Published in final edited form as:

Exp Eye Res. 2006 November ; 83(5): 1005–1016.

Vascular endothelial growth factor receptors: Molecular mechanisms of activation and therapeutic potentials

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Abstract

Angiogenesis-associated eye diseases are among the most common cause of blindness in the United States and worldwide. Recent advances in the development of angiogenesis-based therapies for treatment of angiogenesis-associated diseases have provided new hope in a wide variety of human diseases ranging from eye diseases to cancer. One group of growth factor receptors critically implicated in angiogenesis is vascular endothelial growth factor receptors (VEGFR), a subfamily of receptor tyrosine kinases (RTKs). VEGFR-1 and VEGFR-2 are closely related receptor tyrosine kinases and have both common and specific ligands. VEGFR-1 is a kinase-impaired RTK and its kinase activity is suppressed by a single amino acid substitution in its kinase domain and by its carboxyl terminus. VEGFR-2 is highly active kinase, stimulates a variety of signaling pathways and broad biological responses in endothelial cells. The mechanisms that govern VEGFR-2 activation, its ability to recruit signaling proteins and to undergo downregulation are highly regulated by phosphorylation activation loop tyrosines and its carboxyl terminus. Despite their differential potentials to undergo tyrosine phosphorylation and kinase activation, both VEGFR-1 and VEGFR-2 are required for normal embryonic development and pathological angiogenesis. VEGFR-1 regulates angiogenesis by mechanisms that involve ligand trapping, receptor homodimerization and heterodimerization. This review highlights recent insights into the mechanism of activation of VEGFR-1 and VEGFR-2, and focuses on the signaling pathways employed by VEGFR-1 and VEGFR-2 that regulate angiogenesis and their therapeutic potentials in angiogenesis-associated diseases.

Keywords

vascular endothelial growth factor receptor-1 (VEGFR-1); vascular endothelial growth factor receptor-2 (VEGFR-2); FLT-1; FLK-1; angiogenesis; vasculogenesis; receptor tyrosine kinases; Src kinases; phosphoinositide 3-kinase; PLC-gamma1; c-Cbl; ubiquitination; downregulation; signal transduction; tyrosine phosphorylation

1. Introduction

VEGFR-1/FLT-1 (fms-like tyrosine kinase) and VEGFR-2/KDR/FLK-1 (fetal liver kinase) are the prototypes of a gene family encoding structurally related receptors, FLT-3/FLK-2 and FLT-4/VEGFR-3 and belong to the receptor tyrosine kinase (RTK) subfamily (Hanks and Quinn, 1991; Blume-Jensen and Hunter, 2001). VEGFR-1 and VEGFR-2 are primarily involved in angiogenesis (Yancopoulos et al., 2000) where FLT-3 and FLT-4 are involved in hematopoiesis and lymphogenesis (Jussila and Alitalo, 2000). The diversification of the VEGFR family proteins during evolution, through the advent of multiple ligands and receptors

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has created a decisive signaling network capable of controlling angiogenesis. Through utilization of receptor homo- and heterodimers, activated by specific and common ligands, diverse angiogenic signals are propagated. An additional level of angiogenic signaling diversity is obtained through differential activation of distinct signaling molecules downstream of each receptor.

VEGFR-1 is a kinase-impaired RTK, and may signal in the context of receptor heterodimer (Rahimi et al., 2000; Autiero et al., 2003; Neagoe et al., 2005). In contrast, VEGFR-2 is highly kinase active receptor and activates broad signaling cascades leading to diverse biological responses (Waltenberger et al., 1994; Meyer and Rahimi, 2003; Rahimi, 2006). Three different gene products including, PLGF, VEGF-A, VEGF-B are known to bind VEGFR-1. VEGF-A, VEGF-D and VEGF-C are known to bind VEGFR-2 (Neufeld et al., 1999; Tammela et al., 2005). VEGF-C and VEGF-D also bind to VEGFR-3, an RTK that is expressed by lymphatic endothelial cells and hematopoietic progenitor cells (Jussila and Alitalo, 2000; Tammela et al., 2005). VEGFR-1 and VEGFR-2 are structurally similar, consisting of an extracellular ligand-binding domain with a seven immunoglobulin (Ig)-like motif, a single transmembrane domain, a juxtamembrane domain, a kinase domain split by a kinase insert, and a carboxyl terminus. Overall, there is 43.2% sequence homology between VEGFR-1 and VEGFR-2. The extracellular domain of VEGFR-1 and VEGFR-2 displays 33.3% homology and the cytoplasmic region 54.6%. The kinase domains of VEGFR-1 and VEGFR-2 represent the most conserved region with 70.1% homology. In contrast, the carboxyl terminus represents the most divergent region with only 28.1% sequence homology (Fig. 1).

2. VEGFR-1 and angiogenesis

Initial evidence linking VEGFR-1 to endothelial cell function and angiogenesis was provided by targeted deletion of VEGFR-1, which resulted in early embryonic lethality due to abnormal blood vessel growth (Fong et al., 1995). Although the precise role of VEGFR-1 in endothelial cell functions is still emerging, in recent years several lines of evidence have developed that suggest VEGFR-1 may play both negative and positive roles in angiogenesis. The negative role of VEGFR-1 in angiogenesis was suggested based on the observation that loss of *VEGFR-1/flt-1* in mice caused an increase in the number of endothelial progenitors, resulting in the vascular disorganization (Fong et al., 1995, 1999). How VEGFR-1 contributes to angiogenesis in a negative manner is not fully clear. Some studies have suggested that the negative role of VEGFR-1 in angiogenesis is associated with its ability to alter endothelial cell division (Kearney et al., 2002, 2004). However, work from several laboratories indicates that selective activation of VEGFR-1, either by creating VEGFR-1 chimeras (Rahimi et al., 2000; Zeng et al., 2001) or VEGF mutants is not associated with proliferation of endothelial cells in vitro (Keyt, 1996). In these studies ligand stimulation of VEGFR-1 induced neither cell proliferation nor apoptosis. Selective activation of VEGFR-1 also did not promote cell migration or intracellular calcium release (Meyer et al., 2004a, 2004b). It could be argued that in a defined condition when VEGFR-1 is activated by a ligand that only allows receptor homodimerization and with no cross-talk with other RTKs such as VEGFR-2 and VEGFR-3, VEGFR-1 is not capable of promoting biological responses such as endothelial cell migration, cell proliferation and intercellular calcium release. In line with this notion, in a recent gene targeting study where the entire cytoplasmic region of *VEGFR-1* including its kinase domain was deleted, the knockin truncated *VEGFR-1* mice developed with no aberrant vasculogenesis (Hiratsuka et al., 1998). This was in startling contrast to the *VEGFR-1* knockout mice, which mice died in early development due to overgrowth of endothelial cells (Fong et al., 1995, 1999).

In sum, these observations reinforce the idea that VEGFR-1 may act as a decoy receptor and its function in angiogenesis may involve its ligand binding extracellular region, acting as a VEGF-trap to modulate VEGFR-2 function (Fig. 2).

It should be noted that role of VEGFR-1 in angiogenesis is far more complex than initially thought. Clearly, the ability of VEGFR-1 to modulate angiogenesis is not limited to a mechanism in which it acts as a VEGF-trapping receptor. For example, stimulation of VEGFR-1 with PLGF, (a VEGFR-1 specific ligand) promotes heterodimerization of VEGFR-1 with VEGFR-2 leading to transactivation of VEGFR-2 and angiogenesis (Autiero et al., 2003). Overexpression of PLGF, in transgenic mice is also reported to promote angiogenesis (Odorisio et al., 2002). These findings suggest that VEGFR-1 when paired with VEGFR-2 via a mechanism of heterodimerization its activity positively regulates angiogenesis. Also, it is increasingly apparent that activation of VEGFR-1 in endothelial cells and its final biological function is subject to the microenvironment of endothelial cells, in particular, the presence or absence of the other VEGFR family proteins and neuropilins (Klagsbrun et al., 2002). Likewise, VEGFR-1 homodimerization and heterodimerization may define the inhibitory or stimulatory nature of its signaling relays (Rahimi et al., 2000; Zeng et al., 2001; Autiero et al., 2003).

Another factor that may determine the nature of VEGFR-1 signaling in the endothelium microenvironment is the type of ligand being utilized to activate VEGFR-1. The nature of the signal induced by PLGF, which selectively binds VEGFR-1, might be different than that induced by VEGF-A and VEGF-B. In agreement with this idea, the impaired angiogenesis associated with *plgf* null mice was not rescued by VEGF-B (Carmelito, 2003). Also, a naturally occurring VEGF-PLGF heterodimer may favor the formation of certain heterodimerization complexes among the VEGFR family involving VEGFR-1, VEGFR-2 and neuropilins, which may activate a unique signaling pathway that might not be possible by stimulation with either VEGF or PLGF alone. It should also be noted that endothelial cells are morphologically and genetically different from each other (Cleaver and Melton, 2003) raising the possibility that variation in endothelial cells may ultimately determine or modify the nature of VEGFR-1 signaling. Finally, there are also some evidences that suggest VEGFR-1 signaling in non-endothelial cells might be different than that of the endothelial cells. For example, stimulation of VEGFR-1 with PLGF in non-endothelial cells such as monocytes and trophoblasts induce cell migration and proliferation (Clauss et al., 1996; Athanassiades and Lala, 1998; Dikov et al., 2005). VEGFR-1 activity also is linked to pathological angiogenesis such as cancer and retinopathy of prematurity. It has been reported that activation of VEGFR-2 prevents pathological regression of blood vessels from oxygen-induced retinal vascular degeneration in retinopathy of prematurity (Shih et al., 2003) and VEGFR-1 is also expressed by some carcinomas and is involved in cell migration and cell invasion (Wey et al., 2005).

3. VEGFR-1 is a kinase-impaired RTK

The mechanisms by which most RTKs are activated are well characterized. Transphosphorylation of RTK and their ability to phosphorylate target proteins are the hallmark of RTK activation. In this context, VEGFR-1 is considered to be a kinase-impaired RTK (e.g., VEGFR-1 is poorly tyrosine phosphorylated and its ability to phosphorylate a polypeptide substrate is negligible). All RTKs, including VEGFR-1 contain an evolutionary conserved kinase domain containing GXGXXG, an ATP binding site, HRDLA, a motif essential for catalysis and one or two tyrosine autophosphorylation sites (Hanks and Quinn, 1991). Despite having these motifs, the ligand stimulation of VEGFR-1 results only in minor tyrosine phosphorylation of VEGFR-1 in vivo and in vitro (de Vries et al., 1992; Waltenberger et al., 1994; Rahimi et al., 2000). In some RTKs such as colon carcinoma kinase-4 (CCK4), ErbB3/HER3, KLG, Ror1 and DRL abnormalities in the kinase domain have been suggested to

contribute to the kinase-defective phenotype of these RTKs (Chou and Hayman, 1991; Guy et al., 1994; Mossie et al., 1995; Yoshikawa et al., 2001). VEGFR-1 has no aberrant amino acid replacement in the GXGXXG and HRDLA motifs.

A recent study suggests that the kinase-impaired activity of VEGFR-1 can be rescued, in part, by swapping the carboxyl terminus of VEGFR-1 with that of VEGFR-2. The carboxyl-terminus swapped VEGFR-1 promoted ligand-dependent autophosphorylation of VEGFR-1 and induction of endothelial cell proliferation (Meyer et al., 2004a, 2004b). It appears that this effect is selectively associated with its carboxyl terminus because replacement of the juxtamembrane (JM) region of VEGFR-1 with that of VEGFR-2 does not rescue its kinase-impaired characteristic (Gille et al., 2000). A detailed understanding of the molecular mechanism underlying the carboxyl terminus of VEGFR-1 in its poor tyrosine phosphorylation should wait until the crystal structure of VEGFR-1 is resolved. To date, the crystal structure of VEGFR-1 is not available, only its ligand-binding region is resolved (Wiesmann et al., 1997). How the carboxyl terminus of VEGFR-2 is able to rescue the kinase-impaired activity of VEGFR-1 is not fully understood. However, it is feasible that the carboxyl terminus of VEGFR-1 somewhat obstructs ligand-mediated autophosphorylation of VEGFR-1. In some RTKs, such as Tie-2, deletion of carboxyl terminus has been shown to enhance its autophosphorylation and kinase activation, suggesting that the carboxyl terminus negatively regulates tyrosine autophosphorylation possibly by interacting with activation loop and keeping it in an inhibitory conformation (Niu et al., 2002).

The molecular explanation for the kinase-impaired characteristic of VEGFR-1 is provided by a recent finding that the decoy characteristic of VEGFR-1 is linked to the replacement of a highly conserved amino acid residue, located in its activation loop (Meyer et al., 2006). This amino acid is highly conserved among all the type III RTKs and corresponds to aspartic acid (D) but in VEGFR-1 is substituted to asparagine (N) (Fig. 1). Mutation of asparagine (N1050) within the activation loop to aspartic acid promoted enhanced ligand-dependent tyrosine autophosphorylation and kinase activation in vivo and in vitro. This mutation promotes endothelial cell proliferation, but not tubulogenesis. It also displayed an oncogenic phenotype as its expression in fibroblast cells elicited transformation and colony growth. It has been suggested that the conserved aspartic acid in the activation loop favors the transphosphorylation of the activation loop tyrosines and its absence renders VEGFR-1 to a less potent enzyme perhaps by disfavoring transphosphorylation of activation loop tyrosines. It should be emphasized that although these observations may explain the decoy characteristic of VEGFR-1 (e.g., impaired kinase activation and tyrosine phosphorylation), nevertheless these findings still do not explain mechanisms by which VEGFR-1 contributes to angiogenesis as a kinase-impaired RTK.

Several RTK including, DRL, CCK4, KLG, Ror1 and ErbB3 all display impaired kinase activity, yet they transduce signals and regulate variety of biological functions (Chou and Hayman, 1991; Guy et al., 1994; Mossie et al., 1995; Yoshikawa et al., 2001). The best-studied kinase-impaired RTK is ErbB3, one of the four members of EGFR family (Citri et al., 2003). Data from several laboratories using various systems revealed that signaling of ErbB3 requires kinase activity of another EGFR family including ErbB1, ErbB2 and ErbB4 (Pinkas-Kramarski et al., 1996; Citri et al., 2003). DRL, another kinase-impaired RTK, although it shows no kinase activity in vivo and in vitro, nevertheless its activity is required for axon guidance in *Drosophila* (Yoshikawa et al., 2001). The molecular mechanism of DRL signaling and whether DRL transduces signal like ErbB3 is not known. There is some circumstantial evidence suggesting that VEGFR-1 may signal through other VEGFR family kinases, in particular, through VEGFR-2. Both VEGFR-1 and VEGFR-2 are expressed by endothelial cells, suggesting that under a favorable condition such as availability of appropriate ligand, VEGFR-1 may form heterodimer complexes with the other receptors. Indeed, VEGFR-1 has been shown to

heterodimerize with VEGFR-2 and that heterodimerization leads to autophosphorylation, activation of VEGFR-2, and angiogenesis (Autiero et al., 2003). Thus, the mechanism by which VEGFR-1 supports angiogenesis is complex and likely involves several different mechanisms including VEGF-trapping, activation by heterodimerization and homodimerization (Fig. 2). VEGFR-1 also has a residual kinase activity and its selective activation that results only in homodimerization leads to activation of a limited number of signaling proteins such as p42/44 MAPK (Meyer et al., 2004a, 2004b). Thus, the unique signaling nature of VEGFR-1 and mechanisms by which it stimulates biological responses indicates that we have more to learn about RTK signaling than the current widely accepted and generalized RTK paradigm would suggest.

4. VEGFR-2 activation is essential for angiogenesis

The role of VEGFR-2 in angiogenesis is well established and more comprehensive reviews on the role of VEGFR-2 in angiogenesis have been recently published (Yancopoulos et al., 2000; Carmeliet, 2003; Jain, 2003). In this review we focus on the recent advancement on VEGFR-2 activation and its signal transduction relays.

4.1. Carboxyl terminus is critical for VEGFR-2 activation and its signaling

The mechanisms by which most RTKs transduce signal have been studied extensively at the molecular level. The basic principles of RTKs activation can be summarized as following: ligand-mediated dimerization of receptor monomers, transphosphorylation by dimerized receptors and docking of signaling proteins to receptor phosphotyrosines (Hubbard et al., 1998; Blume-Jensen and Hunter, 2001). The autophosphorylation sites are either involved in the regulation of kinase activity of the receptor or serve as a binding site for SH2- and PTB-containing proteins (Blume-Jensen and Hunter, 2001; Pawson, 2004). Central to ligand-induced RTK activation is the phosphorylation of one or two tyrosine residues within the activation loop (Hubbard et al., 1998). In the unstimulated form, the activation loop orients these tyrosine sites toward the activation sites of the enzyme and thereby sterically prevents binding to ATP-Mg²⁺. It is suggested that phosphorylation of these tyrosines in the catalytic loop orients the inhibitory loop away from the active site enabling the RTK to bind ATP-Mg²⁺ and efficiently phosphorylate substrates (Hubbard et al., 1998; Schlessinger, 2000). The recent crystal structure of VEGFR-2 by McTigue et al. (1999) showed that tyrosines 1054 and 1059, two highly conserved tyrosine residues located in the activation loop of VEGFR-2 are phosphorylated. An unexpected feature of the VEGFR-2 structure is the conformation of the activation loop. Although phosphorylated, the activation loop is disordered in the region that includes phosphotyrosine 1059. This is in contrast to the structures of other tyrosine kinases such as FGFR-1 and the insulin receptor, which are well ordered and participate in specific interactions (McTigue et al., 1999).

In last few years some of the paradigms learned from studying other RTKs revealed that catalytic activity is regulated by other segments of RTKs. For instance, substitution of two tyrosine residues with phenylalanine in the juxtamembrane region of the PDGFR- β drastically reduces autophosphorylation and activation of this receptor (Mori et al., 1993). Similar observations were reported for the MuSK tyrosine kinase, a receptor tyrosine kinase for agrin, which is involved in the functioning of the neuromuscular synapse (Till et al., 2002) and EphB2, a receptor tyrosine kinase for ephrins (Wybenga-Groot et al., 2001; Schlessinger, 2003). Recent crystal structures of MuSK and EphB2 revealed that the juxtamembrane domains of these receptors are directly involved in the regulation of kinase activation. The data obtained from the crystal structures suggest that the juxtamembrane domain interacts with the kinase domain and represses catalytic activity (Schlessinger, 2003). Additional biochemical studies demonstrated that these tyrosines also bind to SH2-containing proteins (Wybenga-Groot et al., 2001). There are two tyrosine residues within the juxtamembrane of VEGFR-2 (tyrosines 799

and 820 in mouse VEGFR-2). Replacement of these tyrosines to phenylalanine in VEGFR-2 does not impair the ligand-dependent activation of VEGFR-2 (Dayanir et al., 2001; Meyer et al., 2002). This suggests that phosphorylation of these tyrosines are not involved in the kinase activation of VEGFR-2.

Unlike juxtamembrane tyrosine phosphorylation sites, however, phosphorylation of tyrosine 1212 (tyrosine 1214 in human VEGFR-2) in the carboxyl terminus plays an important role in autophosphorylation and kinase activation of VEGFR-2. Mutation of tyrosine 1212 to phenylalanine impairs full activation of VEGFR-2 and its ability to efficiently activate signaling proteins. Mutation of the same tyrosine residue to glutamic acid, however, preserved its ability to undergo ligand-dependent autophosphorylation (Meyer et al., 2002). This suggests that tyrosine 1212 regulates VEGFR-2 autophosphorylation in a manner that is similar to that of tyrosine sites in the JM region of MuSK and EphB2. Surprisingly, this mutant receptor as recently reported preserved normal development and angiogenesis in knockin mice (Sakurai et al., 2005). This indicates that either the low level kinase activity of tyrosine 1212 mutant VEGFR-2 is sufficient to promote angiogenesis or that phosphorylation of tyrosine 1212 is not stringently required for function of VEGFR-2 in vivo. It is also conceivable that phosphorylation of other tyrosine sites in VEGFR-2 may compensate the lack of phosphorylation of tyrosine 1212. In agreement with this possibility, the mutant PDGFR- β lacking the binding sites for PLC- γ 1, PI 3 kinase, SHP2 and Ras-GAP has been shown to stimulate gene expression in fibroblast cells almost as efficiently as the wild-type PDGFR- β (Fambrough et al., 1999). Similarly, activation of Src kinases by many laboratories has been shown to be important for PDGFR- β autophosphorylation and its biological function (Mori et al., 1993). Yet, PDGFR- β is fully functional in SYF cells, deficient for Src family kinases (Klinghoffer et al., 1999). This argues that RTK signaling induces activation of signaling proteins with an overlapping role in vivo and thus the role of individual docking sites may be compensated for by the presence of other docking sites.

Further analysis of the carboxyl terminus of VEGFR-2 revealed that this region is engaged in VEGFR-2 activation in multiple ways. First, the carboxyl terminus of VEGFR-2 contains several tyrosine phosphorylation sites including tyrosine 1212, 1173 and 1221. Tyrosines 1212 and 1221 are phosphorylated but the identity of signaling proteins that are recruited to these sites is not known (Meyer et al., 2002; and our unpublished data). As discussed earlier, the presence of tyrosine 1212 is required for full autophosphorylation of VEGFR-2 (Meyer et al., 2002). Tyrosine 1173 is phosphorylated in vivo and recruits p85 of PI3 kinase (Dayanir et al., 2001), PLC γ 1 (Takahashi et al., 2001; Meyer et al., 2003) and regulate phosphorylation of c-Cbl (our unpublished data). In addition to possessing multiple tyrosine phosphorylation sites, the carboxyl terminus of VEGFR-2 also contains putative serine phosphorylation sites. Mutation of serines 1188 and 1191 in the carboxyl terminus of VEGFR-2 is shown to impair the ligand-dependent down regulation of VEGFR-2 (Singh et al., 2005). This might explain why deletion of carboxyl terminus impairs the VEGFR's ability to undergo ligand-dependent downregulation (Meyer et al., 2004a, 2004b). Finally, deletion of the entire carboxyl terminus of VEGFR-2 is shown to impair its ligand-dependent autophosphorylation. It appears that this role of carboxyl terminus is independent of tyrosine 1212 and may involve residues other than tyrosines (Meyer et al., 2004a, 2004b). Altogether, the current literature provides a framework for understanding of regulation of VEGFR-2 activation and further demonstrates that the carboxyl terminus plays various important roles in VEGFR-2 functions ranging from its ability to regulate autophosphorylation and recruitment of signaling proteins to ligand-dependent downregulation. Future work to characterize function of the VEGFR-2 carboxyl terminus will require a detailed analysis including: identification of tyrosine and serine phosphorylation sites and signaling proteins that might interact with these sites. Finally, resolving the crystal structure of VEGFR-2 with its carboxyl terminus intact may provide further insight into regulation of this important protein.

4.2. Role of phosphoinositide 3-kinase in VEGFR-2 signaling and angiogenesis

In the last few years, the phosphoinositide 3-kinase (PI3K) signal transduction pathway has emerged as one of the main signal routes that VEGFR-2 employs to stimulate endothelial cell survival and proliferation. PI3 kinase is a lipid kinase that converts the plasma membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol-3,4,5-triphosphate (PIP₃). Proteins with pleckstrin-homology (PH) domains, such as protein kinase B (PKB/Akt), phosphoinositide-dependent kinase-1 (PDK-1) and PDK-2, bind to PIP₃. Akt is activated by PIP₃, PDK1 and PDK2 leading to phosphorylation of a host of other proteins that affect cell proliferation, cell cycle progression and cell survival (Rameh and Cantley, 1999; Cantley, 2002).

Various experiments using several in vivo and in vitro systems have demonstrated that activation of PI3 kinase by VEGFR-2 promotes endothelial cell survival, proliferation and angiogenesis (Fig. 3). First, Wortmannin and LY294002, the PI3 kinase specific inhibitors and a dominant negative form of Akt, a downstream target of PI3 kinase, all are able to inhibit VEGF-stimulated endothelial cell survival (Gerber et al., 1998). Second, inhibition of PI3 kinase by overexpression of a dominant negative form of p85 of PI3 kinase inhibits endothelial cell proliferation and induction of c-fos (Thakker et al., 1999). Similarly, overexpression of PTEN, a lipid phosphatase that reverses PI3 kinase function by dephosphorylating its phospholipid products, inhibits VEGF-mediated endothelial cell proliferation. Overexpression of phosphatase inactive form of PTEN enhances VEGF-mediated endothelial cell proliferation (Huang and Kontos, 2002). Third, expression of a viral form of PI3 kinase and its target Akt promotes angiogenesis and VEGF expression in chicken embryo (Jiang et al., 2000). The most recent study on PI3 kinase also suggests that it may regulate angiogenesis by regulating expression of Tie-2 receptor (Lelievre et al., 2005).

A mechanistic explanation on how PI3 kinase is activated by VEGFR-2 is demonstrated by a chimeric VEGFR-2 system in which the PI3 kinase binding sites were mutated and its role in VEGFR-2-mediated endothelial cell proliferation was tested. The tyrosine mutant chimeric VEGFR-2 (where tyrosines 799 and 1173 of mouse VEGFR-2 were mutated) abolished the ability of VEGFR-2 to stimulate PI3 kinase activity and cell proliferation (Dayanir et al., 2001). Also, PI3 kinase-dependent proliferation of endothelial cells is linked to activation of S6 kinase. This is supported by the observation that a mutant chimeric receptor that fails to activate PI3 kinase also fails to activate S6 kinase (Dayanir et al., 2001). Consistent with this observation, selective inhibition of S6 kinase inhibits VEGF-induced endothelial cell proliferation and in vivo angiogenesis (Guba et al., 2002; Yamazaki et al., 2005). In agreement with a critical role of PI3 kinase activation in growth and survival of endothelial cells, a recent study also demonstrated that tumstatin, a naturally occurring angiogenesis inhibitor, promotes apoptosis of endothelial cells by inhibiting the PI3 kinase pathway (Sudhakar et al., 2003).

4.3. Role of phospholipase C γ 1 in VEGFR-2 signaling and angiogenesis

Phospholipase C γ 1 (PLC γ 1) hydrolyzes phosphoinositides to generate the second messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ increases intracellular Ca²⁺ levels and DAG activates protein kinase C (PKC) leading to variety of cellular responses (Sekiya et al., 1999). Several lines of evidences indicate that activation of PLC- γ 1 plays a pivotal role in angiogenesis and VEGFR-2 signal transduction (Fig. 3). First, a genetic study shows that lack of PLC γ 1 in mice results in significantly diminished vasculogenesis and erythropoiesis (Liao et al., 2002). Second, in transgenic zebrafish the PLC- γ 1 deficiency shows defects in arterial formation. The PLC- γ 1 deficient embryos also failed to respond to exogenous VEGF, highlighting the importance of PLC- γ 1 for VEGFR-2-mediated signal transduction relay (Lawson et al., 2003). However, the exact role of PLC γ 1 in angiogenesis is not clear. VEGFR-2-mediated activation of PLC- γ 1 in a certain endothelial cellular background is

suggested to stimulate cell proliferation and in other endothelial cells to stimulate differentiation and tubulogenesis (Rahimi et al., 2000; Takahashi et al., 2001; Meyer et al., 2003). Phosphorylation of tyrosines 1006 and 1173 (Y1006 and 1173 correspond to Y1008 and 1175 in human VEGFR-2, respectively) are important for recruitment of PLC γ 1 by VEGFR-2 (Takahashi et al., 2001; Meyer et al., 2003). The tyrosine mutant VEGFR-2 that fails to activate PLC γ 1 also fails to stimulate tubulogenesis in vitro (Meyer et al., 2003). A recent knockin approach in mice showed that mutation of tyrosine 1173 of VEGFR-2 abrogates its ability to promote angiogenesis (Sakurai et al., 2004). Although this finding suggests an essential role for tyrosine 1173 in the regulation of angiogenesis, it is not clear whether the defect is exclusively linked with the inability of the tyrosine 1173 mutant VEGFR-2 to activate PLC γ 1. Tyrosine 1173 of VEGFR-2 is also known to recruit other signaling proteins such as p85 of PI3 kinase (Dayanir et al., 2001) and Shb (Holmqvist et al., 2004).

4.4. Role of Src kinases in VEGFR-2 signaling and angiogenesis

Activation of Src kinases have been implicated in the regulation of a variety of biological responses including cell proliferation, migration, differentiation and cell survival (Bromann et al., 2004). Activation of many RTKs leads to stimulation of Src family kinase activity via tyrosine phosphorylated docking sites on RTKs and the SH2 domain of Src kinases (Boggon and Eck, 2004). Selective activation of VEGFR-2 stimulates Src phosphorylation in endothelial cells (Meyer et al., 2002). However, the precise mechanism involved is not established. In particular, Src binding sites on VEGFR-2 and biological importance of Src in VEGFR-2-mediated signal transduction is not fully understood. A recent study demonstrated that Src kinase activity is required to protect endothelial cells from apoptosis and it appears that Src elicits its anti-apoptotic function via Raf-1 activation (Alavi et al., 2003). It has also been shown that the ability of VEGF to induce vascular permeability requires Src kinases. Curiously, angiogenesis is normal in mice deficient for Src and Yes (Eliceiri et al., 1999). Src activation also is implicated in GPCR (such as nucleotide receptor P2Y₂)-mediated transactivation of VEGFR-2 but not in ligand-mediated VEGFR-2 activation (Meyer et al., 2002; Seye et al., 2004). Indeed, ligand-dependent autophosphorylation of VEGFR-2 is not compromised in SYF cells, deficient in Src, Yes and Fyn and also over-expression of a dominant negative form of Src in endothelial cells has no apparent effect on the ligand-stimulated VEGFR-2 autophosphorylation (Meyer et al., 2002). In addition to the role of c-Src in VEGFR-2-mediated signal transduction, Src also plays a key role in angiogenesis by promoting expression of VEGF in response to hypoxia (Mukhopadhyay et al., 1995). Further studies are required to delineate exact role of Src in VEGFR-2 signal transduction, in particular, its association with VEGFR-2 and tyrosine phosphorylation site on VEGFR-2 that might be involved in recruitment of Src to VEGFR-2.

4.5. Role of serine phosphorylation in VEGFR-2 signaling and angiogenesis

In addition to activating positive signaling pathways, activation of RTKs also induces negative feedback signaling pathways that attenuate or antagonize positive signaling. One mechanism that attenuates RTKs signaling is a rapid depletion of the cellular receptor pool, a phenomenon termed downregulation (Soldi et al., 1999). Recent study indicates that VEGFR-2 undergoes ligand-dependent downregulation and degradation in a novel mechanism involving non-classical PKCs. Singh et al. (2005) demonstrated that the carboxyl terminus is required for PKC-dependent downregulation of VEGFR-2. Surprisingly, the ligand-dependent downregulation and degradation of VEGFR-2 proceeds without involvement of its ectodomain and engagement of γ -secretase activity. Mutation of serine sites at 1188 and 1191 compromised the ability of VEGFR-2 to undergo efficient ligand-dependent downregulation. These findings suggest that serines 1188 and 1191 of VEGFR-2 are phosphorylated either directly by PKC or by serine kinases whose activity is regulated by PKCs. Upon phosphorylation of these sites they may recruit an E3 ligase to VEGFR-2, leading to ubiquitinylation and degradation of

VEGFR-2. The identity of the E3 ligase responsible for ubiquitinylation of VEGFR-2 is not known. It appears, however, that c-Cbl is not the E3 ligase that involved in ubiquitinylation and downregulation of VEGFR-2, although it is phosphorylated upon VEGFR-2 stimulation with ligand (Singh et al., 2005). Since c-Cbl is activated by VEGFR-2 it is interesting to know what role it plays in angiogenesis.

4.6. VEGF, VEGF receptors and ocular angiogenesis

Vascular endothelial growth factor (VEGF) is normally expressed by many cell types of the retina, including retinal pigment epithelium (RPE) cells and ganglion cells (Miller et al., 1997; Ida et al., 2003). VEGF-A is the most potent angiogenic protein known and its expression is abnormally elevated in patients with age-related macular degeneration (AMD), diabetic retinopathy, and retinopathy of prematurity (Campochiaro et al., 1999; Witmer et al., 2003). Expression of VEGF-A and its receptors, VEGFR-1 and VEGFR-2 also play critical role during the embryonic development of retinal, choroidal vasculatures and neural retinal development (Robinson et al., 2001; Fruttiger, 2002).

The survey of current literature indicates that VEGF not only plays a functional role during ocular development but also is involved in normal function of the retina and pathological conditions associated with it. Studies suggest that in Type I diabetes expression of VEGF is altered due to metabolic abnormalities including hyperglycemia, which represent major vascular complications of Type I diabetes (Chiarelli et al., 2000). Aberrant angiogenesis in diabetes is most clinically apparent in proliferative diabetic retinopathy. Hypoxia-induced expression of VEGF and other pro-angiogenic factors leads to altered angiogenesis, which causes major damage to pericytes. These aberrant conditions lead to increased permeability and rupture of retinal vessels (Caldwell et al., 2005). Although the role of angiogenesis is well documented in diabetic retinopathy, altered angiogenesis may also play a key role in diabetic neuropathy, diabetic nephropathy and diabetic wound healing (Isner et al., 2001; Kim et al., 2005). Furthermore, evidence now suggests that VEGF-A also promotes retinal vascular permeability and its presence is associated with the leakiness of retinal vessels in non-proliferative diabetic retinopathy (Murata et al., 1995). While the role of individual VEGF receptors involved in mediating vascular permeability is not fully understood, recent studies suggest that VEGFR-2 may play a prominent role in mediating the ability of VEGF to promote permeability of vascular endothelial cells (Joukov et al., 1998; Stacker et al., 1999). Elevated expression of VEGF-A due to hypoxia is considered to be the primary cause of retinopathy of prematurity (Smith, 2004). In addition, recent study suggests that PlGF, a VEGFR-1 specific ligand may protect against oxygen-induced vessel loss without stimulating angiogenesis (Shih et al., 2003). This finding implies that selective activation of VEGFR-1 but not VEGFR-2 may promote survival of retinal endothelial cells without promoting angiogenesis. It has been shown that VEGF-A plays a key role in choroidal angiogenesis, a major cause of visual loss in AMD. VEGF-A expression found to be significantly higher in patients with AMD (Zarbin, 2004) and its expression is also elevated in RPE cells in patients with AMD and in experimental animal models (Schwesinger et al., 2001).

Altogether, the above-mentioned studies provide ample evidence for the central role of VEGF and its receptors in ocular angiogenesis. Further studies, however, are needed to delineate the exact role of VEGF-A and possible involvement of other VEGF family proteins in ocular angiogenesis. It is also essential to establish the relative contribution of VEGF receptors in the development of these conditions. Finally, it should be noted that signal transduction pathways activated by VEGF receptors in pathological angiogenesis, in particular, in ocular angiogenesis is largely unknown. Thus, it is critical to dissect the molecular mechanisms of signaling aspects of VEGF receptors in various pathological conditions and this certainly will be an important direction for future studies and ultimately new strategies for clinical application.

4.7. Ocular diseases and anti-angiogenesis therapy

Although the idea of angiogenesis therapy was proposed over three decades ago (Folkman, 1971), the use of angiogenesis therapy was only realized in the last few years. Now, angiogenesis therapy is being considered for patients with cancer, age-related macular degeneration, heart diseases and others (Liu and Regillo, 2004; Annex and Simons, 2005; Neri and Bicknell, 2005; Wegewitz et al., 2005). The primary causes of vision loss in Western countries are macular degeneration and diabetic retinopathy. Although the mechanisms of each disease are distinct, they both involve abnormal angiogenesis. In addition, retinopathy of prematurity, a major cause of blindness in children, is associated with abnormal angiogenesis due to oxygen supplementation in the premature infants (Shih et al., 2003). In recent years several classes of anti-angiogenesis agents have been under development, many of these agents either target VEGF (e.g., VEGF antibodies) or VEGFRs (e.g., small molecule kinase inhibitors). Bevacizumab (Avastin; Genentech Inc., South San Francisco, CA) is a humanized monoclonal anti-VEGF-A antibody (Rosen, 2005). Avastin represents the first anti-angiogenesis drug that has recently been approved by the FDA for use in combination with standard chemotherapy in patients with advanced colorectal cancer. Another class of anti-angiogenesis inhibitors that are currently in clinical trials are the small molecule kinase inhibitors such as PTK787, SU11248 and ZD6474 which are potent inhibitors of VEGFR-1 and VEGFR-2 (Kerbel and Folkman, 2002; Rosen, 2005).

To date, the anti-angiogenesis therapy for ocular diseases that have been evaluated are angiogenesis inhibitors that mainly bind to VEGF such as pegaptanib (Macugen), and VEGF-Trap (Saishin et al., 2003; Gragoudas et al., 2004; Zakarija and Soff, 2005). Pegaptanib and VEGF-Trap like Avastin bind to VEGF and prevent binding of VEGF to VEGF receptors. Survey of current anti-angiogenesis therapies indicate that these therapies may in some cases reduces disease progression but by and large they are ineffective in stopping or reversing the diseases that are being tested (Stacker et al., 1999; Kerbel and Folkman, 2002). Another challenge facing the application of these anti-angiogenesis therapies to ocular angiogenesis in diseases such as AMD is the requirement of repeated injection of these drugs into the patient's eye. This represents a serious setback for usefulness of these drugs for patients with AMD. It appears that anti-angiogenesis therapy for ocular diseases may require a long-term administration of anti-angiogenesis drugs. Thus, preparation of these drugs in a sustained-release format may dramatically improve not only their efficacy but also the convenience of their application into the patients' eye. In addition, other strategies such as gene therapy may overcome limitations of the aforementioned anti-angiogenesis drugs. Recent studies using viral vector-mediated delivery of anti-angiogenesis proteins in rodent models provide a new and possibly more effective strategy for treatment of ocular angiogenesis (Reich and Bennett, 2003). Thus, it is clear that further work on the efficacy and safety of these agents as well as the development of more selective, potent and safer anti-angiogenesis agents certainly will provide substantial advances for the treatment of angiogenesis-associated ocular diseases. One avenue toward the development of more selective and better anti-angiogenesis drugs is to target a specific signaling pathway of VEGF receptors.

5. Conclusions

Abnormal angiogenesis is associated with pathology of many diseases including cancer, age-related macular degeneration (AMD), proliferative diabetic retinopathy (PDR), and retinopathy of prematurity (ROP). What is clear is that the more we learn about molecular mechanisms of VEGFR-1 and VEGFR-2 regulation and their signal transduction, the more we continue to learn about angiogenesis and molecules that regulate it. The emerging scenario in VEGFR-1 and VEGFR-2 activation is that the complexity of blood vessel development necessitated more stringent control over VEGFR-1. On one hand VEGFR-1 evolved to have

a higher affinity to various ligands, on the other hand VEGFR-1 lost its potency in enzymatic activity, creating a unique mechanism to control its function. VEGFR-2 plays a broader role in angiogenesis; its activity regulates endothelial cell growth, differentiation, migration and tubulogenesis. The carboxyl terminus of VEGFR-2 plays a central role in its activation, ability to recruit signaling proteins, to undergo downregulation and to promote angiogenesis.

Finally it should be noted that activation and signaling of VEGF receptors appear to be regulated not only by each other in both negative and positive manners but also by other receptors including integrins (Soldi et al., 1999; Yeynolds et al., 2004), cadherins (Rahimi and Kazlauskas, 1999; Bussolino et al., 2001) and neuropilins (Klagsbrun et al., 2002). In addition, the fact that multiple VEGF ligands with different preference and capability bind to VEGFRs and activate them in homo- and heterodimeric manners presents a challenge to study their selective biochemical and cellular signaling in endothelial cell background. Although characterization of specific functions of VEGFR-1 and VEGFR-2 and their signal transduction relays has been a frustrating task, it has nevertheless been illuminating. Continued research into the function of VEGFRs as well as techniques involving creating chimeric VEGFRs in which the extracellular domain of each VEGFR is replaced with that of CSF-1R (Rahimi et al., 2000), and mutant VEGFs that selectively bind to VEGFRs (Keyt et al., 1996; Stacker et al., 1999) continue to provide insight into their specific roles in angiogenesis. Evaluation of targeted mouse knockouts (Fong et al., 1995) and knockin (Sakurai et al., 2005) and the use of RNA interference may provide further insight into the function of this important family of receptors.

Acknowledgements

This work was supported in part through grants from The National Institutes of Health (NIH) EY0137061, EY012997 and a grant from Massachusetts Lions Foundation to N.R. I thank Amrik J. Singh and Rosana D. Meyer for their comments and reading the manuscript.

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Fig. 1.

Schematic representation of VEGFR-1, VEGFR-2 and their corresponding ligands: multiple VEGF ligands bind to VEGFR-1 and VEGFR-2. Placenta growth factor (PLGF) and VEGF-B bind to VEGFR-1 but not to VEGFR-2. VEGF-C and VEGF-D bind VEGFR-2 but not to VEGFR-1. VEGF-A binds to both VEGFR-1 and VEGFR-2. VEGFR-1 and VEGFR-2 are structurally similar, consisting of an extracellular ligand-binding domain with seven immunoglobulin (Ig)-like motifs, a single transmembrane domain (TM) and juxtamembrane (JM) domain, a kinase domain (KD) split by a kinase insert (KI), and a carboxyl terminus. The sequence homology between VEGFR-1 and VEGFR-2 also is shown. The kinase domain of VEGFR-1 and VEGFR-2 represent the most conserved region with 70.1% homology. The carboxyl terminus represents the most divergent region with only 28.1% sequence homology.

Sequence alignment of the catalytic and activation loop regions of human VEGFR-1 and various receptor tyrosine kinases of type III family are shown. The residue corresponding to asparagine (N) 1050 of VEGFR-1 is aspartic acid (D) in type III kinases. The location asparagine (N) 1050 in the activation loop of human VEGFR-1 is indicated by arrow. This residue is highly conserved and is invariable among the type III family kinases except VEGFR-1. Asparagine 1050 of VEGFR-1 is conserved among human (GenBank accession number: NM002019), mouse (GenBank accession number: BAA24498) rat (GenBank accession number: P53767) and chicken (GenBank accession number: BAB84690).

Fig. 2.

Schematic representation of the mechanisms of involvement of VEGFR-1 in angiogenesis: VEGF-trapping: the mechanism by which VEGFR-1 binds to VEGF and prevents it from binding to VEGFR-2. The cytoplasmic domain of VEGFR-1 is not required for its VEGF-trapping function (Hiratsuka et al., 1998). VEGFR-1 may elicit its role in angiogenesis by heterodimerization with VEGFR-2 and VEGFR-3 (Rahimi et al., 2000; Autiero et al., 2003; Dixelius et al., 2003). The third mechanism by which VEGFR-1 regulates angiogenesis is through receptor homodimerization. This type of activation of VEGFR-1 leads to activation of signaling proteins that might regulate angiogenesis in either positive or negative manner (Rahimi et al., 2000; Meyer et al., 2004a, 2004b).

Fig. 3. Schematic representation of VEGFR-2 mediated signal transduction and their role in endothelial biology: VEGFR-2 activation induces tyrosine phosphorylation and activation PLCγ1 via tyrosines 1006 and 1173. Activation of PLCγ1 leads to tubulogenesis and proliferation of endothelial cells. Activation of PI3 kinase by VEGFR-2 is mediated by tyrosines 799 and 1173 and is involved in proliferation and survival of endothelial cells. Tyrosine 1173 of VEGFR-2 also associates with the adaptor protein, Shb. Src family kinases and c-Cbl are also activated by VEGFR-2. Activation of Src is linked to endothelial cell survival and permeability (see text for details). Phosphorylation of tyrosine 1049 in the kinase insert region of VEGFR-2 binds to VRAP (Matsumoto et al., 2005).