [77]. While this approach is intriguing, there are limitations to this system. In mammals, siRNA is not replicated in the cells, so the effect of siRNA is transient. Also, in order to perform, siRNA has to be incorporated into the cells, e.g. *via* lipid-based reagents [77].

In order to tread AMD, short interfering RNA has been developed to silence gene expression of VEGF (Bevasiranib, Cand5, by Acuity Pharmaceuticals) or VEGFR-1 (Sirna-027, Sirna Therapeutics) in a sequence-specific manner.

Sirna-027 was designed using a bioinformatics-based approach with one selection criteria being that the siRNA is conserved between human, mouse and rat, enabling animal testing and clinical trials [78]. (Neither Bevacizumab nor Ranibizumab bind too good to mouse VEGF, as it carries a mutation at AS88 where glycine is changed to serine [79]). The sequences of Sirna-027 is sense:, 5'-BCU GAG UUU AAA AGG CAC CCT TB-3', antisense: 5'-GGG UGC CUU UUA AAC UCA GT_sT-3', and the target sequence is present in *vegfr-1* mRNA for human, mouse, rat, pig and monkey [78]. The siRNA was modified in order to extend its half life. The modifications include inverted abasic moieties at the 5' and 3' ends of the sense strand oligonucleotide (2'-deoxy abasic nucleotides), unpaired deoxythymidines and a single phosphorothioate linkage between the last two nucleotides at the 3' end of the target-complementary (antisense) strand. Sirna-027 reduces the mRNA of VEGFR-1, but not 2, *in vitro* and reduces the CNV lesion size *in vivo* after laser induced rupture of the Bruch's membrane or oxygen induced ischemic retinopathy and in a laser-induced CNV [78].

Bevasiranib is a double stranded RNA oligonucleotide consisting of 21 nucleotides and directed against VEGF with the sense strand sequence 5'-ACC UCA CCA AGG CCA GCA CdTdT-3' and the antisense sequence 5'-G UGC UGG CCU UGG UGA GGUdTdT-3. In animal models, the uptake and down regulation of the target gene has been shown [80,81], and Phase II and Phase III studies to test Bevasiranib in humans are currently conducted.

The mechanism of siRNA-induced gene silencing as a treatment for angiogenesis has recently been challenged. In a recent publication, it is claimed that the CNV inhibition is not a specific response to siRNA targeted against VEGF or VEGFR-1, but a siRNA-class effect. Any siRNA which is 21 base pairs or longer, no matter what target, suppressed CNV in mice to a similar extend as siRNA against VEGF or VEGFR-1 [82]. Applied siRNAs do not enter the target cells, but are extracellularly bound by Toll-like receptor 3 (TLR-3), a sensor for long, double-stranded viral RNA [83]. Inhibition of CNV is conducted by TLR-3 *via* the TLR-3/TRIF/NFkB pathway and induction of IL-12 and IFγ. A knock down of *vegfr-1* mRNA can only occur when the siRNA is aided to enter the cells, while non-targeted siRNA suppresses CNV by means of extracellular TLR3 activation.

CONCLUSION

VEGF antagonists are potent tools in the treatment of AMD. Their main aim is to neutralize VEGF and to prevent the onset of signal transduction pathways that induced angiogenesis. VEGF expression and secretion is tightly regulated by a complex set of pathways, including autokrine mechanisms. Any interference in this system changes the balance and has consequences regarding VEGF expression, secretion and VEGF-induced signal transduction. These mechanisms and consequences need to be elucidated further as well as the effects and the pathways of the medication used. A better understanding of the regulation of VEGF and its manipulation will help to optimize the treatment for wet AMD.

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