Review of Literature
Most of the growth factors are synthesized during development of an individual in response to a signal. Some of these factors include fibroblast growth factor (FGF), transforming growth factor (TGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) etc. Each of these factors has a defined function. For example, EGF is involved in wound healing. TGFs are involved in development, epithelial cell growth, and immune regulation and FGFs are involved in endothelial cell proliferation and migration. Similarly a number of angiogenic factor are involved in several functions.

2.1 Angiogenesis

Angiogenesis is a process of new blood vessel development from pre-existing vasculature both in normal and malignant tissues. It plays an essential role in embryonic development, normal growth of tissues, wound healing, female reproductive cycle (i.e. ovulation, menstruation and placental development) as well as a major role in many diseases (Folkman, 1995) shown in table 2.1. Angiogenesis is also necessary for the spread and growth of tumor cell metastases (Folkman, 1990; Zetter, 1998). Under normal conditions neovascularization is highly regulated via fine tuning of the balance of stimulatory and inhibitory factors. However, these controls may fail and result in persistent and unregulated formation of pathological capillaries during the development of many diseases and pathological conditions such as tumorigenesis, rheumatoid arthritis and diabetic retinopathy (Folkman, 1987).

Numerous angiogenic factors have been discovered including fibroblast growth factors (FGF), (Montesano et al., 1986; Thomas et al., 1985) transforming growth factors
(TGF), (Schreiber et al., 1986; Roberts et al., 1986) angiogenin (Fett et al., 1985) and vascular endothelial growth factor (VEGF) (Leung et al., 1989). Various growth factors and cytokines act as inducers of angiogenesis. One of the most specific and critical regulators of angiogenesis is VEGF that regulates endothelial proliferation, permeability and survival.

**Table 2.1: Diseases characterized or caused by excessive or abnormal angiogenesis**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Disease in mice or human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Different organ</td>
<td>Cancer (activation of oncogenes and loss of tumor suppressor gene), infectious disease (Pathogens express angiogenic gene that induces angiogenic programs, or transformation of Ecs) Auto immuno disorders (Activation of mast cells and other leukocytes).</td>
</tr>
<tr>
<td>Blood Vessels</td>
<td>Vascular malformations, DiGeorge syndrome, Cavernous hemangioma, Atherosclerosis, transplant arteriopathy.</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>Obesity (angiogenesis induced by fatty diet; weight loss by angiogenesis inhibitors.</td>
</tr>
<tr>
<td>Skin</td>
<td>Psoriasis, warts, allergic dermatitis, skin keloids, pyogenis granulomas, blistering disease.</td>
</tr>
<tr>
<td>Eye</td>
<td>Persistent hyperplastic vitreous syndrome, diabetic retinopathy, retinopathy of permaturity, choroidal neovascularisation.</td>
</tr>
<tr>
<td>Lung</td>
<td>Primary pulmonary hypertension, asthma, polyps.</td>
</tr>
<tr>
<td>Intestine</td>
<td>Inflammatory bowls and periodontal disease, ascites, peritoneal adhesions.</td>
</tr>
<tr>
<td>Reproductive</td>
<td>Endometriosis, uterine bleedings, ovarian cysts, ovarian hysteimulation.</td>
</tr>
<tr>
<td>System</td>
<td>Arthritis, synovitis, osteomyelitis, osteophyte formation.</td>
</tr>
</tbody>
</table>

21
Cytokine is a soluble glycoprotein secreted by most of the living cells to regulate the metabolism and works non-enzymatically in picomolar to nanomolar concentrations. Cytokines are a fourth major class of soluble intercellular signaling molecules, along side endocrine hormones, neurotransmitters and autacoids. (Autacoids released from the cells in response to various types of stimulation to elicit normal physiological responses locally). After secretion they diffuse into the extracellular matrix and act locally in a paracrine manner unlike hormones which are distributed throughout the circulation. They act on specific cells and direct their activity. They regulate different functions in tissues through cell signaling like cell migration, proliferation, matrix synthesis and remolding etc. (Nathan and Sporn, 1991). The central role of cytokines is to controls the remodeling of tissues that includes inflammation, infection, wound healing and repair (Vlassara et al., 1988).

Many growth factors are rich in extended β-sheet characteristic for group 2 cytokines (fig. 2.1). Binding of growth factor to its corresponding cell surface receptor activates complex multistep signal transduction pathways involving changes in protein phosphorylation, ion fluxes, metabolism, gene expression, protein synthesis and ultimately a biological response (Nicola, 1994). PDGF (Platelet derived growth factor) family of cytokines enhances vascularization and angiogenesis in vitro and in vivo. VEGF forms a subfamily within the PDGF family of growth factors (Joukov et al., 1996) which itself belongs to the cystine knot class of cytokines (McDonald and Hendrickson, 1993).
Figure 2.1: Classification of Cytokines: VEGF classified in group 2 β-sheet rich cytokines. VEGF form a subfamily within the PDGF family of growth factors (McDonald and Hendrickson, 1993).
2.1.1 Mechanism of Angiogenesis

Angiogenesis is mediated by endothelial cells that line the blood vessels (Daniel et al., 2000). Angiogenesis is a multistep process involving both the endothelium and the extracellular matrix (Risau, 1997). Angiogenesis requires the coordination of a variety of cellular and extracellular matrix components. The most important of these is the endothelium itself since most angiogenic growth factors and modulatory peptides ultimately mediate their effects on endothelial cells. The angiogenic signals from these factors direct the endothelium through a series of characteristic steps that include endothelial protease production, basement membrane breakdown, endothelial migration, proliferation, capillary morphogenesis and subsequent vascular maturation. Although a variety of different substances can promote angiogenesis from small molecules to relatively large polypeptides, a selected few acts specifically on endothelial cells.

Many molecules have been implicated as positive regulators of angiogenesis (Angiogenic growth factors). Angiogenic growth factors have ability to induce the proliferation of various cells \textit{in vitro} which contribute to the process of angiogenesis \textit{in vivo} as demonstrated by studies of animal models. It has been difficult to correlate such activity with the physiological or pathological regulation of blood vessel growth. These growth factors are produced by various cell types and include a diverse range of proteins. Cognate receptors of these proteins that have been identified include a number of stimulators such as VEGF (Ferrara and Davis-Smith, 1997; Petrova et al., 1999), FGF (Nugent et al., 2000), angiopoietins (Davis et al., 1996), activators of integrins (Eliceiri and Cheresh 1999), inhibitors such as thrombospondin (Roberts, 1996), angiostatin and endostatin (O'Reilly et al., 1997).
The sprouting angiogenesis is a complex process involving many cell types and signaling pathways (Risau, 1997; Han and Liu, 1999; Conway et al., 2001; Carmeliet, 2003). The process of sprouting has been divided into five sequential steps (Folkman, 1985) as outlined.

- **Local degradation of the vascular basement membrane.** The importance of proteolytic activity increases with the development of the endothelial basal lamina and is certainly crucial in adult angiogenesis. Capillary endothelial cells are capable of secreting several proteases, which hydrolyze the basal lamina of endothelial cells and certain extracellular matrix (ECM) components (Moscatelli and Rifkin, 1988).

- **Migration of endothelial cells along angiogenic gradients.** The extremely high migratory potential of endothelial cells during development has been shown in quail-chick grafting experiments. Gradients of soluble angiogenic factors, ECM-bound angiogenic factors and the specific composition of the ECM have been identified as key factors in the directed migration of both embryonic and adult endothelial cells (Wilting and Christ, 1996).

- **Lumen formation.** Two opposite mechanisms for lumen formation have been described, inter- and intracellular lumen formation. Intercellular lumen formation is the extension of the lumen of the pre-existing capillary. Intracellular lumen formation as a result of vacuolization seems to be of minor importance.

- **Mitosis of endothelial cells in the midsection of capillary sprouts.** Although vessel formation seems to be possible without mitosis, it is restricted to at least two levels of vascular loops, whose capacity to proliferate is destroyed by
irradiation/poisoning (Koolwijk et al., 1996; Sholley et al., 1984). Whether the initial steps of angiogenesis take also place without concurrent mitosis of the involved endothelial cells is still a subject of discussion.

- **Fusion of adjacent sprouts and loop formation.** It is unknown, whether individual capillary sprouts find each other by a directed mechanism or by trial-and-error migration (Folkman, 1985). A directed mechanism would involve concentration gradients and/or adhesion molecules and, indeed, several candidate molecules have been proposed, e.g. Tek/Tie and their ligands (Dumont et al., 1994).

### 2.2 Angiogenesis in Physiological and Pathological Conditions

In physiological angiogenesis after the embryonic and postnatal development rapid proliferation of the endothelial cells is down regulated almost to quiescence (Denekamp, 1993). However, the capillary endothelial cells maintain their proliferative potential throughout the life of an organism. The only circumstance, proliferation is upregulated during female reproductive cycle and pregnancy. In pathological angiogenesis different pathological conditions involve the neovascularization of tissues (Folkman, 1995). Neovasularization can be an accelerator of disease progression (e.g. neoplastic diseases) or a mediator of pathogenesis (e.g. in diabetic retinopathy).

### 2.3 Vascular Endothelial Growth Factor (VEGF)

VEGF gene family is one of the most specific and critical regulators of angiogenesis that regulates endothelial proliferation, permeability and survival. There is
strong evidence that this family plays a fundamental role in the growth and differentiation of vascular as well as lymphatic endothelial cells. VEGF belongs to the VEGF/ PDGF (platelet-derived growth factor) group of the cystine-knot superfamily of hormones and extracellular signaling molecules (Vitt et al., 2001). This family have several members including VEGF-A (referred to as VEGF here) that was discovered in 1983 (Senger et al., 1983) and termed as VEGF in 1989 (Ferrara and Hengzel, 1989; Plouet et al., 1989; Leung et al., 1989). Other members of the family includes placental growth factor (PLGF)(Maglione et al., 1991), VEGF-B (Olofsson et al., 1996b), VEGF-C (Joukov et al., 1996), VEGF-D (Achen et al., 1998), VEGF-Es (Ogawa et al., 1998) and VEGF-Fs (Yamazaki et al., 2005).

All VEGF family members have a common homology domain and are characterized by the presence of conserved cysteine residues forming the typical cystine-knot motif with eight invariant cysteine residues involved in inter and intramolecular disulfide bonds at one end of a conserved central four-stranded-β sheet within each monomer which dimerize in an antiparallel side by side orientation (Neufeld et al., 1999; Ortega et al., 1999). Solution structure of VEGF has shown that VEGF forms an antiparallel homodimer covalently linked by two disulfide bridges between Cys-51 and Cys-60 (fig. 2.2). This mode of dimerization is similar to that of the PDGF monomers. The dominant feature within the VEGF monomer is the cystine knot motif that is found in other growth factors (Muller et al., 1997). Thus the folding pattern of these proteins is likely to be similar resulting in a typical compact cystine-bonded knot and physical properties like relative stability against heat, acids and mild proteolysis (Thomas, 1996). The VEGF monomers have a single glycosylation site at Asp 75 of the mature protein but

27
glycosylation is not necessary for biological activity (Claffey et al., 1995; Yeo et al., 1991) although it is important for the efficient secretion of VEGF.

2.4 Phylogenetic Relationship

The VEGF/PDGF group is evolutionarily related to other groups within the cystine-knot superfamily notably the glycoprotein hormone, mucin-like protein families and more distantly the transforming growth factor-β (TGF-β) family. Such protein have evolved in multicellular organisms with tissue level organization to perform hormonal and extracellular signaling functions and these are absent in unicellular organisms. The known members of the human VEGF family have been found in all vertebrate and are highly conserved among species. VEGF-A has been found in teleost fish (zebrafish \textit{Danio rerio}, puffer fish \textit{Fugu rubripes}), frogs (\textit{Xenopus laevis}), birds (\textit{Gallus gallus}) and mammals (Gong et al., 2004). VEGF-A related sequence has been reported in \textit{Drosophila} also (Cho et al., 2002; Duchek et al., 2001). Comparison of human VEGF with PDGF and related sequences from \textit{Drosophila} and Orf virus are shown in fig. 2.3.
Figure 2.2: Ribbon representation of the receptor-binding domain of VEGF isoforms showing a monomer in fig. 2.2a and dimer in fig. 2.2b. The two disulfide bonds are shown as black lines. Helix a1 consists of residues 16 to 24, and the central four-stranded b-sheet is formed by b1 (residues 27–34), b3 (51–58), b5 (73–83), and b6 (89–99), with the characteristic cysteine knot motif at one end [strands b4 (67–69) and b7 (103–105)], and a short three-stranded b-sheet [strands b2 (46–49), b5, and b6] at the other end. VEGF monomers dimerize in an antiparallel way (Takahashi and Shibuya, 2005).
Figure: 2.3 Comparison of human VEGFs with PDGFs and related sequences from Drosophila and Orf virus. An alignment of the deduced amino-acid sequences of the VEGF/PDGF homology domain (VHD) from various human, Drosophila and Orf virus VEGFs and PGDFs. Residues that are conserved in at least 50% of the aligned sequences are shaded in green; those fully conserved are in yellow. The eight cysteine residues that constitute the cystine-knot structure are denoted by asterisks below the sequences.
2.5 Genomic Structure and Splice Variants of VEGF

The human VEGF gene is located in chromosome 6p.12 (Vincenti et al., 1996; Wei et al., 1996). It has a single major transcriptional start site 1038 bp upstream from the ATG initiation codon. The 5'-UTR and 2.4 kb of the TATA-less promoter have been sequenced revealing three specific protein-1 (SP-1) binding sites immediately in front of the transcriptional start site and four Activator protein-1 (AP-1) and two AP-2 binding sites located around the transcriptional start site (Tischer et al., 1989). The VEGF gene is organized into eight exons separated by seven introns and its coding region spans approximately 14 kb (Tischer et al., 1989).

Alternative exon splicing of the VEGF-A mRNA results in at least 6 different isoforms consisting of 121, 145, 165, 183, 189 and 206 amino acids (termed VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206 respectively) (Houck et al., 1991; Tischer et al., 1991; Robinson and Stringer, 2001). All transcripts contain exons 1-5 and 8 with diversity generated through the alternative splicing of exons 6 and 7. The shortest isoform, VEGF121 is encoded by exons 1-5 and 8, lacks exons 6 and 7. VEGF165 includes additionally exon 7 and lacks exon 6. Another splice variant of human VEGF-A was identified as VEGF-A163b. Exon 6 encodes a heparin binding domain, exons 7 and 8 encode a NRPI/heparin-binding domain with the exception of VEGF-A121 and all isoforms are thought to bind polysaccharide heparin. VEGF-A165 binds to NRPI and NRP2 whereas VEGF-A145 binds only to NRP2 (Soker et al., 1998; Gluzman-Poltorak et al., 2000). Exon structure of the VEGF mRNA and its functional domains are shown in figure 2.4A.
2.5.1 VEGF A

VEGF-A (also known as vascular permeability factor VPF, commonly termed VEGF) is a glycoprotein that assembles into a disulphide linked homodimer. The main form has a molecular weight of approximately 45 kDa and it is composed of the 165 amino acid isoform and is structurally related to platelet derived growth factors (Ferrara and Henzel, 1989).

The majority of transcripts for VEGF\textsubscript{165} contain 1.9 kb of 3'-UTR (2.2 kb for murine mRNA) resulting in mRNA transcripts around 3.9 kb (Conn et al., 1990) although four potential polyadenylation sites are present and differential termination of transcription has been reported (Levy et al., 1995). VEGF\textsubscript{165} was thought to be the predominant isoform in all tissues except placenta where VEGF\textsubscript{121} is the most abundant species. Although VEGF\textsubscript{186} have been shown to predominate in the majority of cells and tissues expressing the VEGF gene (Bacic et al., 1995). VEGF\textsubscript{121} seems to be expressed by several tissues and cell lines. The longest isoform VEGF\textsubscript{206} was expressed in cell culture that has been identified only in a human fetal liver cDNA library (Houck et al., 1991). Less frequent splice variants have been also reported such as VEGF\textsubscript{145} and VEGF\textsubscript{183} (Neufeld et al., 1999). Different isoforms of human VEGF-A are shown in table 2.2.
Table 2.2: Isoforms of human VEGF-A

<table>
<thead>
<tr>
<th>Isoforms</th>
<th>Size (Amino acids)</th>
<th>Coding exons</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A_{121}</td>
<td>121</td>
<td>1-5, 8</td>
<td>Secreted</td>
</tr>
<tr>
<td>VEGF-A_{145}</td>
<td>145</td>
<td>1-6, 8</td>
<td>Binds NRP2 but not NRP1; secreted</td>
</tr>
<tr>
<td>VEGF-A_{165}</td>
<td>165</td>
<td>1-5, 7, 8</td>
<td>The most abundant and biologically active isoform; secreted; binds NRP1 and NRP2</td>
</tr>
<tr>
<td>VEGF-_{165b}</td>
<td>165</td>
<td>1-5,7, alternative exon 8</td>
<td>Secreted, endogenous inhibitory form of VEGF-A_{165}</td>
</tr>
<tr>
<td>VEGF-A_{183}</td>
<td>183</td>
<td>1-5, short exon 6, 7, 8</td>
<td>Sequestered in ECM but released by cleavage</td>
</tr>
<tr>
<td>VEGF-A_{189}</td>
<td>189</td>
<td>1-8</td>
<td>Sequestered in ECM but released by cleavage</td>
</tr>
<tr>
<td>VEGF-A_{206}</td>
<td>206</td>
<td>1-8 plus additional exon 6-encoded sequence</td>
<td>Sequestered in ECM but released by cleavage</td>
</tr>
</tbody>
</table>

The minor human VEGF isoforms contain 121 and 189 amino acids following signal sequence cleavage. Compared with VEGF_{165}, VEGF_{121} lacks 44 amino acids. VEGF_{189} has an insertion of 24 largely cationic amino acids and VEGF_{206} has an additional insertion of 17 amino acids resulting in different affinities for endogenous polyanions such as cell surface heparan sulphates (Park et al., 1993). VEGF_{165b} has same number of amino acids as VEGF_{165} but its lacks exon 6 while VEGF_{165b} contains an alternative exon 8 with 6 amino acids in the C-terminal region as shown in fig. 2.4B (Bates et al., 2002b). The C-terminal of VEGF_{165} is essential to regulate mitogenic signaling therefore changes in this region are likely to influence function. Woolard et al., (2004) termed this VEGF_{165b} alternative exon as an exon 9. Unlike the other VEGF
isoforms that stimulate angiogenesis, VEGF_{165}^{b} is an endogenous inhibitory form of VEGF which decreases VEGF induced proliferation and migration of endothelial cells.

VEGF_{121} is a freely diffusible protein while VEGF_{165} has intermediary properties as it is secreted but a significant fraction remains bound to cell surface and extracellular matrix (ECM) (Cao et al., 1996). VEGF_{189} and VEGF_{206} are almost completely sequestered into ECM. The ECM bound isoforms may be released in all diffusible from by plasmin cleavage at the C- terminus which generates a bioactive fragment consisting of the first 110 N- terminal amino acids (Houck et al., 1992). Loss of the heparin binding domain results in a significant loss of the mitogenic activity of VEGF (Keyt et al., 1996). These findings demonstrated that VEGF_{165} has optimal characteristics of bioavailability and biological potency. All four mRNA isoforms contain the sequence encoding the hydrophobic signal peptide of 26 amino acids for secretion (Houck et al., 1991; Tischer et al., 1989).

2.5.2 VEGF-B

VEGF-B is a highly basic heparin binding growth factor. VEGF-B, structurally similar to VEGF and PLGF and approximately 40 % identical in amino acid sequence to VEGF. This member of the VEGF gene family is composed of 188 amino acids and can be expressed as homodimer or heterodimer with VEGF-A (Joukov et al., 1997). VEGF-B gene situated on chromosome 11q13 is composed of seven exons. Exons 3 and 4 encode for invariant cysteine residues that are responsible for a cysteine knot motif with two disulfide bridges. Alternate splicing results in two isoforms VEGF-B_{167} (21 kDa) and VEGF-B_{189} (32 kDa). Both isoforms is different only in their C-terminal domains (Olofsson et al., 1996a; Olofsson et al., 1996b).
VEGF-B binds to receptor VEGFR-1 (Olofsson et al., 1998). VEGF-B
transcripts the use of an alternative splice acceptor site in exon 6 introduces a frame shift
resulting in an alternative exon 6 (Olofsson et al., 1996b) encoding an NRP1/heparin
binding domain. VEGF-B_{186} transcripts contain the entire exon 6 and encode a soluble
isoform. Conversely the C-terminal domain of VEGF-B_{186} is hydrophobic modified by
O-linked glycosylation and requires limited proteolysis to bind to neuropilin-1. VEGF-
B_{167} is bound to the cells surface or pericellular heparan sulfate proteoglycans whereas
VEGF-B_{186} is secreted freely (Holmes et al., 2005).

Both VEGF-B isoforms are able to form heterodimers with VEGF and perhaps
with other growth factors. During development, VEGF-B may modulate the biological
activities of VEGF either by forming heterodimers or by controlling the bioavailability of
VEGF (Olofsson et al., 1998; Jussila and Alitalo, 2002). VEGF-B is produced in large
quantities by the developing myocardium, muscle, bone, pancreas, adrenal gland and the
smooth muscle cell layer of several large vessels. VEGF-B is likely to act in a paracrine
fashion as its receptor is almost exclusively located on endothelial cells. VEGF-B is a
very weak endothelial cell mitogen when produced in mammalian cells (Olofsson et al.,
1998; Nash et al., 2006).

2.5.3 VEGF-C

VEGF-C is a protein composed of 419 amino acids with a predicted molecular
mass of 47 kDa whose gene is located on chromosome 4q34 (Joukov et al., 1997; Chilov
et al., 1997). VEGF-C/VEGF-related protein contains a region sharing ~31% amino acid
identity with VEGF_{165} (Lee et al., 1996; Joukov et al., 1996). The gene for VEGF-C
spans more than 40 kb of DNA and consists of seven exons. The VEGF homology
domain of VEGF-C is encoded by exons 3, 4, 5 and 7 encode cysteine rich motifs (Joukov et al., 1996, Witzenbichler et al., 1998; Li and Eriksson, 2001). In adult tissues, it is expressed most prominently in heart, placenta, skeletal muscle, ovary, small intestine and the thyroid gland (Kukk et al., 1996; Enholm et al., 1998; Wartiovaara et al., 1998). VEGF-C participates in the formation and maintenance of the lymphatic venous systems, promotes lymphatic endothelial cell proliferation and vessel enlargement (Kaipainen et al., 1993; Kaipainen et al., 1995; Fitz et al., 1997). At higher concentrations than VEGF, VEGF-C increases vascular permeability and stimulates the migration of endothelial cells as well as endothelial cell proliferation.

They are synthesized as precursor proteins requiring proteolytic processing at the C- and N-termini to release the VEGF-homology domain (Joukov et al., 1996). This might control receptor specificity because incompletely processed VEGF-C binds VEGFR-3 with lower affinity but does not bind VEGFR-2 at all (Joukov et al., 1997). VEGF-C lacks the NRP/heparin binding domain like some other VEGF isoforms not to bind NRPs. VEGF-C is mitogenic for lymphatic endothelial cells and promotes lymphatic endothelial cell survival through VEGFR-3 (Lee, et al., 1996; Achen, et al., 1998; Makinen, et al., 2001).
Figure 2.4: An Exon structure of the VEGF mRNA. The functional domains are also shown. 2.4B Comparison of structures of the VEGF family: Differential splicing results in different isoforms with differing properties. There are at least 6 different isoforms of VEGF-A, which arise by alternative exon splicing. All isoforms contain exons 1-5. Numbers on the right side of structures indicate identities with VEGF165 at the amino acid level. Arrows denote positions of proteolytic cleavage that give rise to mature VEGF-C or VEGF-D. (Bates et al., 2002a; Takahashi and Shibuya, 2005)
2.5.4 VEGF-D

VEGF-D is a member of the mammalian VEGF family. shares 30 % homology with VEGF-A and its gene is located on chromosome Xp22.31 (table 2.3) (Yamada et al., 1997; Achen et al., 1998). The human VEGF-D cDNA encodes a protein of 354 amino acids. It is synthesized as a preproprotein which requires proteolytic processing in both the N- and C-terminal regions for activity and the fully processed growth factor is a non-covalent dimer.

Proteolytic processing also appears to regulate VEGF-D biological activity and receptor specificity (Stacker et al., 1999). The mature form binds to and activates VEGFR-2 and VEGFR-3 (Flt-4; a member of the VEGFR family that does not bind VEGF), is mitogenic for cultured endothelial cells and appears to be capable of stimulating lymph angiogenesis (Karkkainen et al., 2000). VEGF-D lack the NRP/heparin binding domain found in some VEGF isoforms and appear to be unable to bind NRPs. It is found in adult tissues particularly lung, heart, skeletal muscle, colon and small intestine. In embryonic tissues it is abundant in the developing lung (Yamada et al., 1997; Ortega et al., 1999; Li and Eriksson, 2001). It has been shown to be able to stimulate the proliferation of endothelial cells and shows angiogenic properties in vitro and in vivo (Marconcini et al., 1999).

2.5.5 VEGF-E

VEGF-E refers to a group of VEGF related proteins encoded by the Orf virus (Meyer et al., 1999). Orf virus is a parapox virus that infects sheep, goats and occasionally humans that share between 16 and 27 % amino acid identity to mammalian
VEGF (Lytle et al., 1994). Presumably the VEGF gene was acquired from a host genome and has subsequently undergone genetic drift. Some biological functions appear to be retained because viral infection results in skin lesions with acute microvascular proliferation and dilation. All VEGF-E variants studied bind to VEGFR-2 and stimulate angiogenesis, vascular permeability, therefore enhancing viral infection (Savory et al., 2000) but not VEGFR-1 or VEGFR-3 (Ogawa et al., 1998; Meyer et al., 1999; Wise et al., 1999). One variant was further shown to bind neuropilin-1. These viral proteins seem to be as potent as VEGF165 at stimulating endothelial cell proliferation despite lacking heparin-binding ability.

Table 2.3: Different properties of human VEGF family proteins

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosomal location</th>
<th>Soluble VEGF isoforms</th>
<th>Heparin-binding</th>
<th>Protein Size (kDa)</th>
<th>Identities %</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A</td>
<td>6p12</td>
<td>VEGF-A165, VEGF-A115, VEGF-A121</td>
<td>VEGF-A189, VEGF-A206 weakly: VEGF-A121, VEGF-A145.</td>
<td>21</td>
<td>100</td>
</tr>
<tr>
<td>VEGF-B</td>
<td>11q13</td>
<td>VEGF-B167</td>
<td>VEGF-B167</td>
<td>21, 30</td>
<td>40.9</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>4q34.1-q34.3</td>
<td>Yes</td>
<td>-</td>
<td>20-21</td>
<td>31.6</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>Xp22.31</td>
<td>No</td>
<td>Yes</td>
<td>20-21</td>
<td>30.9</td>
</tr>
<tr>
<td>PLGF</td>
<td>14q24-q31</td>
<td>No</td>
<td>No</td>
<td>-</td>
<td>46.3</td>
</tr>
</tbody>
</table>

2.5.6 Placental Growth Factor

The first VEGF-related protein placenta growth factor (PLGF) discovered in 1991 owes its name to the predominance in placental tissue as well as expressed in heart and lung. It was later identified as a member of the VEGF family as the molecule shares 46 % of a homologous domain with the PDGF-like region of VEGF (Maglione et al., 1991).
Its expression is restricted to the placenta where alternative mRNA splicing generates three isoforms: PLGF-1 (PLGF$_{131}$), PLGF-2 (PLGF$_{152}$) and PLGF-3 (PLGF$_{183}$) (Maglione et al., 1993; Cao et al., 1997).

2.5.7 VEGF-F

Recently novel VEGFs was reported from snake venom designated as VEGF-Fs (Yamazaki et al., 2005). VEGF family proteins have been identified in snake venom including svVEGF from Bothrops insularis ((Junqueira et al., 2001) and $Tj$svVEGF (Trimeresurus flavoviridis svVEGF ) (Takahashi et al., 2004) from pit vipers in addition to HF (hypotensive factor) (Komori, et al., 1999), ICPP (increasing capillary permeability protein), (Gasmi et al., 2002) and vammin (Yamazaki et al., 2003) from vipers. svVEGFs function as dimer and each chain comprises approximately 110–122 amino acid residues. The cysteine knot motif a characteristic of the VEGF family of proteins is completely conserved in svVEGFs and the sequence identity with human VEGF$_{165}$ is approximately 50 %. Vammin does not bind VEGFR-1 but binds VEGFR-2 with high affinity as well as VEGF$_{165}$ (Yamazaki et al., 2003). However $Tj$svVEGF binds VEGFR-1 with high affinity and VEGFR-2 with low affinity compared with VEGF$_{165}$ leading to a strong enhancement of vascular permeability but weak stimulation of endothelial cell proliferation (Takahashi et al., 2004). Both vammin and $Tj$svVEGF are unable to bind VEGFR-3 or NRP-1, but $Tj$svVEGF binds heparin. The svVEGFs may contribute to the enhancement of toxicity in envenomation, but they seem to have individual biological characteristics reflecting divergence in the classification of the host snake.
2.6 VEGF Receptors

Three VEGF tyrosine kinase receptors have been identified: The fms-like tyrosine kinase Flt-1 (VEGFR-1/Flt-1), the kinase domain region, also referred to as fetal liver kinase (VEGFR-2/KDR/Flk-1) and Flt-4 (VEGFR-3). VEGFR-3 (fms-like tyrosine kinase (Flt-4) is a member of the same family of RTKs and it binds to VEGF-C and VEGF-D. Each receptor has seven immunoglobulins like domains in the extracellular domain, a single trans-membrane region and a consensus tyrosine kinase sequence interrupted by a kinase insert domain (Ortega et al., 1999). Immunoglobulin homology domains of the second and third are critical for ligand binding and the first three domains are necessary for establishment of full binding affinity. Neuropilins originally identified as a receptor for the semaphorin family of neuronal guidance mediators and was shown to act as an isoform specific receptor for VEGF165 (Soker et al., 1998). Different VEGF receptors, ligands and their functions are shown in table 2.4.

2.6.1 VEGFR-1

VEGFR-1 was the first RTK to be identified as a VEGFR more than a decade ago (Shibuya et al., 1990; de Vries et al., 1992). The human gene for VEGFR-1 is located on chromosome 13q12 (Rosnet et al., 1991). VEGFR-1 (Flt-1) is a 180 kDa trans-membrane protein which binds VEGF-A, PLGF and VEGF-B. It was originally cloned from a placental cDNA library (Shibuya et al., 1990). Alternative splicing produces a shorter soluble form (soluble Flt-1, sVEGFR-1) which can act as an inhibitor of VEGF. VEGFR-1 is expressed in endothelial cells as well as a range of non-endothelial cells including osteoblasts, monocytes, macrophages, placental trophoblasts, and also in some
hematopoietic stem cells (Zachary and Gliki, 2001; Sawano et al., 2001; Hattori, 2002; Ferrara et al., 2003). The affinity of VEGFR-1 for VEGF is ten-fold higher than VEGFR-2 (Kd =10–30 pM) but its tyrosine kinase activity is ten-fold weaker than VEGFR-2. During development, VEGFR-1 is first expressed in angioblasts and in the endothelium although less strongly than VEGFR-2. VEGFR-1 expression subsides during later embryonic development (Fong et al., 1995). VEGFR-1 gene targeted mice die at embryonic day 8.5 (E-8.5) due to disorganization of blood vessels and overgrowth of endothelial cells (Ferrara et al., 2003).

Table 2.4: VEGF receptors, ligands and their functions

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR-1</td>
<td>VEGF-A121, VEGF-A165, VEGF-B, PLGF-1, PLGF-2</td>
<td>Promotion of cell migration, Organization of blood vessels. Gene expression of monocytes and macrophages</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>VEGF-A121, VEGF-A145, VEGF-A165, VEGF-C, VEGF-D</td>
<td>Mitogenesis, differentiation of endothelial cells. Promotion of cell migration.</td>
</tr>
<tr>
<td>VEGFR-3</td>
<td>VEGF-A145, VEGF-A165, VEGF-A189, PLGF-2, VEGF-D167</td>
<td>Enhancement of vascular permeability, Remodeling of primary capillary vasculature.</td>
</tr>
<tr>
<td>Neu-1</td>
<td>VEGF-A165, PLGF-2</td>
<td>VEGF-A165 embryonic cardiovascular development, VEGF-A189 Regulation of growth and maintenance of lymphatic system, Development of cardiovascular system</td>
</tr>
<tr>
<td>Neu-2</td>
<td>VEGF-A165</td>
<td>Organization of peripheral nerve fibers. Development of vascular networks</td>
</tr>
</tbody>
</table>
al., 1998; Ortega et al., 1999; Andre et al., 2000; Partanen et al., 2000; Bernatchez et al., 2002). PAF has many crucial roles in the induction of angiogenesis. During the development VEGFR-2 is expressed by the primitive endoderm, embryonic angioblasts and in the blood islands as well as in angiogenic vessels (Kaipainen et al., 1993). VEGFR-2 gene targeted mice die at embryonic day 8.5-9.5 (E-8.5-9.5) due to lack of development of the blood islands, embryonic vasculature and hematopoietic cells (Shalaby et al., 1995; Ferrara et al., 2003). VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF-A.

2.6.3 VEGFR-3

VEGFR-3 (Flt-4) was first cloned from human erythroleukemia cells (Aprikian et al., 1992; Pajusola et al., 1992) and placental cDNA libraries. VEGFR-3 is also known as fms insert-like tyrosine kinase 4 (Flt-4) and its extracellular domain is 80% homologous to the other VEGFRs. The VEGFR-3 gene is encoded in the chromosomal region 5q34–q35 (Galland et al., 1992). VEGFR-3 is a 195 kDa glycosylated precursor which has high-affinity receptor for VEGF-C and VEGF-D but not for VEGF. The second immunoglobulin-like domain in the extracellular domain of VEGFR-1 and VEGFR-3 is responsible for specific ligand recognition. VEGFR-3 is proteolytically cleaved within the fifth extracellular Ig loop into a 125 kDa and a 70 kDa. The 125 kDa C-terminal fragments spans the cell membrane and contains the tyrosine kinase domain whereas the 70 kDa N-terminal fragment forms a major part of the extracellular domain. These two forms are linked by a disulphide bridge (Pajusola et al., 1994). Flt-4 is predominantly expressed in lymphatic endothelium in adult tissue and is thought to
control lymph angiogenesis however in most vascular endothelial cells low levels of VEGFR-3 are detectable.

VEGFR-3 mRNA is found in venous endothelium in early stages of embryonic development but soon becomes restricted to developing lymphatic vessels in later stages of development. Disruption of VEGFR-3 has led to a defective remodeling of the primary vascular plexus and cardiovascular failure after embryonic day 9.5 (E-9.5), but differentiation of endothelial cells formation of primitive vascular networks and vascular sprouting occurred normally.

2.6.4 Neuropilla 1 and 2

Neuropilin-1 (Nrp-1), a 130–140 kDa cell-surface glycoprotein previously characterized as a neuronal receptor for certain secreted members of the collapsin/semaphoring family (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Soker et al., 1998). These receptors recognize the exon-7 encoded domain of VEGF and therefore bind VEGF₁₆₅ but not VEGF₁₂₁ (Soker et al., 1996). Nrp-1 is also able to bind VEGF-B, PLGF-2 and some VEGF-E variants whereas Nrp-2 can bind VEGF₁₄₅, VEGF₁₆₅, PLGF-2 and VEGF-C. Nrp-2 was identified by virtue of its sequence homology with NRP-1 and shares 44 % identity at the amino acid level with Nrp-1 (Neufeld et al., 2002). Nrp-1 acts as a co-receptor enhancing VEGFR-2 interactions forming complexes with VEGFR-1 and augmenting tumor angiogenesis in vivo.

The neuropilins (Nrp-1 and Nrp-2) not only play important roles in immunology and neuronal development but they are also involved in angiogenesis (Klagsbrun et al., 2002; Bagri et al., 2002). Studies on mouse embryos suggest it also has a role in
angiogenesis and vasculogenesis. Nrp-1 over expression causes death at embryonic day 17.5 with an excessively high density of blood vessels which are dilated and prone to hemorrhaging $NRPI/-$ mice die at embryonic day 10.5-12.5 (E-10.5-12.5) from cardiovascular anomalies (Kitsukawa et al., 1995; Kitsukawa et al., 1997).

2.7 Regulation of VEGF Expression

Regulation of VEGF expression has been reported to occur at the transcription, translation and post translational levels. Transcriptional regulation of VEGF expression has been studied extensively because the impact of most genetic and epigenetic factors on VEGF expression is realized by controlling VEGF gene transcription. Computer-based sequence analysis of the VEGF gene promoter structure revealed a number of potential binding sites in the 5'-flanking region of the VEGF gene for specific protein-1 (Sp1), hypoxia-inducible factor 1 (HIF-1), signal transducer and activator of transcription-3 (Stat3), activator protein-1 (AP-1), Egr-1, activator protein-2 (AP-2), nuclear factor IL6, and many others (Tischer et al., 1991; Lander et al., 1996; Benckert et al., 2003) indicating the diverse complexity of VEGF transcriptional regulation (fig. 2.5). Among the many transcription factors, Sp1, HIF-1, Stat3 and AP-1 appear to be the key factors in regulation of VEGF expression and have been well characterized. Even though transcriptional regulation represents the most important mechanism of VEGF expression and regulation, posttranscriptional regulation also plays an important part in VEGF expression.
Figure 2.5: Structure of VEGF promoter. The major putative transcription factor recognition elements were marked in the human VEGF promoter. The arrow downstream of the EcoI enzyme restriction site indicates the start site of transcription (+1). (Xie et al., 2004)
stabilization. Subsequently, the protein responsible
HuR, an AU-rich element binding protein (Levy et al., 1998).
mechanisms exerted at the translational level the use of alternative codons
means of increasing gene diversity by expressing several proteins from a single mRNA.
At least five isoforms of VEGF-A are generated in this way. Different VEGF isoforms
have distinct activities at different anatomical sites and the microenvironment of different
tissues affects the expression and function of VEGF isoforms (Guo et al., 2001; Prats and
Parts, 2002).

2.7.1 Hypoxia

Hypoxia or low oxygen tension occurs when the growth of neoplastic cells
outpaces the rate of new functional blood vessel formation (i.e. inadequate tumor
angiogenesis) due to the limitations of oxygen diffusion. As a result, tumor cells lying far
from the nearest functional blood vessels experience chronic hypoxia. Because the
diffusion distances of glucose and many other critical nutrients are similar to those of
oxygen, these cells also experience nutritional deficiencies (Vaupe1 and Hoeckel, 1999).
The most prominent feature of VEGF regulation is the upregulation of VEGF by hypoxia
involving a putative oxygen sensitive heme-protein, whose reduced state can be
mimicked by cobalt or manganese. Hypoxia occurs in wounds, ischemic organs or around
necrotic areas of tumors. Hypoxia is the most prominent factor for VEGF induction in
vitro and in vivo. VEGF was originally identified as the hypoxia inducible angiogenic
factor because in vitro its mRNA was dramatically induced by exposing cell cultures to
hypoxia and \textit{in vivo} its expression was higher in tumor cells adjacent to necrotic areas where they were believed to be under hypoxic conditions (Shweiki \textit{et al.}, 1992).

2.8 Role of VEGF in Physiological Angiogenesis

2.8.1 The Female Reproductive Cycle

VEGF plays an important role in the female reproductive cycle as one of the primary angiogenic factors regulating follicular and luteal vascular development. In contrast to pathological conditions growth and angiogenesis in the female reproductive cycle is highly regulated and well coordinated (Reynolds and Redmer, 1998). VEGF expression is highest in early luteal phase as the development of new blood vessels is required for the corpus luteum declines after the mid-luteal phase when the vasculature is established and is absent in the late corpus luteum (Otani \textit{et al.}, 1999).

It is present exclusively in the luteal connective tissue and perivascular cells. Follicular growth and the development and endocrine function of the ovarian corpus luteum (CL) are dependent on the proliferation of new capillary vessels. Subsequently the blood vessels regress suggesting the coordinated action of inducers and inhibitors of angiogenesis in the course of the ovarian cycle. Follicles with a high content of dissolved oxygen also contain the highest follicular fluid concentration of VEGF-A. These follicles display better fertilization rates and embryo development than do oocytes from severely hypoxic follicles. Hormonally regulated angiogenesis occurs also in the proliferating and regressing endometrium and especially prominently upon implantation of the embryo (Cullinan-Bove and Koos, 1993).
2.9 Role of VEGF in Pathological Angiogenesis

2.9.1 Tumor Angiogenesis

During tumorigenesis, neoplastic lesions initially undergo an avascular growth phase to a size not much greater than 2 to 3 mm. The cells in the center become too far from existing blood vessels to receive necessary oxygen and nutrients. This phase is followed by a second event that distinguishes a growing tumor from one that is dormant: the switch from the avascular to vascular phenotype or “the angiogenic switch”. This initiates a cascade of events that results in the expansion of tumor volume and subsequent metastasis. The angiogenic switch is regulated by the net balance between positive and negative regulators of new capillary growth (Folkman, 1985; Hoeben et al., 2004). Molecular sensors within these “starved” cells recognize the decrease in oxygen and initiate processes for producing angiogenic growth factors. VEGF is secreted from the tumor and binds to high affinity signaling receptors on the endothelial cells of existing blood vessels. This leads to the formation of new blood capillaries which provide the necessary nutrients for tumor cell survival and tumor growth.

Various studies suggested that VEGF is a key mediator of angiogenesis in cancers. VEGF is expressed in most tumors and its expression correlates with tumor progression. In addition to tumor cells tumor-associated stroma is also an important source of VEGF-A. Many tumor cell lines secrete VEGF in vitro, suggesting the possibility that this diffusible molecule may be a mediator of tumor angiogenesis (Ferrara et al., 1992).
2.9.2 Ocular Angiogenesis

Angiogenesis is crucial in the development of the eye as well as in the pathogenesis of a variety of ocular diseases. Ocular neovascularization is the most common cause of blindness and dominates many eye diseases among others diabetic retinopathy (Olk and Lee, 1993) and age-related macular degeneration (AMD) (Bressler et al., 1994). VEGF stimulated new capillaries of the retina invade the vitreous, bleed and cause blindness (Adamis et al., 1994; Aiello et al., 1994; Malecaze et al., 1994). Hypoxia plays an important role in the development of diabetic retinopathy. Diabetes mellitus, occlusion of central retinal vein or prematurity with subsequent exposure to oxygen can all be associated with retinal ischemia and intraocular neovascularization, which may result in vitreous hemorrhages, retinal detachment, neovascular glaucoma and blindness (Ferrara et al., 2003). By virtue of its regulation by hypoxia, VEGF was a potential mediator of such neovascularization. Excessive secretion of VEGF in the retina leads to ocular neovascularisation.

Neovascularization and vascular leakage are also causes of visual loss in the AMD, the overall leading cause of blindness. Earlier studies demonstrated the immuno histochemical localization of VEGF in surgically choroidal neovascular membranes from AMD patients (Lopez et al., 1996). Whether such VEGF up-regulation is hypoxia related is unclear (Campochiaro and Hackett, 2003). Anti-angiogenic therapy (using either neutralizing antibodies against VEGF or a soluble form of the VEGFR-1 receptor) successfully inhibited ocular neovascularization in a mouse and a monkey model (Adamis et al., 1996; Aiello et al., 1995). In June 2006, Ranibizumab (Lucentis®, Genentech), a humanized monoclonal antibody fragment that binds to vascular
endothelial growth factor was approved by the USFDA for the treatment of patients with neovascular age-related macular degeneration (Pieramici et al., 2006; Narayanan et al., 2006).

2.9.3 VEGF in Non-Malignant Disease

2.9.3.1 Inflammatory Diseases

VEGF up-regulation has been implicated in various inflammatory disorders (Dvorak, 2002). VEGF acts as a pro-inflammatory cytokine by increasing the permeability of endothelial cells inducing the expression of endothelial adhesion molecules and via its ability to act as a monocyte chemo attractant VEGF is strongly expressed by epidermal keratinocytes in wound healing and psoriasis conditions that are characterized by increased microvascular permeability and angiogenesis (Detmar et al., 1995).

2.9.3.2 Rheumatoid Arthritis (RA)

Rheumatoid arthritis (RA) is an autoimmune disease which affects the peripheral and synovial joints. RA is a polyarticular disease characterized by proliferation of the synovial lining of cells, thickening of the synovial membrane (an increase in thickness of six to eight cell layers), villus projections and dense infiltration of the synovia with macrophages, lymphocytes and plasma cells (Walsh et al., 1998; Lee et al., 2001). Angiogenesis associated with the proliferation of inflammatory synovial tissue is most likely VEGF-mediated. Synovial tissue and macrophages could be identified as sources of elevated VEGF levels (Koch et al., 1994). Inhibition of angiogenesis was suggested as a possible treatment of the disease based on an animal model (Oliver et al., 1994).
2.9.4 Cardiovascular Disease

Angiogenesis is more critical in cardiovascular disease. Various angiogenic agents are in clinical trials for treating ischemic heart disease. However, one growth factor may not be sufficient by itself but may require additional growth promoting cytokines. VEGF and PLGF have been shown to stimulate angiogenesis and collateral growth with comparable efficiency in the ischemic heart and limb ischemia. VEGF and other cytokines can be administered as a natural recombinant human protein or by gene transfer to promote the development of collateral blood vessels which may constitute endogenous bypass conduits around occluded native arteries (Isner, 1998; Freedman and Isner, 2002). VEGF is useful for attempts to increase collateral vessel formation in ischemic heart disease (IHD) or critical limb ischemia.

2.10 Functions of VEGF₁₆₅

2.10.1 Endothelial Cell Growth and Proliferation

VEGF has been shown to be expressed by endothelial cells and by non-endothelial cells such as epithelial cells, monocytes, smooth muscle cells, macrophages and trophoblast cells. VEGF acts in an autocrine and in paracrine fashion (Connolly et al., 1989a; Berse et al., 1992; Plate et al., 1992; Pierce et al., 1995; Gitay-Goren et al., 1996; Brown et al., 1997; Ferrara, 2004). It binds to its receptors that are found predominantly on endothelial cells but also on non-endothelial cells including trophoblast cells (Charnock-Jones et al., 1994; Ahmed et al., 1995), monocytes (Clauss et al., 1990; Barleon et al., 1996) and tumor cell lines (Cohen et al., 1995; Boocock et al., 1995).
Although some non endothelial cells, such as human myometrial smooth muscle cells (Brown et al., 1997) has been shown to respond to VEGF (Thomas, 1996).

The mitogenic activity of VEGF has first been demonstrated towards capillary endothelial cells. *In vitro* half-maximal stimulation of bovine capillary endothelial cell growth was obtained at 100-300 pg/ml (2-6 pM) and a maximal stimulation at 1-5 ng/ml (22-110 pM) (Ferrara and Henzel, 1989; Plouët et al., 1989). As a potent endothelial mitogen, VEGF has been shown to elicit an angiogenic response in a range of *in vivo* models including the chick chorio-allantoic membrane (Leung et al., 1989; Connolly et al., 1989), the rabbit cornea (Phillips and Knighton, 1995), the primate iris (Tolentino et al., 1996) and rabbit bone (Connolly et al., 1989a). The mitogenic activity of VEGF has been shown to be important in tissue repair from injury (Thomas, 1996). VEGF stimulates DNA synthesis and proliferation via VEGFR-2 and extracellular-regulated kinase-1/2 (ERK1/2). Activation of ERK 1/2 is mediated by Ras-Raf-MEK-ERK pathway (Pedram et al., 1998; Parenti et al., 1998, Xie et al., 2004). The mitogen activated protein kinase (MAPK) pathway is also implicated in cell proliferation in response to VEGF.

2.10.2 Cell Migration and Invasion

The fact that VEGF acts as a chemo-attractant for endothelial cells suggests that it plays a role in migration. In addition to endothelial cells, VEGF also stimulates migration of vascular smooth muscle cells, monocytes, mononuclear phagocytes, polymorphonuclear cells (Barleon et al., 1996), migration and invasion of some tumor cells such as breast and leukemia. The VEGF receptors VEGFR-1, VEGFR-2 and the NRPs have all been implicated in VEGF-mediated cell migration and invasion (Barleon et al., 1996;
Grosskreutz et al., 1999; Dias et al., 2000; Price et al., 2001). VEGF induces cell migration by activating factors such as focal adhesion kinase (FAK) and Paxillin and also via the PI3 Kinase/Akt pathway. FAK activation is mediated by the c-terminal region of VEGF-R2 (Qi et al., 2001). VEGF activation of the p38/MAPK stress pathway is also implicated in cell migration and p38 inhibitors decrease cell migration (Rousseau et al., 1997).

2.10.3 Role in Vascular Permeability

VEGF increases vascular permeability and it was originally called vascular permeability factor (VPF). VEGF has both permeability inducing and mitogenic activity (Shibuya et al., 1995; Bates et al., 2002a). On a weight-to-weight basis VEGF is 50,000 times more potent than histamine in increasing vascular permeability (Dvorak et al., 1995). It does not work via histamine release from mast-cells (Gruber et al., 1995), instead VEGF triggers direct fenestration of endothelial cells, even those resistant to classical inflammatory mediators such as platelet activating factor (PAF) or fibrin breakdown products (Roberts and Palade, 1995). Related effects of VEGF on the cardiovascular system are vasodilatation in vitro (Ku et al., 1993; Doi et al., 1996) resulting in tachycardia and hypotension in vivo (Yang et al., 1996). VEGF induces vascular leakage in vivo (Senger et al., 1983; Keck et al., 1989; Jakeman et al., 1992). Intradermal or topical administration results in fenestrations or openings in endothelial cells lining small venules and capillaries consequently increasing their permeability (Roberts and Palade, 1995). In vivo mitogenic and permeability-inducing activities of VEGF are closely linked (Dvorak et al., 1995). Apart from VEGF, no other angiogenic factor is able to induce vascular hyper permeability which indicates that VEGF could be a
general "immediate" angiogenic agent which is activated by indirect angiogenic factors (Pertovaara et al., 1994).

2.10.4 Breakdown of the Endothelial Basement Membrane

Angiogenesis by capillary sprouting involves the breakdown of the basement membrane by enzymes, "primarily Matrix metalloproteinases (MMP), Plasminogen activator (PA), Tissue inhibitor of metalloproteinase (TIMP) and Plasminogen activator inhibitors (PAI) inhibit the actions of these enzymes. VEGF has been shown to induce expression of the MMP collagenase and PA suggesting that VEGF stimulates factors responsible for the breakdown of the endothelial basement membrane" (Han and Liu, 1999). VEGF has also been shown to induce expression of PAI and it is understood to provide a negative regulatory step that serves to balance the proteolytic process (Pepper et al., 1991). It certainly appears that a fine balance in VEGF expression is required for appropriate rather than inappropriate angiogenesis (Ferrara and Davis-Smyth, 1997).

2.10.5 Wound Healing

In wound healing, angiogenesis is most prominent during the first seven days after wounding and this correlates temporarily, spatially with the expression of angiogenic factors. The formation of new blood vessels provides a route for oxygen and nutrient delivery as well as a conduit for components of the inflammatory response during the healing of wounds. Pro-angiogenic treatments have shown remarkable promise in the healing of wounds in pathological conditions. In wound healing, activated platelets release several cytokines including VEGF upon injury. VEGF then attracts circulating neutrophils and monocytes to the site of injury as part of the normal inflammatory...
response. It is also released by monocytes, keratinocytes and endothelial cells at the
wound site where it can act on capillaries (Hoeben et al., 2004). VEGF also increases
permeability by affecting the endothelial cell junction proteins which could enhance the
formation of granulation tissue (Dvorak et al., 1995).

2.11 Angiogenesis Therapy

2.11.1 Therapeutic Angiogenesis for Cardiovascular Disease

Therapeutic angiogenesis offers promise as a novel treatment for ischemic heart disease.
The concept of therapeutic angiogenesis or stimulation of new vessels growth to restore
blood supply to ischemic tissue has been studied in patients with advanced coronary and
peripheral arterial disease. Angiogenesis related research in cardiovascular medicine has
initially been linked to ischemic heart disease and atherosclerosis. Therapeutic
angiogenesis can reduce tissue ischemia by simulating the natural process of
angiogenesis by using VEGF and other angiogenic protein (Tirziu and Simons, 2005).
Besides direct administration of angiogenic proteins, plasmids and viral vectors carrying
angiogenic genes have been used. Animal experiments have shown promise with
evidence of neovascularization and improved perfusion in the target myocardium (Ye et
al., 2004). However, one growth factor may not be sufficient by itself, but may require
additional growth promoting cytokines.
Figure 2.6: Therapeutic angiogenesis in heart by using VEGF.
Two major avenues for achieving therapeutic angiogenesis are currently under intense investigation.

Protein therapy: The direct administration of the growth factors (protein) which is responsible for angiogenesis (Freedman and Isner, 2002).

Gene therapy: The introduction of new genetic material into somatic cells to synthesize target protein for specific therapeutic purposes (Isner et al., 1996).

Protein-based therapy with cytokines including VEGF and fibroblast growth factor (FGF) demonstrated functionally significant angiogenesis in several animal models. However, clinical trials have yielded largely disappointing results. The attenuated angiogenic response seen in clinical trials of patients with coronary artery disease may be due to multiple factors including endothelial dysfunction, particularly in the context of advanced atherosclerotic disease and associated comorbid conditions, regimens of single agents, as well as inefficiencies of current delivery methods (Freedman and Isner, 2002; Rosingberg et al. 2004).

Gene therapy has several advantages over protein therapy and recent advances in gene transfer techniques have improved the feasibility of this approach (Isner et al., 1996; Isner, 2002). Despite much promise in animal models and in early open-label trials, protein and gene therapy approaches to therapeutic angiogenesis so far have uniformly failed in larger, double blind controlled trials. However, even with poor data regarding efficacy, current clinical trials have demonstrated safety of angiogenic therapies in various patient populations thereby enabling further explorations of these therapies. Currently VEGF based therapy for therapeutic angiogenesis is under different stage of clinical trials (table 2.5).
Table 2.5: VEGF based therapy under different developmental stages of clinical trials

<table>
<thead>
<tr>
<th>Delivery</th>
<th>Types of Study</th>
<th>Patient No</th>
<th>Developmental Stage</th>
<th>Results</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhVEGF Protein (IC)</td>
<td>Solo therapy</td>
<td>15</td>
<td>Phase I</td>
<td>Tolerated</td>
<td>Henry et al., 2001</td>
</tr>
<tr>
<td>VEGF Gene</td>
<td>Solo therapy</td>
<td>14</td>
<td>Phase I</td>
<td>Dose dependent effect</td>
<td>Hendel et al., 2000</td>
</tr>
<tr>
<td>VEGF\textsubscript{165} (IM)</td>
<td>Solo therapy VIVA trial</td>
<td>178</td>
<td>Phase II/III</td>
<td>Tolerated, no improvement</td>
<td>Henry et al., 2003</td>
</tr>
<tr>
<td>Plasmid (VEGF-2) (IM, NOGA Guided)</td>
<td>Solo therapy</td>
<td>5</td>
<td>Phase I</td>
<td>Safe, improved myocardial perfusion</td>
<td>Losordo et al., 1998</td>
</tr>
<tr>
<td>Adenovirus (VEGF\textsubscript{165})(IM)</td>
<td>Solo therapy</td>
<td>20</td>
<td>Phase I</td>
<td>Safe, symptomatic improvement</td>
<td>Symes et al., 1999</td>
</tr>
<tr>
<td>Adenovirus (VEGF\textsubscript{165}) adenovirus or VEGF\textsubscript{165} plasmid liposome (IC)</td>
<td>Solo therapy</td>
<td>13</td>
<td>Phase I</td>
<td>Safe, improved myocardial perfusion</td>
<td>Vale et al., 2000</td>
</tr>
<tr>
<td>Adenovirus (VEGF\textsubscript{165}) adenovirus or VEGF\textsubscript{165} plasmid liposome (IC)</td>
<td>Solo therapy</td>
<td>6</td>
<td>Phase I</td>
<td>Safe, feasible</td>
<td>Vale et al., 2001</td>
</tr>
<tr>
<td>Adenovirus (VEGF\textsubscript{165}) adenovirus or VEGF\textsubscript{165} plasmid liposome (IC)</td>
<td>Solo therapy Adjunct or CAD/G solo therapy</td>
<td>29</td>
<td>Phase I/II</td>
<td>Safe, reduction in angina class</td>
<td>Vale et al., 2001</td>
</tr>
<tr>
<td>Adenovirus (VEGF\textsubscript{165}) adenovirus or VEGF\textsubscript{165} plasmid liposome (IC)</td>
<td>Solo therapy</td>
<td>21</td>
<td>Phase I</td>
<td>Tolerated</td>
<td>Losordo et al., 2002</td>
</tr>
<tr>
<td>Adenovirus (VEGF\textsubscript{165}) adenovirus or VEGF\textsubscript{165} plasmid liposome (IC)</td>
<td>Solo therapy</td>
<td>103</td>
<td>Phase II</td>
<td>Safe, no difference in clinical restenosis rate, better myocardial perfusion in Ad-VEGFgroup</td>
<td>Fortuin et al., 2003</td>
</tr>
<tr>
<td>Adenovirus (VEGF\textsubscript{165}) adenovirus or VEGF\textsubscript{165} plasmid liposome (IC)</td>
<td>Solo therapy</td>
<td></td>
<td></td>
<td></td>
<td>Rosengart et al., 1999</td>
</tr>
<tr>
<td>Adenovirus (VEGF\textsubscript{165}) adenovirus or VEGF\textsubscript{165} plasmid liposome (IC)</td>
<td>Solo therapy</td>
<td></td>
<td></td>
<td></td>
<td>Hedman et al., 2003</td>
</tr>
</tbody>
</table>
The developments of VEGF as therapeutic agents (protein, monoclonal antibody, receptor inhibitor etc.) are under different phases of clinical trials (table 2.6). Bevacizumab (rhuMab VEGF, Avastin®, Genentech Inc. USA) a humanized monoclonal antibody directed against VEGF expression for the treatment of cancer is first drug in the market. Recently RNA interference (RNAi) has re-invigorated the therapeutic prospects for inhibiting VEGF gene expression and promises many advantages over binding inhibitors, including high specificity which is essential for targeted therapeutics for the cancer (Patrick et al., 2005).

Table: 2.6 Various VEGF/VEGF receptor inhibitors under clinical investigation for the treating of different cancers.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target/mechanism of action</th>
<th>Current status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bevacizumab</td>
<td>VEGF monoclonal antibody</td>
<td>Approved for use with fluorouracil-based chemotherapy for 1st-line metastatic CRC. Ongoing evaluation in NSCLC and breast cancer shows promising efficacy data in combination with other chemotherapies.</td>
</tr>
<tr>
<td>Ranibizumab (Lucentis)</td>
<td>Lucentis is an antibody fragment to VEGF</td>
<td>Approved by U.S. Food and Drug Administration (FDA) for the treatment of neovascular wet age-related macular degeneration.</td>
</tr>
<tr>
<td>PTK787</td>
<td>VEGFR-1,-2 TKI</td>
<td>Ongoing phase III evaluation with FOLFOX for 1st- and 2nd-line treatment of metastatic CRC— reports indicate that both trials are unlikely to demonstrate a significant survival benefit.</td>
</tr>
<tr>
<td>SU11248 (Pfizer)</td>
<td>VEGFR-1,-2, PDGFR TKI</td>
<td>Ongoing phase III evaluation in second-line RCC and phase II in NSCLC.</td>
</tr>
<tr>
<td>ZD6474</td>
<td>VEGFR-1,-2,-3, EGFR TKI</td>
<td>Ongoing phase II evaluation in NSCLC and other cancer types.</td>
</tr>
<tr>
<td>VEGF-trap</td>
<td>Soluble VEGF receptor</td>
<td>Ongoing phase I evaluation in solid tumors. Preliminary data only shows evidence of stable disease.</td>
</tr>
</tbody>
</table>
2.12 Cloning of VEGF

The first human VEGF cDNAs were cloned from a phorbol ester-activated HL-60 pro-myelocytic leukemia cell library (Leung et al., 1989) and a histiocytic lymphoma cell line U937 library (Connolly et al., 1989b). The VEGF\textsubscript{165} was cloned in a baculovirus based expression system (Cohen, et al., 1992; Wu, et al., 2004; Lee, et al., 2006). Cohen et al., (1992) cloned and expressed VEGF cDNA encoding the 165 amino acid long isoform of VEGF in insect cells using the baculovirus based expression vector. Wu, et al., (2004) used baculovirus-silkworm expression system for the cloning and expression of VEGF. For cloning total RNA was extracted from the human lung tissue and VEGF\textsubscript{165} gene amplified by RT-PCR. VEGF\textsubscript{165} amplified gene cloned into pCR2.1 vector and sub-cloned into baculovirus transfer vector pBlueBacHisA. Recently Lee, et al., (2006) cloned human VEGF\textsubscript{165} from the ovarian carcinoma cell line (OVCAR3) and expressed in insect cells using the baculovirus expression vector system. The cDNA encoding the human VEGF\textsubscript{165} was amplified by RT-PCR and cloned into vector pGEM-T Easy and sub-cloned in the baculovirus expression vector pBacPAK9.

VEGF gene was also cloned in yeast based expression system (Mohanraj et al., 1995a; Mohanraj et al., 1995b; Kondo et al., 1995; Ma et al., 2001). Mohanraj, et al., (1995; Mohanraj et al., 1995b) first time cloned VEGF\textsubscript{121} and VEGF\textsubscript{165} in yeast expression system. The coding region of VEGF\textsubscript{165} was amplified from U937 cell by RT-PCR and then cloned in to yeast expression vector pHLS1 and VEGF\textsubscript{121} pPIC9 in yeast expression vector (Mohanraj, et al., 1995b; Yan, et al., 2000). The methanotrophic yeast \textit{Pichia pastoris} was used to express VEGF under the control of AOX 1 promoter. Kondo, et al., (1995) cloned the shortest isoform of human VEGF/VPF (yVEGF/VPF\textsubscript{121}) in
Saccharomyces cerevisiae. Ma, et al., (2001) cloned cDNA of hVEGF<sub>165</sub> in Pichia pastoris expression vector pPIC9K containing AOX1 promoter. The recombinant expression plasmid pPIC9K/hVEGF<sub>165</sub> was transformed to yeast host strain KM71 for expression.

Siemeister, et al., (1996) cloned VEGF<sub>165</sub> and VEGF<sub>121</sub> in E. coli. VEGF<sub>121</sub> was cloned with His<sub>6</sub>-tag in pET vector under the control of T7 RNA polymerase. VEGF<sub>165</sub> was cloned without His tag under the control of T7 RNA polymerase in pET3d. Zhou, et al., (2002) cloned gene encoding the human VEGF<sub>121</sub> and VEGF<sub>165</sub> from human lung tissue of a 4-month-old fetus into pMD18-T vector and sub-cloned into the expression plasmid pcDNA3.1 and expressed this recombinant plasmid in COS-7 cells. Zheng, et al., (2002) cloned human VEGF<sub>165</sub> cDNA from human ovarian carcinoma and inserted into eukaryotic expression vector pcDNA3.1 and expressed in COS-7.

2.13 Purification of VEGF

The cloned VEGF<sub>165</sub> was purified from conditioned medium of transfected Chinese hamster ovary cells (Ferrara and Henzel, 1989; Leung et al., 1989, Ferrara, 1993). Keyt et al., (1996) purified VEGF from Chinese hamster ovary by cation exchange chromatography and metal-chelating chromatography followed by hydrophobic interaction chromatography. In E. coli VEGF<sub>165</sub> is expressed as inclusion bodies. Keyt et al. (1996) have solubilised inclusion bodies and subsequently purified using cation exchange chromatography. The hVEGF<sub>165</sub> expressed in Pichia pastoris was purified by Heparin agarose resin and Heparin-Sepharose CL6B affinity chromatography (Mohanraj et al., 1995a; Ma et al., 2001). The baculovirus expressed VEGF<sub>165</sub> was purified by three steps.
2.14 Assays to Study Angiogenesis

The methods used for the detection of activity of angiogenic factors including VEGF include the followings in vitro and in vivo assays developed by various workers.

Lists of in vitro and in vivo assay used are listed in table 2.7.

Table 2.7: Assays to Study Angiogenesis

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Types of assay</th>
<th>Assay Method</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>in vitro assays</td>
<td>Cell proliferation</td>
<td>Mosmann, 1983</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cell migration and invasion</td>
<td>Harris and Thorgeirsson, 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tube Formation</td>
<td>Madri et al., 1988</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The aortic ring assay</td>
<td>Nicosia and Ottinetti, 1990</td>
</tr>
<tr>
<td>2.</td>
<td>in vivo assay</td>
<td>The CAM Assay</td>
<td>Auerbach et al., 1974</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The corneal angiogenesis assay</td>
<td>Gimbrone et al., 1974</td>
</tr>
<tr>
<td></td>
<td></td>
<td>The Matrigel Plug Assay</td>
<td>Passaniti et al., 1982</td>
</tr>
</tbody>
</table>