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SIGNAL RELAYS IN THE VEGF SYSTEM

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1. ABSTRACT

A considerable progress has been made during the past years in elucidating the molecular actors of angiogenesis. Vascular endothelial growth factor turned out to represent the major inducer of angiogenesis. Optional splicing of its pre messenger RNA generates various isoforms which differ not only by their storage in the extracellular matrix but also by their signaling pathways. VEGF binds and activates two tyrosine kinase receptors called VEGFR1 and VEGFR2 and neuropilin-1. The elucidation of the transduction pathways of each receptor suggests that VEGFR1 mediates cell migration whereas VEGFR2 mediates cell proliferation. The construction of internal images of VEGF by the anti-idiotypic antibody strategy allowed us to determine that quiescent endothelial cells need to be activated by so far unknown factors to become competent to respond to mitogenic signals and acquire an angiogenic phenotype. The discovery of the mechanisms of action of the VEGF system has allowed the design of promising drugs which already entered the pre-clinical or clinical assays.

2. INTRODUCTION

In adult mammals the vasculature is quiescent except during the physiological cycles of reproduction. Endothelial cells are among those exhibiting the lowest replication level in the body with only 0.01% cells engaged in cell division at any time. Additional requirements in oxygen or nutrients, as in the course of tumor progression, diabetic retinopathy or rheumatoid arthritis, result in the sprouting of new capillaries from pre-existing vessels, or angiogenesis. This local hypervascularization is thought to result from the release of soluble mediators by the involved tissues, which induce the switch of the quiescent endothelial cell phenotype to an activated one, so that the endothelial cells are then able to respond to mitogenic signals. The release of mitogenic growth factors switches the activated phenotype to an angiogenic phenotype. The interactions of angiogenic growth factors with their receptors provide the signals for cell migration, proliferation and differentiation to form new capillaries.

It was postulated half a century ago that hypoxia preceded the retinal neovascularizations observed in diabetic retinopathy or retinopathy of the premature (1). The hypothesis that tumoral progression was dependent on angiogenesis also led to the concept of "tumor angiogenic growth factors" (2). The search for hypoxic retina and tumor angiogenic factors proved misleading until purification and cloning of the genes encoding angiogenic growth factors was achieved. During the past decade it has become clearer that the angiogenic growth factors which are up-regulated during pathological angiogenesis are similar to those promoting physiological angiogenesis or vasculogenesis during embryogenesis. Several candidates have been identified such as acidic fibroblast growth factor, basic fibroblast growth factor, transforming growth factor α, hepatocyte growth factor and interleukin 8, all of which are able to induce neovascularization in avascular organs such as the cornea and mimic *in vitro* all the steps of the angiogenic cascade (induction of proteases, cleavage of the extracellular matrix, proliferation, migration and differentiation into capillary-like tubes). It is now evident that the biological factor purified in 1989, and designated vascular endothelial growth factor (3), vascular permeability factor (4) or vasculotropin (5) represents a key regulator of angiogenesis.

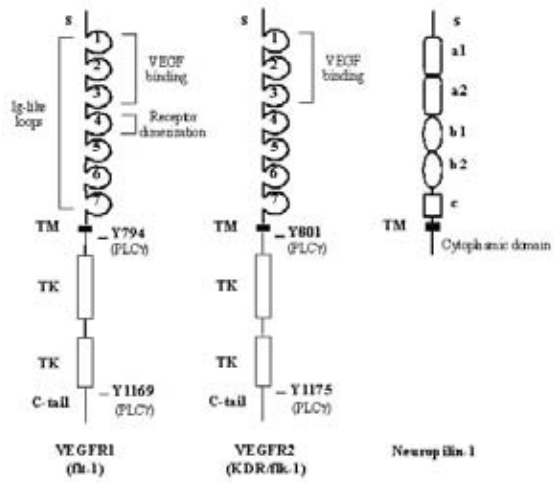

Figure 1: Molecular structure of the VEGF receptors. Diagram displaying the modular structure of VEGF receptors VEGFR1 and VEGFR2 (s), signal peptide ; (1-7) extracellular domain containing 7 immunoglobulin-like loops ; (TM) transmembrane domain ; (TK) tyrosine kinase domain.Neuropilin-1(s), signal peptide ; (a1 and a2), complement C1r/s homology domain (CUB domain) ; (b1 and b2), regions of homology to coagulation factors V and VIII, MFGPs and the DDR tyrosine kinase ; (c), MAM domain.

3. MOLECULAR BIOLOGY OF THE VEGF SYSTEM

3.1. VEGF ligands

Vascular endothelial growth factor (VEGF) has been purified from the conditioned medium of a variety of cell types including bovine folliculostellate cells (3;6), tumoral cell line AtT-20 derived from mouse anterior pituitary (5), guinea pig tumor (4), and a rat glioma cell line (7). This growth factor, also known as vasculotropin (5), was primarily described as a specific mitogen for vascular endothelial cells derived from large or small vessels, regardless of their species origin. It is angiogenic *in vivo* in the chick chorioallantoïc membrane assay (5) and the corneal pocket assay (8). VEGF is identical to the previously described vascular permeability factor (9; 4).

Molecular cloning of the cDNA showed that VEGF shares an overall homology of 18% with the B chain of platelet-derived growth factor (10; 11; 12). The human VEGF gene is organized in eight exons separated by seven introns (13). Alternative splicing of a single gene transcription product can generate multiple species. The full transcript encodes a 189 amino acids isoform (V189). A longer molecular species, (V206), which contains an additional 17 codons following the 24 codons encoded by exon 6 appears to be expressed only in embryonal tissue (14). The transcript encoding the 165 amino acids form, deleted of exon 6, is expected to generate the 45 kDa peptide following signal peptide cleavage (V165). A fourth transcript, deleted of exon 7, encodes a 145 amino acids isoform (15). A shorter transcript, deleted of exons 6 and 7 encodes a 121 amino acids isoform which compared to V165 presents a deletion of 44 amino acids between positions 116 and 159.

Over the past few years four VEGF-related genes have been identified: VEGF-B (16; 17), VEGF-C (18), VEGF-D (19) or VEGF-related protein (VRP), and placenta growth factor (20). These four VEGF-related genes generate glycosylated dimers which are secreted after cleavage of their signal peptide. They contain the eight cysteine residues that are highly conserved within the VEGF and the PDGF family.

3.2. THE VEGF RECEPTORS

3.2.1. The VEGF binding sites

Two classes of high-affinity binding sites were initially described on microvascular derived endothelial cells, with dissociation constant (Kd) values of 2-5 pM and 50-100 pM, `respectively (21; 22). Cross-linking experiments showed that the molecular mass of these cellular binding sites corresponds to 180-200 kDa. These two classes of binding sites have also been found in cells which are not of endothelial origin, namely retinal pigment endothelial cells (23), stromal cells cultured from neonatal hemangiomas (24) or hair dermal follicle cells (25). However other cells such as lens epithelial or corneal endothelial cells (26) bind VEGF on a single high affinity binding site with a Kd in the 10 pM range and a molecular mass of 120 kDa. These cells migrate or differentiate, but do not proliferate when VEGF is added. Recent reports have stated that other binding sites specific for the exon 7-containing VEGF isoforms exist on cancer cells (27; 28). The content or the structure of membrane heparansulfate proteoglycans is likely to modulate the affinity for VEGF. Although heparin increases the binding of iodinated VEGF to endothelial cells (29), it does not modulate the biological activity of VEGF (30). Ligand autoradiography studies of fetal or adult tissues depicted a specific binding of VEGF on endothelial cells regardless of their proliferation status (31; 32). The distribution of VEGF and its binding sites during cyclical growth of corpus luteum suggests that they are hormonally regulated (32; 33; 34).

3.2.2. The VEGF receptors genes

Two VEGF tyrosine kinase receptors have been identified. The fms-like-tyrosine kinase (35) Flt-1, also called VEGFR1, and the kinase domain region (36) KDR, also called VEGFR2, or its murine homologue fetal liver kinase-1, Flk-1 (37; 38). Both of these tyrosine kinase receptors bind VEGF with high affinity. Both receptors have seven immunoglobulin-like domains in the extracellular domain, a single transmembrane region and a consensus tyrosine kinase sequence that is interrupted by a kinase insert domain (figure 1). The recent identification in the rat retina of a second VEGFR2 transcript truncated of the COOH-terminal half of the intracellular kinase domain and the carboxyl region has demonstrated that these sequences are not necessary for transphosphorylation and function (39). The two promoters of VEGFR1 and VEGFR2 contain a 5' flanking sequence essential for endothelial specific expression (40; 41). The VEGFR2 promoter is sufficient to induce a 10 fold enhancement in

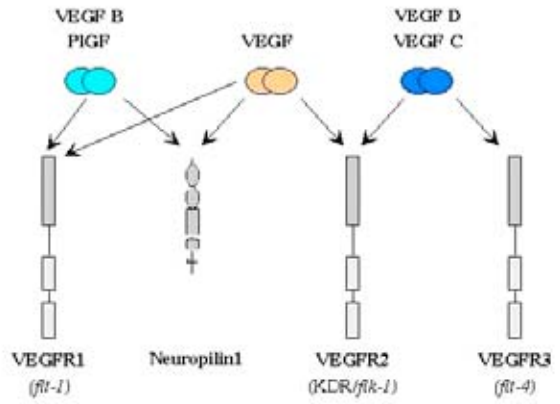

Figure 2: Interactions of the five VEGF ligands with the four VEGF receptors. Schematic representation of the distinct binding of the members of the VEGF family to the 3 tyrosine kinases receptors containing 7 immunoglobulin-like loops. Splice variants which contain basic domains (VEGF 165 aa, VEGF 189 aa, PlGF 152 aa) bind also to Neuropilin-1.

the expression of foreign genes in endothelial cells compared to fibroblasts (42).

VEGF binds to VEGFR1 with a Kd of 20 pM (35), whereas the Kd observed for PlGF1 or PlGF2 is significantly higher, in the 100-200 pM range (43). This receptor is expressed in cells which do not proliferate but migrate in response to VEGF, such as corneal endothelial cells or monocytes (44). Constitutive expression of VEGFR1 allows cells to migrate in response to VEGF or PlGF (45). Lower affinity (Kd of 100-700 pM) has been reported for the binding of VEGF to VEGFR2 in transfected cells (46). However the affinity of dimeric receptors is 100 fold higher. Down regulation of VEGFR2 achieved by VEGFR2 antisense oligonucleotide transfection reduced the overall binding of iodinated VEGF to human umbilical vein endothelial cells or stromal cells derived from neonatal hemangiomas by only 10%, but this reduction appeared to concern a high (Kd =1 pM) affinity binding site (24). VEGF-C, providing it is cleaved by proteases (47), binds and activates VEGFR2. Recently the HIV-1 transactivator Tat-1 has also been demonstrated as a VEGFR2 ligand inducing its phosphorylation (48). VEGFR2 constitutive expression allows cells to proliferate upon addition of VEGF (46).

A third member of this family of type III tyrosine kinases is represented by Flt-4, also called VEGFR3 (49; 50). Its expression is restricted in adults to lymphatic endothelial cells which suggests a role in lymphangiogenesis. It does not bind VEGF or PlGF but binds VEGF-C (47) and VEGF-D (51) in its native or cleaved forms.

Neuropilin-1, the previously identified neuronal cell guidance receptor of the semaphorin ligands (52), has been recently identified as a VEGF receptor (53). Neuropilin-1 modulates the interaction of VEGF with VEGFR2, and therefore the mitogenic activity of VEGF and it might also been involved in endothelial cell guidance (53) (figure 2).

3.2.3. Structural mapping of VEGF/VEGFRs interactions

The production of specific VEGFR2 agonists by anti-idiotypic strategy led to the hypothesis that critical VEGF determinants for each of its receptors should be distinct (54). Site-directed mutagenesis was used to localize the determinants that mediate VEGF binding to its two receptors (55). A model based on the crystal structure of PDGF-BB was used to perform alanine-scanning analysis. Although single mutations of Lys 82, Arg 84 and His 86 located in a hairpin loop displayed a moderate decrease in KDR binding, the triple mutation yielded to a strong inhibition of VEGF binding. Another study revealed that a second region (Ile 46, Ile 43) is critical for binding to the receptor and to a neutralizing monoclonal anti-VEGF antibody (56). Mapping of these determinants to the structure of the VEGF dimer shows that these two hot spots are located at each pole of the molecule. Similarly, negatively charged Asp 63, Glu 64 and Glu 67 are responsible for VEGFR1 binding. These mutants are still mitogenic whereas those which do not bind to VEGFR2 are not.

A third domain, encoded by exon 7 of VEGF is necessary and sufficient to allow the binding of VEGF to neuropilin-1 (53,57). This receptor also interacts with exon 6 of VEGF and PlGF1 (58).

The construction of various mutants of the Ig-like domains of each VEGF receptor has provided useful tools for analysis of their ligand binding domains. VEGFR1 mutants deleted of the second Ig-like domain fail to bind VEGF or PlGF, whereas the exchange of this domain with the second Ig-like domain of VEGFR2 restores VEGF binding similar to that observed in native VEGFR2, which means that this mutant no longer binds PlGF (59). However maximal VEGF binding to VEGFR1 only requires the presence of the 1-3 Ig-like domains (59) or only the domains 2 and 3 (60) whereas maximal transphosphorylation also requires the presence of the 4-7 Ig-like domains (61).

The crystal structure of VEGF has shown that its dimerization is similar to that of PDGF and symetrical binding sites for VEGFR2 are located at each pole of the homodimer (56). Each site contains two functional hot spots composed of binding determinants presented across the subunit interface. The crystal structure of VEGF complexed with the second domain of VEGFR1 shows predominant hydrophobic interactions with the poles of the VEGF dimer. Five of the seven VEGF residues critical for tight binding to VEGFR2 are buried in the interface with the domain 2 of VEGFR1 in the complex (60).

3.2.4. Signal transduction

Although pioneer experiments using endothelial cells expressing each VEGF receptor constitutively led to the conclusion that VEGFR2 mediates cell proliferation, migration and actin reorganization whereas VEGFR1 does

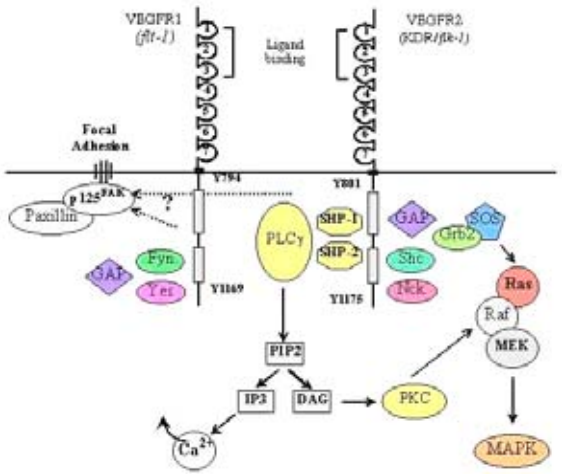

Figure 3: Signal transduction of the VEGF receptors. Schematic representation of the interactions of cellular adaptors to VEGF tyrosine kinases receptors.

not signal for VEGF (46), it is now emphasized that VEGFR1 expression in naive cells mediates cell migration (44; 62).

Although both VEGF receptors are tyrosine phosphorylated (46; 64) *in vitro*, it seems that the tyrosine phosphorylation of VEGFR1 is not required for its function *in vivo*. The deletion of the tyrosine kinase domain of VEGFR1 does not impair blood vessel formation in homozygotous mice (63). Several proteins are phosphorylated in endothelial cells (65; 66). Both receptors have the potential to signal through PLCγ (figure 3) via phosphotyrosine residues located in the juxtamembrane and carboxyl tail regions (67; 68). Although the use of the two hybrid system has demonstrated that VEGFR1 associates with PI3 kinase (69), this association has not been found in endothelial cells. Several adaptators of the Src family, such as Fyn and Yes are phosphorylated in response to VEGF/VEGFR1 but not to VEGF/VEGFR2 interactions (64). In contrast phosphorylated VEGFR2 associates with Shc, Grb2 and Nck, but also with the phosphatases SHP-1 and SHP-2 (70), activates the phosphorylation of the MAPK cascade (71) via the stimulation of Raf (72). VEGF also induces the tyrosine phosphorylation and recruitment of focal adhesion kinase and paxillin (73). Although the transduction mechanisms of the other ligands of the VEGF receptors remain unclear, it seems that tat-1 mimicks VEGF in inducing the phosphorylation of VEGFR2, MAPK and paxillin on Kaposi derived cells (74) (figure 3).

3.2.5. Regulation of gene expression

It was postulated half a century ago that retinal neovascularization was a consequence of hypoxia, and that hypoxic retina contained angiogenic factors (1; 75). Similarly several tumors with extensive vascularization, such as multiform glioblastoma, exhibit hypoxic areas in the periphery of necrotic foci. These physiopathological observations led to a search for hypoxia-regulated angiogenic factors. Low oxygen tension dramatically up-regulates VEGF expression in tumoral cell lines (76; 77). However it is not clear whether hypoxia regulates VEGF receptors. It seems that VEGFR1 is upregulated whereas the expression of VEGFR2 is not (78; 79). Hypoxia induces the expression of VEGF in human endothelial cells which in turn activates VEGFR2 phosphorylation and cell proliferation (80). The up-regulation of VEGFR2 observed in endothelial cells surrounding hypoxic tissues seems to require the release of a yet unidentified paracrine factor (81).

The up-regulation of VEGF that occurs in virtually any tumoral cell line has led to many studies aimed to decipher the mechanism of VEGF overexpression during the switch of a normal cell to a transformed one. Although both VEGF receptors are expressed in normal adult endothelial cells (82, 83), their proliferation rate is very low. This is yet to be explained. However VEGF overexpression might in turn activate the expression of VEGFR1 (79) and TNFα increase that of VEGFR2 and neuropilin 1 (84).

4. BIOLOGICAL FUNCTIONS MEDIATED BY EACH VEGF RECEPTOR

4.1. VEGF activity *in vitro*

The identification of VEGF transcripts in cultured vascular smooth muscle cells (13; 14) and the response of endothelial cells suggested that VEGF was a typical paracrine factor involved in proliferation and/or survival of the endothelium.

Although most endothelial cells do not express VEGF, the endothelial cells derived from brain and retina capillaries secrete and respond to exogenously added VEGF (85). This functional autocrine loop suggests that in neural tissues, VEGF might contribute to maintenance of the fenestration in the blood brain barrier (86). VEGF up-regulates the expression of plasminogen activator and plasminogen activator type I inhibitor (87), tissue factor (30), interstitial collagenase and promotes tube formation of endothelial cells cultured in three dimensional collagen gels (30; 88).

The pioneer observation by Dvorak's group that several tumoral cell lines increased microvessel permeability, leakage of plasma proteins and formation of a fibrin gel facilitating endothelial cell sprouting led to the discovery of the permeability action of VEGF (9). This effect is thought to constitute a crucial step in tumoral angiogenesis and wound healing. It seems to be more likely exerted through an increase of the transcellular pathway inducing the formation of vesiculo-vacuoles (89) rather than through an opening of tight junctions leading to an increase of paracellular permeability. Inhibition of MAP kinase pathway but not protein kinase C or PI3 kinase prevents the permeability action of VEGF.

Since adult endothelial cells express VEGFR1 (91, 92), it is tempting to speculate that VEGF may be involved in endothelial survival. Indeed recent reports demonstrate that VEGF delays the senescence of human dermal endothelial cells (93). A unique effect of VEGF has been observed on the deposition of a scaffolding allowing these cells to attach, proliferate and escape apoptosis (94).

When endothelial cells are coated on gelatin gels, VEGF prevents cell apoptosis induced by TNFα (95) or ionizing radiations (96).

Further studies have demonstrated that the vascular endothelial cell is not the sole target of VEGF which is a true interleukin since it stimulates the proliferation of IL2 dependent lymphocytes (97) the colony formation of granulocyte-macrophage progenitor cells (98) and inhibits the differentiation of dendritic cells (99). VEGF is also an autocrine growth factor for retinal pigment epithelial cells (23), hair dermal papilla cells (25), and stromal cells cultured from neonatal hemangiomas (24). In addition, VEGF is chemotactic for monocytes (44), lens epithelial or corneal endothelial cells (26), as well as a differentiation factor for osteoblasts (100) and 3T3 cells (101).

A recent report focused attention on the ability of VEGF to induce cell transformation. Constitutive expression of VEGF in rat retinal pigment epithelial cells leads to cell transformation; which is dramatically increased by FGF2 (102). This transformation is linked to a major overexpression of FGFR1. However neutralizing antibodies directed against VEGF or FGF2 have no effect on FGFR1 expression or cell transformation, suggesting that the target site of VEGF is intracellular.

4.2. Developmental regulation of the VEGF receptors

VEGFR1 (91) and VEGFR2 (37; 38) are selectively expressed in embryonic endothelial cells, first in the yolk sac and intraembryonic mesoderm, later in angioblasts, endocardium and in small and large vessels. The observation that VEGFR2 expression decreases at the end of gestation, whereas VEGFR1 persists in adult quiescent endothelial cells led to the hypothesis that they might be involved in different functions: VEGFR2 mediating the vasculogenic and angiogenic activities and VEGFR1 mediating vessel survival.

This hypothesis was confirmed by gene knockout experiments demonstrating that both receptors were essential for development of the vasculature. Null embryos for VEGFR1 fail to organize normal vessel channels, although endothelial cells proliferate, and die between day 8.5 and 9.5 (103). Both endothelial and hematopoietic cells fail to develop in VEGFR2 null mutant embryos which die at the same stage from distinct malformations (104). Complementation experiments have shown that VEGF is required for endothelial differentiation (105), and the analysis of null VEGFR2 embryo stem cells has shown that endothelial cells fail to proliferate and migrate from the primitive streak to the yolk sac and to intraembryonic sites of early angiogenesis (106).

4.3. Physiological activation of VEGF receptors in normal adults

The persistence of VEGF and VEGF receptors in organs where angiogenesis does not occur, such as brain choroid plexus and ventricular epithelium (107) or kidneys (82) has led to numerous hypothesis. The most widespread hypothesis speculates functions of permeability or survival. However long term treatment of mice with neutralizing antibodies reduces tumor growth but has no effect on kidney or brain ultrastructure (108). *In vivo* VEGF induces vasodilatation through an NO-dependent pathway leading to acute and severe hypotension. This effect is distinct from its angiogenic effect since VEGFR2 agonists mediate angiogenesis but not hypotension (109).

In situ hybridization demonstrates that VEGF expression is spatially and temporally expressed during the menstrual cycle in the ovaries and uterus (110). The concomitant expression of VEGF receptors suggested that the VEGF system plays a role in hormonally regulated angiogenesis. This hypothesis has been recently confirmed by the same group by the demonstration that the selective decrease of VEGF bioavailability impairs corpus luteum angiogenesis (111). Hair follicle growth also requires cyclical angiogenesis to initiate the proliferation and survival of the dermal papilla. Immunohistochemical studies have provided insight into the cyclical expression of VEGF in the hair follicle where this latter may in addition act as an autocrine factor for hair dermal papilla cells. This hypothesis is in good accordance with the report of a concomitant decrease in VEGF expression and the onset of alopecia (112).

4.4. Activation of VEGF receptors in experimental angiogenesis

Any experimental model of angiogenesis associates the local delivery of the putative angiogenic factor and an inflammation resulting from the surgical trauma necessary to insert the device (slow releasing pellet or producing cells) containing the factor. Therefore the straightforward demonstration that VEGF acts by activating a VEGF receptor and not by releasing other growth factors from the extracellular matrix raises several methodologic points. For the instance it has been shown that basic synthetic peptides corresponding to the VEGF exon 6 sequence are angiogenic through the release of FGF2 from the corneal stroma (45). Although progress has been made in this area, the large number of heparin binding growth factor receptors makes screening of the distinct functions mediated *in vivo* by each receptor a difficult task.

The expression of VEGF receptors in adult endothelial cells which do not proliferate remains puzzling and raises serious questions about the function of their translation products in quiescent endothelial cells. Although the delivery of growth factors is impaired by their inability to reach their targets as a result of being sequestered in the extracellular matrix, systemic injections of VEGF do not induce the proliferation of endothelial cells of large vessels unless they had been previously activated by a trauma such as artery ligation or angioplasty (113). Accordingly, the ability of VEGF to bind to the proteoheparansulfates of the vascular wall prevents it from reaching tumors and modulating tumoral angiogenesis (114). It seems therefore that the target of VEGF is not the quiescent endothelial cell, but rather the endothelial cells activated by inflammation. However a prerequisite to demonstrate that a growth factor activates *in vivo* a receptor

is the obtention of circulating agonists specific for this receptor. We constructed circulating agonists mimicking the distinct domains of VEGF interactions with each receptor by the use of the anti-idiotypic strategy (108).

These internal images selective for VEGFR2 induced the tyrosine phosphorylation of a set of proteins similar to the one observed upon VEGF addition, and induced cell proliferation but not migration. These VEGFR2 agonists stimulated the outgrowth of capillaries from the limbal vessels in rabbit and rat corneal pocket assays, demonstrating that VEGFR2 homodimerization was sufficient to induce angiogenesis and was not species-dependent. Corneal angiogenesis can also be mediated by the homodimerization of VEGFR1 since PlGF1 or PlGF2 alone may elicit angiogenesis (115; our unpublished results). Histology of the corneas demonstrated that VEGF led to the formation of corneal edema surrounding the pellet whereas homodimerization of VEGFR2 did not. This edema resulted probably from an increase of VEGF-driven permeability which we have shown to be mediated by the activation of VEGFR1 in the Miles assay.

These anti-idiotypic antibodies induced corneal angiogenesis in the absence of a local delivery of growth factor, suggesting that in this animal model of controlled angiogenesis, the proliferating phenotype switch of the endothelial cells is not linked to the presence of angiogenic growth factors. It is tempting to speculate that the functional expression of VEGFR2 on limbal endothelial cells is triggered by corneal cytokines released following the local trauma occuring during the graft of the pellet. Despite the fact that VEGFR2 mRNA are expressed in adult glomeruli and retinal pigment epithelial cells, no microscopic modification could be detected in kidneys or retinas thus indicating that VEGFR2 gene translation products are not functional in healthy organs. VEGFR2 appears as a functional marker of the endothelial cells which have switched to the angiogenic phenotype occuring in controlled and uncontrolled angiogenesis.

4.5. VEGF receptor activation in tumoral angiogenesis

The demonstration that VEGF mRNA is expressed in cancer cells, whereas the VEGF protein is also accumulated in endothelial cells located in the vicinity of cancer cells (116) has paved the way to numerous studies confirming the essential role of VEGF in tumoral angiogenesis (117; 118). VEGF is usually overexpressed in the hypoxic periphery of necrotic areas, whereas VEGFR1 and VEGFR2 are overexpressed in contiguous endothelial cells. In accordance with the previous finding that VEGF stimulates the proliferation of IL2-dependent lymphocytes, VEGF is also expressed in tumor infiltrating lymphocytes (99). Counting endothelial cells has been proposed as an independent factor reflecting the metastatic potential of prostate and breast cancer (119). Indeed a good correlation between vascularity and VEGF expression has been found in tumor samples, and VEGF seems to represent a useful independent prognosis marker. VEGF immunoreactivity is increased in the serum of cancer patients (120) and its decrease might indicate an efficacy of the chemotherapy used (121). However more studies are required to evaluate the potential interest of blood VEGF measure as an indicator of cancer metastasis or treatment efficacy since VEGF is present in platelets and therefore its blood content might in part result from the release of the platelets during the formation of the blood clot.

5. POTENTIAL VEGF RECEPTORS BASED THERAPEUTIC AGENTS

The genetic stability of endothelial cells constitutes an advantage for drug targeting because it is unlikely that such cells will acquire mutations and become resistant. This concept was developed in 1971 by Judah Folkman (2) and recently has been confirmed by the same group (122). Nude mice bearing tumors were treated with conventional chemotherapy. After three cycles of treatment some clones escaped and the treatment became inefficient. Conversely nude mice treated with the potent anti-angiogenic agent endostatin responded to each cycle and even ceased to relapse after a various number of cycles. Several attempts to inhibit VEGF bioavailability have proved to be successful: monoclonal neutralizing antibodies (123), antisense oligonucleotides (124), soluble VEGFR1 fusion proteins (125) or expressed by adenovirus carrying the cDNA sequence (126), anti-VEGFR2 antibodies (127) or immunotoxins targeted on VEGFR2 (Sordello, unpublished results) have been shown to inhibit tumoral progression or retinal neovascularization, as well as metastasis (128). It seems that the immunoneutralization of VEGF decreases the permeability of tumoral vessels without affecting the maintenance of the normal vasculature. Phase I and II clinical trials of humanized anti-VEGF antibodies are currently in progress. A synthetic inhibitor of VEGFR2 phosphorylation which has been reported as not affecting the tyrosine phosphorylation of other tyrosine kinases receptors (SU 5416) is also in phase II clinical trials. Although the specificity for angiogenic endothelial cells of some potential anti-angiogenic compounds remains to be demonstrated, they could act at least in part through interfering with VEGF signaling. For instance the 16K prolactin fragment inhibits VEGF-dependent MAPK activation (71) and angiostatin the VEGF-dependent tyrosine phosphorylation of paxillin (129) (figure 4).

The angiogenic activity of VEGF delivered either through protein infusion (113) or naked DNA intramuscular injection (130) seems to improve the recovery of limb or myocardial ischemia.

6. PERSPECTIVES

Experimental angiogenic assays have already ascribed an angiogenic activity to so many factors that it would be illusive to attempt to control angiogenesis by interfering with only one angiogenic pathway. However the growth factors tested in these assays act in an inflammatory context created by a surgical trauma which might overstate the actual role of a compound in pathological angiogenesis. It has been proved that several so-called angiogenic factors act by releasing endogenous angiogenic factors from macrophages or from the extracellular matrix. These assays

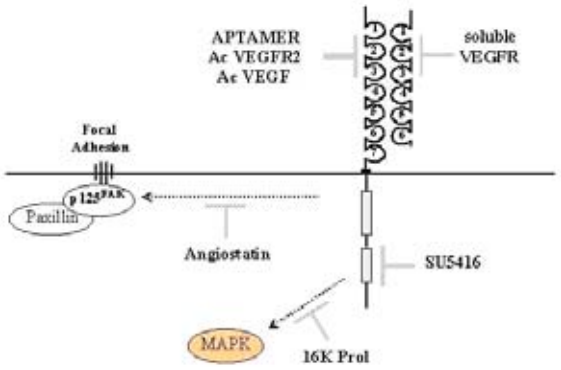

Figure 4: VEGF receptors based potential therapeutic. agents. Representation of the molecular targets of potential anti-angiogenic agents interfering with VEGF signal transduction.

therefore constitute a better reflection of controlled angiogenesis, as observed in wound healing or cyclical growth of the ovary, than the uncontrolled pathological angiogenesis observed in tumoral progression or diabetic retinopathy. Meanwhile the discovery of VEGF led to major insights in the comprehension of development of the vascular system as well as its role in physiological and pathological angiogenesis. Further studies using inducible gene knockouts of one or all the VEGF isoforms, and then tempting to recover the phenotype by activating VEGFRs signal transduction by injection of circulating receptors agonists such as anti-idiotypic antibodies should help to determine where and when the VEGF system is critical. The elucidation of the signal transduction properties of the tyrosine kinases VEGFR1, VEGFR2, VEGFR3 and their modulation by the recently identified neuropilin should also help to understand the mechanism of VEGFR2 silencing in quiescent endothelial cells or VEGFR2 transduction in angiogenic endothelial cells. Clinical trials of VEGF inhibition in cancers and VEGF infusion in myocardial ischaemia should also delineate the beneficial and adverse effects of each treatment.

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