VASCULAR ENDOTHELIAL GROWTH FACTOR IN HEALTH AND DISEASE: A REVIEW

Niranjan Gopal[1], Srinivasan A. R[2], Prakash H. Muddegowda[3], Sathish Babu Murugaiyan[4], Srikanth K[5], Ramesh Ramaswamy[6], Anitha Rajendiran[7]

[1]Associate Professor, Department of Biochemistry, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed University Accredited with 'A' Grade by NAAC), Pillaiyarkuppam, Puducherry.
[2]Professor, Department of Biochemistry, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed University Accredited with 'A' Grade by NAAC), Pillaiyarkuppam, Puducherry.
[3]Professor, Department of Transfusion Medicine, Vinayaka Mission's Kirupananda Variyar Medical College and Hospitals, Salem, Tamilnadu, India.
[4]Associate Professor, Department of Biochemistry, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed University Accredited with 'A' Grade by NAAC), Pillaiyarkuppam, Puducherry.
[5]Professor and HOD, Department of Ophthalmology, Mahatma Gandhi Medical College & Research Institute (MGMC & RI), Sri Balaji Vidyapeeth (Deemed University accredited with 'A' grade by NAAC), Pillaiyarkuppam, Puducherry.
[6]Professor HOD, Department of Biochemistry, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed University Accredited with 'A' Grade by NAAC), Pillaiyarkuppam, Puducherry.
[7]Final Year Postgraduate, Department of Biochemistry, Mahatma Gandhi Medical College and Research Institute, Sri Balaji Vidyapeeth (Deemed University Accredited with 'A' Grade by NAAC), Pillaiyarkuppam, Puducherry.

ABSTRACT

BACKGROUND

Vascular Endothelial Growth Factor (VEGF) has been implicated as a key molecule, which regulates physiological angiogenesis. VEGF exerts its molecular actions through Receptor Tyrosine Kinases (RTKs) VEGFR-1 and VEGFR-2, differ in signaling properties. VEGF plays a vital role in embryogenesis, growth and reproduction. VEGF as a mediator of pathological angiogenesis is associated with proliferation and micrometastasis of various tumours, ocular disorders involving neovascularisation, pre-eclampsia, etc. The pathogenesis of micro and macrovascular complications of diabetes mellitus and the role of VEGF is being studied extensively. Currently, several pharmacological interventions based on VEGF inhibitors and receptor antagonists have been tried to combat the pathological angiogenesis in a wide gamut of disorders. This review attempts to put together important properties, mechanism of action and the role of VEGF in common diseased states.

CONCLUSION

Effects of VEGF are widespread and have been implicated in several disease states. Therapeutic modalities targeting VEGF have been tried with success in recent years.

KEYWORDS

Neovascularisation, VEGF, Diabetes Mellitus, Pre-Eclampsia, Neoplasia.

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INTRODUCTION: The role of vasculature is cardinal to the comprehension of a wide variety of physiological, biochemical and pathological events.[1] This is mainly attributed to the fact that the blood vessels function as a conduit of nutrients and a portal for vital parameters besides affording a suitable exit platform for waste and toxic substances, both endogenous and exogenous.[2] Yet another important physiological attribute is the role of vasculature in immune surveillance, which warrants our immediate attention.[3]

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Corresponding Author:
Dr. Srinivasan A. R,
Professor, Department of Biochemistry, Mahatma Gandhi Medical College and Research Institute (MGMC and RI), Sri Balaji Vidyapeeth (Deemed University Accredited with 'A' Grade by NACC), Pillaiyarkuppam, Puducherry-607403, India.
E-mail: raghavan.drvars2000@gmil.com
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a) Endothelium-It's role and implications: The endothelium assumes an important role. It is nothing, but a simple squamous epithelium that carefully lines the innermost layer of the vessels as in cardiovascular and lymphatic systems.[4] A major anatomical consideration is that it is continuous with the endocardial lining of the heart.[5] The role of endothelium and its participation in the physiological and pathological processes are variegated.[6] Among them are mechanical factors that influence blood flow, transport of macromolecules and blood components from the interstitium to the lumen of the vessel.[7] The contractile state of the overlying smooth muscle and capillary permeability also deserve mention. By virtue of possessing smooth luminal surface, the endothelium allows efficient blood flow.[8] Differences in the architecture of the endothelium arise due to the distinctive features associated with venous endothelia and differences between larger and smaller blood vessels.[9]

b) Embryological Considerations: Endothelial cells originate from the hemangioblast, a mesoderm derived progenitor cell that gives rise to haematopoietic stem cells and to angioblasts.[10] The angioblasts are the precursors of endothelial cells.[11] The hemangioblasts swell and yield clusters or blood islands. The primordial clusters depict the earliest distinction between the outer endothelial cells and the inner haematopoietic cells; the outer cells give rise to endothelium, while the inner cells differentiate into haematopoietic cells.[12]

Organs, viz. kidney and liver possess circular windows in the endothelial cell body and these fenestrations allow transport of water and small hydrophilic molecules. The blood-brain barrier is unique and significant and is made up of a non-fenestrated endothelium.[13] The tight junctions that are in place culminate in impermeable connections between endothelial cells, thereby separating the cerebral capillaries from the brain, referred to as Blood-Brain Barrier.

The development of blood vessels: The terms vasculogenesis and angiogenesis should not be confused owing to the fact that they are two temporally distinct processes, which influence blood vessel development.[14] During the committed process of vasculogenesis, an intrinsic network of blood vessels is created de novo based on the specification of embryonic endothelial cells, which then undergo proliferation-guided migration and systematic coalescence prior to forming the lumen. During angiogenesis, the primary vascular plexus is remodeled into a branched network of large and small vessels (arteries and veins). The Notch family determines the fate of choice between arterial and venous endothelial cells and the choice is made following angioblast specification. Whereas, the lymphatic endothelium is essentially composed of a unilayer of endothelial cells that lines the lymph vessels and originates from the venous endothelial cells. The lymphatic endothelium is pronouncedly more permeable than the vascular endothelium.

c) Why are endothelial cells cardinal? Since the endothelial cells line the inner surface of vessels, they support tissue growth and repair. The network provides nourishment to all the tissues.[15] Hence, as a corollary, structural or functional vessel aberrations are implicated in several diseases.[16,17] Inadequate and less than physiological maintenance/growth causes decreased supply of oxygen (ischaemia) as manifest in clinical conditions including Acute Myocardial Infarction (AMI), cerebrovascular stroke and neurodegenerative disorders.[17] On the contrary, excessive vascular growth or over exuberant remodelling promotes processes that include inflammation and carcinogenesis. It must also be remembered that vessels provide the favoured routes for tumour cells to spread to other tissues.[18] During embryonic life, new vessels form de novo through the congregation of mesoderm-derived endothelial precursors (angioblasts) as described before that differentiates into a primitive vascular labyrinth (vasculogenesis). Subsequent vessel sprouting (angiogenesis) creates a network that eventually remodels into arteries and veins. The pericytes and vascular smooth muscle cells that enwrap nascent endothelial cell tubules provide stability and controls perfusion (arteriogenesis). The vessels are quiescent in the adults and the endothelial cells are endowed with plasticity to sense and respond to angiogenic signals.[19] Studies carried out during the last five years have provided newer insights into several aspects of angiogenesis.[20] A probable mechanism is one where endothelial cells exhibit motility attracted by proangiogenic signals.[21] The motile cells with protruding filopodia spearhead new sprouts. Following tip cells, stalk cells extend fewer filopodia, but promulgate a lumen and proliferate to support sprout elongation. Tip cells anastomose with the neighbourhood to build vessel loops. The advent of blood flow, formulation of a basement membrane and the recruitment of mural cells stabilise new connections. The sprouting process occurs repeatedly until proangiogenic signals abate and quiescence is re-established. Other mechanisms by which vessels grow have also been postulated.[22]

d) The pro and anti-angiogenic responses-Role of VEGF: The significance of angiogenesis ushered in hopes as related to the manipulation of this process. This could in effect afford pharmacological approaches pertaining to chemotherapeutic modalities. However, such endeavours are hampered by certain limitations. Putative strategies, for instance, proangiogenic cell therapies enable new modalities, but require further concrete evidence based on research ultimately augmenting the existing armamentarium.

Vascular Endothelial Growth Factor (VEGF) is one such candidate that deserves mention. Many anti-angiogenic approaches are essentially focussed on vessel growth in eye diseases and cancer. Presently, certain therapeutics targeting Vascular Endothelial Growth Factor (VEGF) are available, though instances have been observed, wherein tumours evolve mechanisms of resistance or are absolutely refractory toward VEGF (receptor) inhibitors. In view of the mixed response as told above, it is a matter of conjecture as to whether anti-angiogenic treatment may trigger more invasive and metastatic tumours ending in a paradox.[23-28]

e) The VEGF-NOTCH pathway considerations: Several multicellular organisms possess a highly conserved cell signaling system, namely the Notch signaling pathway.[29] Mammals exhibit different Notch-Receptors, referred to as NOTCH1, NOTCH2,

NOTCH3 and NOTCH4.[30] Structurally, the Notch receptor is a single-pass transmembrane receptor protein. Single-pass membrane protein essentially refers to such of those proteins that cross the membrane.[31] The receptors are composed of a large extracellular portion, which associates in a calcium-dependent, non-covalent interaction with a smaller piece of the Notch protein composed of a short extracellular region, a single transmembrane-pass and a small intracellular region.[32-34] VEGF and Notch cooperate in an integrated intercellular feedback that functions as a "branching pattern generator."[35] VEGF stimulates tip cell induction and filopodia formation through VEGF receptor-2 (VEGFR2), whereas VEGFR2 blockade causes sprouting defects with blunt-ending channels. VEGFR3 is expressed in the embryonic vasculature, but later becomes confined to lymphatics.[36] However, tip cells re-express VEGFR3 and its pharmacological inhibition diminishes sprouting. Loss of VEGFR1 increases sprouting and vascularisation.[37] A soluble variant or a kinase-dead mutant of VEGFR1 rescues vascular defects caused by VEGFR1 deficiency suggesting that this receptor functions as a VEGF trap. VEGFR1 is predominantly expressed in stalk cells and involved in guidance and limiting tip cell formation.[36-38]

The feedback loop between VEGF and Notch involves regulation of all VEGFRs by Notch. VEGF/VEGFR2 enhances DLL4 expression in tip cells. DLL4-mediated activation of Notch in neighbouring ECs inhibits tip cell behaviour in these cells by down regulating VEGFR2, VEGFR3 and NRP1 while up regulating VEGFR1. ECs at the angiogenic front dynamically compete for the tip position through DLL4/Notch signalling.[38] Following VEGF exposure, all cells up regulate DLL4. However, ECs that express DLL4 more hurriedly or at higher levels have an advantage to become a tip cell as they activate Notch signaling in neighbouring cells more effectively. Precise regulation of DLL4 expression is achieved through a TEL/CtBP repressor complex at the DLL4 promoter,[39] which is transiently disassembled upon VEGF stimulation, allowing a formation, whereas Rac1 regulates lamellipodia formation.

f) Role of VEGF in Lumen Formation: Studies on intersomitic vessels indicate that ECs form a lumen by facilitating coalescence of intracellular vacuoles.[40] This process of pinocytosis and subsequent nexus with vacuoles from neighbouring endothelial cells are significant. Endothelial cells are endowed with the capacity to adjust their shape and facilitate their junctions to open up a lumen. The apical (luminal) membranes are impregnated with sialic acid rich glycoproteins. By virtue of the repulsive signal, the lumen gets opened.[41] Subsequent changes in the morphology of endothelial cells are mediated by VEGF and Rho-Associated Protein Kinase (ROCK), synonymous with the expansion of the lumen. Other proteins are also required for this process. The mechanisms of lumen formation are based on the type of vessel formation.[42]

g) Resting Endothelial cells and VEGF: Quiescent endothelial cells form a barrier between the blood and surrounding tissues. This helps in regulation of exchange of fluids and solutes and transmigration of immune cells. A variety of transmembrane proteins is implicated. Instances include iVE-cadherin, N-cadherin, occludins, etc. The Junctional Adhesion Molecule (JAM) localised at tight junctions also plays a role. Function wise, the adherens and tight junction molecules are different. The tight junctions are involved in the maintenance and regulation of paracellular permeability, whereas adherens junction molecules mediate several important events including cell-cell adhesion and intracellular signaling. Teaming up with VEGFR2, VE-cadherin maintains the quiescence of endothelial cells through phosphatases that act on VEGFR2. This covalent modification restrains VEGF signaling. VE-cadherin-based adherens junctions establish endothelial cell adhesion or facilitate its separation and movement.[43] Activation of TIE-2 by ANG1 protects vessels from VEGF-induced leakage by inhibiting VEGFs ability to induce endocytosis of VE-cadherin. It must be noted that angiopoietin (ANG) 1 is a ligand for endothelium-specific receptor tyrosine kinase TIE-2. Signaling by TIE-2 and ANG1 also controls survival and vessel quiescence. ANG1 clusters TIE-2 junctionally at interendothelial junctions, thereby promoting survival and endothelial quiescence.[44]

h) Role of metabolic sensors and regulators in vascular growth: In actively metabolising tissues, the uptake of nutrients is linked to energy demand in order to confer homeostasis. High levels of VEGF-B, a VEGF member with poor angiogenic activity are found in metabolically active tissues, where it is coexpressed with genes including VEGF, stimulating mitochondrial biogenesis and controls transendothelial uptake of fatty acids into other tissues. VEGF-B prepares tissues for fatty acid consumption.[39]

An increase in cellular levels of AMP induces VEGF-driven angiogenesis through activation of AMPK. Vascular growth is regulated by LKB1, an activating kinase of AMPK and regulator of metabolism.[45] The vascular-metabolic interface is further regulated by FoxO transcription factors, which are upregulated during fasting and restrict angiogenic behaviour. FOXO1 and Notch1 are controlled by SIRT1, a deacetylase activated by NAD^+ under conditions of energy distress and nutrient deprivation.[39]

i) **Biomedical implications:** Despite progress in understanding the molecular basis of angiogenesis and successful translation of VEGF blockade for the treatment of age-related macular degeneration and certain types of cancers, challenges must be overcome to improve the overall efficacy of antivascular strategies to combat cancer more efficiently.

I. **VEGF and vascular complications of T2DM:** Vascular Endothelial Growth Factors (VEGFs) are the group of protective cytokines, which promote cell survival and proliferation even under the extreme stress. Various factors such as hypoxia, hypertension, free radicals, advanced glycosylation end products, Insulin-like Growth Factor-1 (IGF-I), angiotensin II and a few other hormones are known to induce the VEGF genes. However, hyperglycaemia in T2DM is the most potent stimulus for opening up of VEGF genes.[46] Poor glycaemic control is regarded as an important metabolic factor responsible for the various micro and macrovascular complications including retinopathy, maculopathy, neuropathy and nephropathy in T2DM.[47] Studies on different populations are presently available implicating VEGF gene polymorphisms and their association with the severity of microvascular complications of T2DM.[48,49]

VEGF165, a prominent member of the VEGF-A family has two isoforms, namely VEGF165a and 165b. VEGF165a has potent angiogenic properties while VEGF165b isoform has anti-angiogenic properties and hence it is protective and healthy isoforms. High level of Insulin-like Growth Factor (IGF) in T2DM is known to down regulate expression of VEGF 165b isoforms, thereby promoting angiogenesis.[50]

a) **VEGFs and Retinopathy:** Retinal neovascularisation is the pathognomonic feature of all the ischaemic retinal diseases namely diabetic retinopathy, retinal vein occlusion and retinopathy of prematurity, etc. It could lead to vitreous haemorrhage, retinal detachment and/or glaucoma if not detected at the early stages. VEGF-A plays an important role in promoting angiogenesis and causing vascular permeability. Among five VEGF molecules and three VEGF receptors known, VEGF-A and VEGF-D with receptors Flt1, Kdr and Flt4 are considered important.[51] Hence, anti-VEGF agents are beneficial in the treatment of vascular leakage and macular oedema due to retinopathy.[52] Reactive oxygen species generated from hyperglycaemia are also involved in the pathogenesis of retinal vascular complications, which promotes ligand-independent phosphorylation of vascular endothelial growth factor receptor 2. Hence, supplementation of antioxidants could be beneficial in T2DM cases in preventing the vascular complications.

b) **VEGFs and Nephropathy:** Diabetic nephropathy is the leading cause of End-Stage Renal Disease (ESRD). VEGFs are implicated in proteinuria and mesangial proliferation, the early features of glomerular pathology observed in several renal diseases.[53] VEGFs are the independent predictors of ESRD in diabetics over systolic blood pressure and albuminuria.[54] Few studies referring to polymorphism of VEGF genes and their association with the severity of nephropathy exists. Polymorphism-460[55] and -2549 in the western world[56] and +405 in Japanese population are notable.[57]

c) **Diabetic neuropathy and VEGFs:** In contrast to retinopathy, reduced VEGF levels accelerate the degenerative process of neurons. VEGFs also have been implicated in various neurological disorders such as diabetic and ischaemic neuropathy, nerve regeneration, Parkinsonism, Alzheimer's disease and multiple sclerosis. These findings have recently provoked interest in studying the therapeutic potential of VEGFs for the various neurodegenerative disorders.[58]

Hyperglycaemia-induced changes in endoneurial metabolism assume a key role in the pathogenesis of diabetic neuropathy. In addition, flux of excess glucose into polyol pathway, advanced glycation end products, lipid peroxides, hyperactivity of protein kinase C, defective essential fatty acid metabolism culminate in endothelial and neural cell damage, especially the Schwann cells. In addition, a toxic milieu is created in the nerve cells due to defective neurotropic factors, accumulation of sorbitol and reduced Na+/K+ATPase activity, microvascular damage and hypoxia due to nitric oxide deficit and increased oxygen free radical activity are all implicated in the pathogenesis of neuropathy in diabetes mellitus. Also, polymorphism of the VEGF gene at position -7*C/T, recently has been documented in diabetic neuropathy.[59]

II. **VEGF and Angiogenesis in Skin Disease:** Recent studies have shown VEGF as one of the most potent proangiogenic factor in the skin particularly during the process of wound healing. Injured skin, macrophages, fibroblasts and mast cells express VEGF probably secondary to hypoxia. VEGF is also believed to repair the epidermal barrier during wound healing by stimulating keratinocytes.[60] Abnormal or excessive angiogenesis in skin culminate in psoriasis, warts, allergic dermatitis, scar keloids, pyogenic granuloma, blistering disease, Kaposi sarcoma in HIV patients, etc. In case of insufficient angiogenesis, it can cause hair loss, skin

purpura, telangiectasia and venous lake formation.[60]

VEGF in therapy use for wound healing is beneficial for wound closure. However, the same can lead to abnormal scarring. More evidence based research on mass lines is deemed necessary.[60]

III. **VEGF and Angiogenesis in Neoplasia:** In any solid tumour, we observe cancer cells and its supporting stroma. New blood vessels are the components of the stroma, which through the neovascular fenestrated endothelium allows hyperpermeability. There is a general saying "tumours are wounds that do not heal" as more angiogenesis is seen. The 'angiogenic switch' is responsible for tumour shifting from its avascular growth phase to the vascular growth phase, a key change through angiogenesis and circumvents the limitations of oxygen and nutrient exchange. Further, the tumour can spread to distant organs (micrometastasis).[61] Also, VEGF is known to upregulate expression of anti-apoptotic protein BCL-2 and Survivin protein allowing enhanced endothelial survival. It is known to affect dendritic cells impairing host antitumour immune response thereby promoting generation of autoimmune tumour cells. VEGF-A level is suggested to be a useful marker of tumour status and prognosis.[62]

IV. **VEGF and Pregnancy:** Pregnancy is a physiologically relevant metabolic state where there are several developmental changes right from the date of fertilisation of ovum, implantation of embryo.[63] Placenta is a highly vascular tissue, it acts as a barrier for materno-foetal junction.[64] Poorly formed placenta is seen in different diseases.[65] Placental angiogenesis is one of the most important adaptations observed during pregnancy, which plays a critical role for ensuring adequate blood flow.[66] Vasodilation is an important phenomenon in the blood vessels, which is mediated by VEGF and other peptides derived from endothelium.[67] Imbalance in these molecules are considered harmful for both maternal and foetal survival.[68] There are two types of angiogenesis (branching and non-branching). In normal pregnancy branching, angiogenesis occurs predominantly in the first and second trimester, while non-branching angiogenesis is facilitated during the third trimester.[69]

a) **Factors influencing the Placental Angiogenesis:** Physiological hypoxia: It is an important stimulator of placenta development and angiogenesis. Low oxygen stimulates the production and release of a variety of transcription factors, which enhance the production of growth factors such as VEGFs, Fibroblast growth factor-2, etc.,[70,71]

b) **Pre-eclampsia and VEGF:** Pre-eclampsia affects multiple organs and remains to be one of the leading causes for maternal mortality and poor foetal outcome worldwide.[72] Amongst numerous causes of pre-eclampsia, one of the most important causes is oxidative stress, which affects the release of the angiogenic growth factors.[72]

c) **Oxidative stress and VEGF:** Oxidative stress is a state of imbalance in pro-oxidant and antioxidant balance of a system. Oxidative stress mediated endothelial dysfunction is noted in several common disease states, such as metabolic syndrome, diabetes mellitus and coronary artery disease, etc., Synergistically hypoxia and oxidative stress cause impairment of trophoblastic invasion during pregnancy leading to increase in placental vascular resistance.[73] Several predisposing conditions such as obesity, previous history of pre-eclampsia, gestational diabetes mellitus, etc., could interfere with the placental angiogenesis. Vascular resistance of umbilical vessels, which can be easily detected by Doppler study can be an early marker. Early appearance of angiogenic factor such as VEGF, sFLT-1 and PlGF-3 is seen in various placental vascular diseases.[73] Levels of fms-like tyrosine kinase receptor (VEGF receptor 1) in pre-eclampsia are low.[73] These are the potential markers to predict pre-eclampsia.

Studies on placenta have shown that abnormality in the formation of placental vessels is linked to alterations in VEGF and its receptor up regulation. Several transcription factors of oxidative stress like Nrf2, NfKB and Ets-1 have been linked to VEGF.[74] Upregulation of sFlt-1 and unbalanced PlGF/VEGF production associated with increased oxidative stress are consequences of hypoxia as observed in placental trophoblast cells, which induce these growth factors [75]. Hypoxia-induced increase in soluble Flt-1 production correlates with enhanced oxidative stress in trophoblast cells from the human placenta.[75]

d) **Inflammation and VEGF:** Redman et al proposed an association between systemic inflammation and pre-eclampsia.[76] But, in recent times the 'excessive inflammation' and the 'angiogenic imbalance' theories have been highlighted as the primary causes for pre-eclampsia. It is the imbalance in angiogenic factors that cause pre-eclampsia; in particular, the increase in soluble fms-like tyrosine kinase receptor-1 (sFlt-1) and soluble endoglin (sEng) and the decrease in PlGF (placenta growth factor).[77] Maternal circulatory sFlt-1 is elevated 5-10 weeks and sEng as early as 11–13 weeks of gestation prior to the development of pre-eclampsia.

Decrease in urinary PlGF also precedes the onset of pre-eclampsia. Elevated levels of inflammatory cytokines are seen associated with endothelial dysfunction, increased placental apoptosis, decreased angiogenesis and renal impairment.[77]

Greer et al have shown that neutrophil activation is confined to the maternal circulation in pre-eclampsia,[78] but certain studies have also proved that the levels of inflammatory parameters including TNFα, IL-6 and IFNγ were not elevated in women who later developed pre-eclampsia compared with healthy controls. The lack of a temporal relationship between the inflammatory cytokines and the maternal syndrome of pre-eclampsia raises the ambiguity of pre-eclampsia is caused by 'excessive inflammation.' A recent study has demonstrated that sFlt-1 acts synergistically with pro-inflammatory mediators to activate endothelial cells compared with endothelial cells treated with TNFα alone. Treatment with sFlt-1 or VEGF-neutralising antibodies or blockade of VEGFR-1 and VEGFR-2 reduced the phosphorylation of Akt and Endothelial Nitric Oxide Synthase (eNOS) leading to reduced Nitric Oxide (NO) bioavailability and increased endothelial dysfunction.[79] Furthermore, a study demonstrated that adenoviral overexpression of sFlt-1 in endothelial cells resulted in a pronounced inhibition of eNOS phosphorylation and NO production as well as a decrease in VEGFR-2 phosphorylation in pre-eclamptic placenta.[78,79]

In normal gestation, recognition of foetal HLA-C by receptor inhibitory killer cell immunoglobulin-like receptors of uterine natural killer cells triggers the release of cytokines by uNK cells. These include transforming growth factor-beta (TGF-β), placenta growth factor (PIGF) and VEGF, whose participation in immune regulation and angiogenesis have been well established. Conversely in pre-eclampsia, when KIR-AA of maternal uNK cells recognise the HLA-C of the extravillous trophoblast, uNK cells display a poorer expression of these mediators as well as an overexpression of anti-angiogenic factors like sEng and sFLT1 kinase-1 factor.[80]

sEng inhibits TGF-β1 from binding to the surface of its receptors and diminishes nitric oxide-mediated endothelial signaling. SFLT1 binds to angiogenic proteins VEGF and PIGF and blocks their actions.[29] Interestingly, significantly lower PIGF levels, but with higher sFLT1 and sEng concentrations have been demonstrated before 30 weeks of gestation in the serum or plasma of pregnant women who have developed pre-eclampsia if compared with pregnant women who have not developed this disease. Therefore, they can be used as predictor markers of pre-eclampsia.[79-81]

Studies document that not only angiogenesis, but also an impairment in lymphangiogenesis in the decidua, not in the placenta are associated with pre-eclampsia. There are evidences for relating development of hypertension and cardiovascular disease in the offsprings who had pre-eclampsia during pregnancy. Study by Escudero C et al has documented that is mediated by adenosine.[80,81] Galectin family has been shown to exert several roles in the context of reproduction and is involved in embryogenesis and angiogenesis.[82] Subakir BS et al have shown that beta-hCG enhance VEGF activity to induce angiogenesis.[83]

V. The Current status and strategies of anti-VEGF Therapy: Various modalities to block the VEGF signal transduction are under study, which include development of neutralising anti-VEGF monoclonal antibodies, receptor antagonists, soluble receptors, antagonistic VEGF mutants, inhibitors of VEGF receptor function, etc. These agents can be divided in two broad classes, namely agents designed to target the VEGF activity and the surface receptor function.[84]

a) Systemic anti-VEGF therapy: Systemic therapy with oral fenofibrates, PKC inhibitors, Peroxisome Proliferator-Activated Receptors (PPAR) agonists, Forskolin (which binds GLUT1 receptor), minocycline (for its anti-inflammatory effect) and celecoxib (a Cox-2 inhibitor) have been shown to pause the angiogenesis through their anti-VEGF effects.[85] The glucose-induced expression of VEGFs can be successfully inhibited by the various Protein Kinase C (PKC) inhibitors namely ruboxistaurin, Forskolin, 2-amino-3-carboxy-4-phenylthiophene, etc., several more are in the pipeline and waiting for USA-FDA approval.[86] The Action to Control Cardiovascular Risk in Diabetes Mellitus (ACCORD) trial documented that oral fenofibrate therapy (Originally, a hypolipidemic agent), significantly helped in delaying the onset of Diabetic Retinopathy (DR) through its effect on the VEGF pathway.[87] It also has antioxidant[88] and anti-inflammatory properties.[89] In addition, it also regulates the survival of retinal endothelial cells.[90] Lin CC et al recently have demonstrated that Lysophosphatidic Acid (LPA) induces VEGF-C gene expression by promoting the generation of reactive oxygen species. NADPH oxidase inhibitors like diphenyleneiodonium was used to block LPA-induced ROS production. Some siRNAs are also known to inhibit PKC and phospholipase C.[91]

b) Anti-VEGF Agents for Intravitreal Injection: LASER photocoagulation still remains the preferred modality for treating DR. But, it is destructive and can be used only for already established cases of DR.[92] Intravitreal injections of several anti-VEGF agents including steroids have been tested and many are now proved successful. VEGF-inhibition is also being studied as a strategy for the prevention of angiogenesis, vascular leakage and visual loss in macular degeneration due to many causes including T2DM.[93] These anti-VEGF injections for local use have been tried in many conditions with success. Although, the dose of anti-VEGF agents used for

treating eye diseases is small compared with that used intravenously, it could lead to systemic absorption and reduce serum VEGF levels too. Several systemic side effects such as hypertension and cardiovascular complications have been reported. Renal complications of intravenous administration of anti-VEGF are noticed and include a variety of renal pathological damage, which can induce proteinuria and hypertension.

c) **Antibodies against VEGFs:** Intravitreal injections of anti-VEGF-A monoclonal antibodies such as bevacizumab (Avastin; Genentech, Vacaville, CA) and pegaptanib sodium (Macugen; OSI Pharmaceuticals, Melville, NY) were successfully used to treat persistent proliferative retinopathy and optic neuropathy. Aptamer (pegaptanib) used in treatment of neovascular age-related macular degeneration is in phase 2 clinical trials.[94] Anti-VEGF antibodies in experimental models significantly reduce hyperfiltration, albuminuria and glomerular hypertrophy, which are mostly due to the VEGFs.[95] However, authors suggest that normalising the glycaemic status and maintaining it is the gold standard anti-VEGF therapy.

d) **Antioxidants as Anti-VEGF Agents:** Warren CM et al recently have demonstrated that the reduced cell surface abundance of VEGFR2 in diabetic mice was reversed by treatment with the antioxidant N-acetyl-L-cysteine. This suggests the role of higher degree of oxidative stress in T2DM, which causes elevation of VEGFs.[96]

e) **Novel Anti-VEGF Therapy for Neuropathies:** Local VEGF application also protected against paclitaxel and diabetes-induced neuropathies causing little side effects. A small synthetic VEGF mimicking pentadecapeptide (QK) exerted similar effects on cell cultures: the peptide reduced ATF3 expression in vitro and ex vivo in paclitaxel and hyperglycaemia-induced models of neuropathy to a similar extent as the full-length recombinant VEGF protein. Overall, these studies underscore the potential of VEGF and VEGF-derived peptides for the treatment of peripheral neuropathies.[97,98] Meticulous glycaemic control could delay the onset or slow down the progression of diabetic neuropathy in patients with T2DM, but it does not completely prevent the progression of the disease, a matter of great concern.

f) **Anti-tumour anti-VEGF Therapy:** Due to the vast effect on angiogenesis, therapeutic applications are underway like VEGF-A neutralising antibodies, VEGF tyrosine receptor kinase inhibition, toxins inhibiting stimulation effect of VEGF-A, DNA vaccine, rapamycin, combination therapy, etc.[61,62] However, mare focussed and evidence based research has to be promulgated in objectivising anti-VEGF agents as the viable modality to manage various pathological events involving neovascularisation.

VI. Quantification of VEGF Levels, its Receptors and Expression of Gene: VEGF is measured in supernatants and lysates of different cell types and in tumour tissue samples. Serum VEGF levels reflect its levels in different systems. ELISA is commonly used to detect human and rat VEGF up to a minimum detection limit of 100 pg/mL with a little cross-reactivity with related proteins. Its soluble receptors VEGFR-2 and VEGFR-3 won't restrict the sensitivity of the assay. Cell lines secrete VEGF in very low amounts (<1 ng/mL) whereas VEGF-C transfected cells can secrete up to 50 ng/mL VEGF-C into the supernatant.[99] Fabrication and utilisation of capture-antibody immobilised macroporous poly (ethylene) glycol diacrylate hydrogel microspheres for quantitative and reproducible measurement of VEGF has been successful in improving the assay sensitivity and specificity. Induction of porosity using PEG porogen improves the sensitivity of this simple hydrogel microsphere based system with a detection limit of 2.5 pg/mL.[100] A specific indirect ELISA for the quantification of VEGFR-3 in different human cell and tissue lysates has been published recently.[101]

A combination of the goat polyclonal anti-VEGFR-3 antibody and the mouse monoclonal anti-human VEGFR-3 antibody was used. The assay was highly sensitive and reproducible with a detection range of 0.2-25 ng/mL.[101] The assay was specific for VEGFR-3 with no cross-reactivity to VEGFR-1 or VEGFR-2. Complex formation with VEGF-C and VEGF-D had no effect on the sensitivity of the assay. The VEGFR-3 concentration in the lysates of cultured human dermal microvascular endothelial cells was 14-fold higher than in the lysates from human umbilical vein endothelial cells. In human kidney, breast, colon, gastric and lung cancer tissues the protein levels of VEGFR-3 were in the range of 0.6-16.7 ng/mg protein. Importantly, the level of VEGFR-3 protein detected in the ELISA correlated significantly with the number of VEGFR-3 positive vessels observed in histochemical sections suggesting that the ELISA assay maybe a reliable surrogate of measuring VEGFR-3 positive vessel density.[101] VEGF gene expression: Quantified Competitive RT-PCR (QC RT-PCR) and real-time quantitative reverse transcription-PCR (RTQ RT-PCR) are used to quantify the VEGF mRNA expression. RTQ RT-PCR is found to be more accurate and sensitive than QC RT-PCR.[102] Recent studies showed that VEGF gene -460 C/T and +936 C/T polymorphisms are found to be suitable genetic markers of oral malignancy in the Indian population.[103,104]

CONCLUSIONS: VEGF has widespread actions and hence has been directly implicated in the pathogenesis of many

disease states, viz. pre-eclampsia, complications of T2DM and even many tumours. Therapeutic modalities targeting VEGF have been tried with success and found beneficial in many such conditions. Further research on the expression of VEGF gene and its polymorphism could be studied in detail that would enhance the objectivity and reliability of pharmacological interventions.

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