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Anne Marie Byrne
Royal College of Surgeons in Ireland

David J. Bouchier-Hayes
Royal College of Surgeons in Ireland

Judith H. Harmey
Royal College of Surgeons in Ireland

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Angiogenic and cell survival functions of Vascular Endothelial Growth Factor (VEGF)

Anne Marie Byrne, D.J. Bouchier-Hayes, J.H. Harmey *

Royal College of Surgeons in Ireland, Department of Surgery, Education and Research Centre, Beaumont Hospital, Dublin, Ireland

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Abstract

Vascular endothelial growth factor (VEGF) was originally identified as an endothelial cell specific growth factor stimulating angiogenesis and vascular permeability. Some family members, VEGF C and D, are specifically involved in lymphangiogenesis. It now appears that VEGF also has autocrine functions acting as a survival factor for tumour cells protecting them from stresses such as hypoxia, chemotherapy and radiotherapy. The mechanisms of action of VEGF are still being investigated with emerging insights into overlapping pathways and cross-talk between other receptors such as the neuropilins which were not previously associated with angiogenesis. VEGF plays an important role in embryonic development and angiogenesis during wound healing and menstrual cycle in the healthy adult. VEGF is also important in a number of both malignant and non-malignant pathologies. As it plays a limited role in normal human physiology, VEGF is an attractive therapeutic target in diseases where VEGF plays a key role. It was originally thought that in pathological conditions such as cancer, VEGF functioned solely as an angiogenic factor, stimulating new vessel formation and increasing vascular permeability. It has since emerged it plays a multifunctional role where it can also have autocrine pro-survival effects and contribute to tumour cell chemoresistance. In this review we discuss the established role of VEGF in angiogenesis and the underlying mechanisms. We discuss its role as a survival factor and mechanisms whereby angiogenesis inhibition improves efficacy of chemotherapy regimes. Finally, we discuss the therapeutic implications of targeting angiogenesis and VEGF receptors, particularly in cancer therapy.

Keywords: VEGF • angiogenesis • cell survival • tumour angiogenesis • chemotherapy • hypoxia

* Correspondence to: J.H. HARMEY,
Royal College of Surgeons in Ireland, Dept of Surgery, Education and Research Centre, Beaumont Hospital Dublin 9, Ireland.
Tel.: 353-1-809 3858
Fax: 353-1-809 3335
E-mail: jharmey@rcsi.ie

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Introduction

Vascular endothelial growth factor (VEGF) was originally described as a homodimeric 34–42 kD protein that increased vascular permeability in the skin [1]. It was identified by partial purification from the ascites fluid and cell culture supernatants of a guinea-pig hepatocarcinoma cell line and termed vascular permeability factor (VPF). VPF was the most potent permeability enhancing factor known and was responsible for the vascular hyperpermeability and the accumulation of plasma-protein-rich fluid in solid and ascites tumours [1]. In 1989 Ferrara and Henzel identified a growth factor for endothelial cells in conditioned medium from bovine follicular pituitary cells and called it VEGF [2]. This was subsequently sequenced and found to be identical to VPF [3, 4]. VEGF is required for growth and differentiation of endothelial cells [3]. In addition, it is chemotactic for monocytes, attracting these cells into sites of inflammation and tumours [5].

VEGF family and isoforms

The VEGF gene family consists of VEGF-A (hereafter referred to as VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PLGF). These glycoproteins belong to a structural superfamily of growth factors which includes PDGF. VEGF-A is mainly involved in angiogenesis while VEGF-C and VEGF-D are involved in lymphangiogenesis. VEGF mediates its signals *via* high affinity receptor tyrosine kinases which have structural and functional similarities to the platelet derived growth factor (PDGF) family, suggesting the VEGF and PDGF receptor sub families are evolutionarily linked [6].The human VEGF-A gene is organized into eight exons and alternative exon splicing results in at least 5 different isoforms, the more common isoforms consisting of 121, 145, 165, 189, 206 amino acids (termed $VEGF_{121}$, $VEGF_{145}$ $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$, respectively) (Fig. 1 and [7]). Other isoforms have also been reported consisting of 148, 162 and 183 amino acids (termed $VEGF_{148}$, $VEGF_{162}$, $VEGF_{183}$) and a more recently identified variant of $VEGF_{165}$, which is termed $VEGF_{165}b$. The splice variants are distinguished by their heparin and heparin-sulfate binding ability. The amino acids encoded by exons 1-5 are conserved in all isoforms but alternative splicing can occur in exons 6 and 7. These exons encode 2 heparin-binding domains, which influence receptor binding and solubility. The isoforms that encode this exon 6 are tightly bound to the cell surface ($VEGF_{145}$, $VEGF_{189}$ $VEGF_{206}$). The isoforms lacking exon 6 are diffusible. $VEGF_{165}$ which lacks only exon 6 is moderately diffusible where as $VEGF_{121}$ lacks both exon 6 and 7 and is therefore highly diffusible [8]. $VEGF_{165}$ is the most predominant isoform and is also the most potent in terms of stimulating angiogenesis. It can bind to both heparin and the extracellular matrix [9]. The more recently identified splice variant $VEGF_{165}b$ has the same number of amino acids as $VEGF_{165}$ but 6 amino acids in the COOH terminal region usually coded for by exon 8 are different. The COOH terminal of $VEGF_{165}$ is necessary for determining mitogenic signaling, therefore changes in this region are likely to influence function. The authors termed this new open reading frame exon 9 [10]. Unlike the other VEGF isoforms, which stimulate angiogenesis, $VEGF_{165}b$ is an endogenous inhibitory form of VEGF, which decreases VEGF-induced proliferation and migration of endothelial cells. Although it can bind to VEGFR-2 (Flk-1/KDR) $VEGF_{165}b$ binding does not result in receptor phosphorylation or activation of the downstream signaling pathways [10].

VEGF-B is a highly basic heparin binding growth factor, which is structurally similar to VEGF-A and PLGF. It is highly abundant in tissues such as heart, skeletal muscle and pancreas and may act in paracrine fashion to regulate endothelial cell function [11]. VEGF-B expression has been found in astrocytomas [12] and squamous oral cancer [13]. High levels of VEGF-B and C have been associated with lymph node metastasis of colorectal cancer [14] but its functional role in tumours has not been fully investigated.

VEGF-C was isolated from the conditioned medium of PC3 prostate cancer cells and identified as a specific activator of VEGF receptor 3 (VEGFR-3). VEGF-C binds VEGFR-3 and induces tyrosine phosphorylation of VEGF receptor 2 (VEGFR-2) and VEGFR-3 [15]. VEGF-D is also a ligand for both VEGFR-2 and VEGFR-3. Both VEGF-C and D are mitogenic for lymphatic

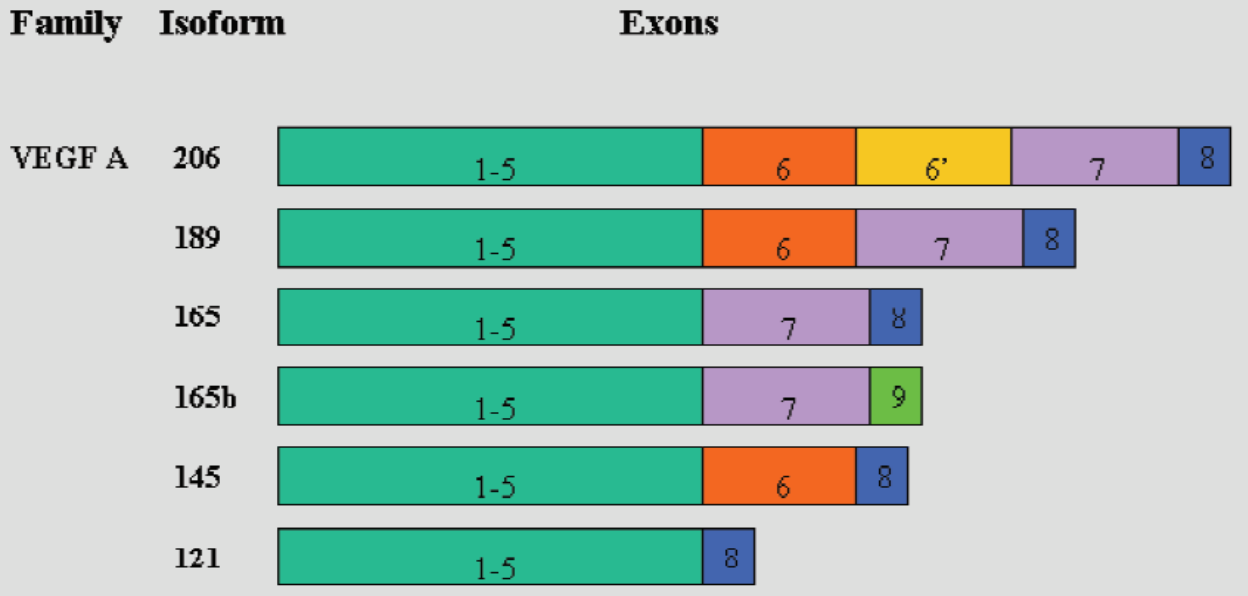

Fig. 1 VEGF-A isoforms. There are at least 6 different isoforms of VEGF-A, which arise by alternative exon splicing. VEGF165 is the most potent in terms of inducing angiogenesis whereas VEGF165b inhibits angiogenesis. All isoforms contain exons 1-5.

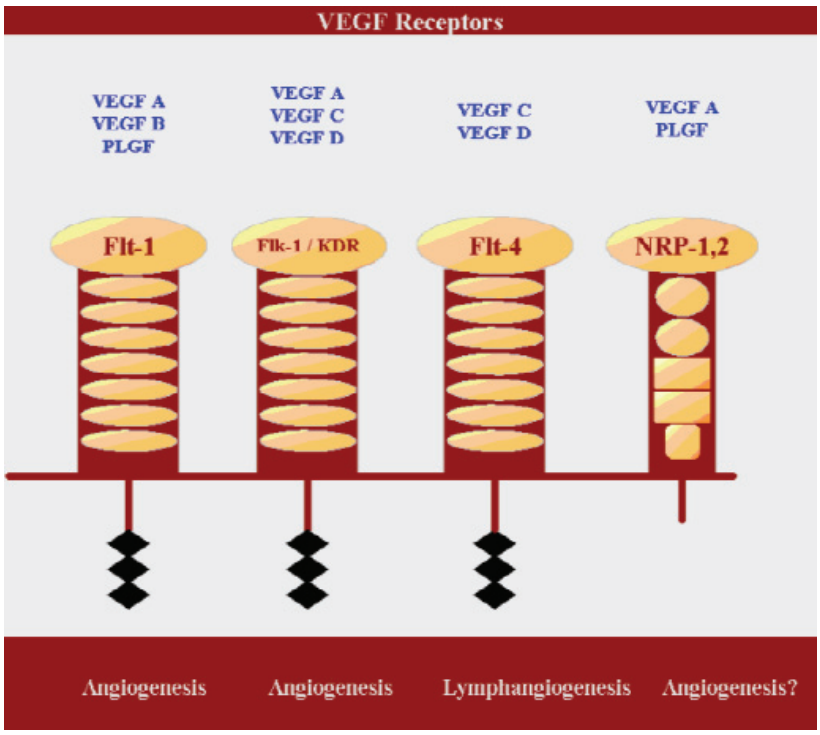

Fig. 2 VEGF receptors and their ligands. The VEGF receptors Flt-1 (VEGFR-1), Flk-1/KDR (VEGFR-2), Flt-4 (VEGFR-3), Neuropilin-1 and 2 (NRP-1,2) and the VEGF family members they bind are shown . Flt-1, Flk-1/KDR and the NRPs play a role in angiogenesis whereas Flt-4 is involved in lymphangiogenesis. Flt-1, Flk-1/KDR and Flt-4 all have tyrosine kinase activity which mediates VEGF signaling. However, the NRPs have no tyrosine kinase domain and the VEGF signaling pathway *via* NRPs is currently unknown.
VEGFR-1 VEGFR-2 and VEGFR-3 all have IgG domains.
The NRPs have an a1/a2 domain b1/b2 domain , C domain.

endothelial cells and promotes lymphatic endothelial cell survival *via* VEGFR-3 [16, 17, 18]. VEGF-C also induces hyperplasia of pre-existing lymphatic vessels [19].

VEGF-E is the viral VEGF homologue encoded by the Orf virus, which is structurally similar to VEGF-A. VEGF-E binds to and activates VEGFR-2 but not VEGFR-1 and is a potent stimulator of angiogenesis [20].

VEGF receptors

VEGF receptors were initially identified on endothelial cells. VEGF binds to the three receptor tyrosine kinases, flt-1 (fms-like tyrosine kinase, VEGFR-1), Flk-1/KDR (fetal liver kinase 1-murine homologue/Kinase insert Domain containing Receptor-human homologue, VEGFR-2) and flt-4 (VEGFR-3). VEGFR-1 and VEGFR-2 are primarily found on the vascular endothelium whereas VEGFR-3 is mostly found on the lymphatic endothelium. These receptors all have an extracellular domain, a single transmembrane region and a consensus tyrosine kinase sequence interrupted by a kinase-insert domain [21, 22]. More recently neuropilin (NRP-1), originally identified as a receptor for the semaphorin/collapsin family of neuronal guidance mediators, was shown to act as an isoform specific receptor for $VEGF_{165}$ (Fig. 2 and [23]).

VEGFR-1 (flt-1) is a 180 kD transmembrane protein, which binds VEGF-A, PLGF and VEGF-B [24]. It was originally cloned from a placental cDNA library [21]. Alternative splicing produces a shorter soluble form (soluble flt-1, sVEGFR-1) which can act as an inhibitor of VEGF [25]. The affinity of VEGFR-1 for VEGF is ten-fold higher than VEGFR-2 but its tyrosine kinase activity is ten-fold weaker than VEGFR-2. VEGFR-1 can also act as a decoy receptor preventing VEGF binding to the more mitogenic receptor VEGFR-2 [24]. In addition to endothelial cells, VEGFR-1 is also expressed by monocytes, osteoblasts, macrophages, pericytes, hematopoietic stem cells, vascular smooth muscle cells and more recently VEGFR-1 was identified on colorectal tumour cells [26–29]. VEGF signaling has been shown to occur in cells that solely express VEGFR-1, indicating that it does have mitogenic properties and in these cases sVEGFR-1 may act as the regulator for VEGF activity by preventing VEGF binding to the membrane bound VEGFR-1 receptor [30]. VEGFR-2 (KDR/flk-1) was first identified from a human endothelial cell cDNA library [22]. It is a 230 kD glycoprotein and it binds VEGF, VEGF-C and VEGF-D and has a lower affinity for VEGF than VEGFR-1. However, VEGFR-2 is the primary mediator of VEGF signaling as demonstrated by selective activation of either VEGFR-1 or VEGFR-2. Using novel highly selective VEGF mutants with substantially increased selectivity for either VEGFR-1 or VEGFR-2, it was demonstrated that VEGFR-2 is the primary mediator of VEGF signaling [31]. In addition to endothelial cells, hematopoietic stem cells, megakaryocytes, retinal progenitor cells and vascular smooth muscle cells express VEGFR-2. More recently, along with VEGFR-1, VEGFR-2 has been identified on some tumour cell lines, non-small cell lung carcinomas (NSCLCs), breast, neuroblastoma and gastric cancer cells [6, 28, 32–34]. VEGFR-3 (flt-4) is a 170 kD glycosylated protein which was first cloned from human erythroleukemia cells and placental cDNA libraries [35]. It binds the full length and mature forms of VEGF-C and is expressed in embryonic endothelial cells but during development its expression on blood vessels decreases and becomes restricted to the lymphatic endothelium in adult tissue [36].

Neuropilin-1 (NRP-1) was originally identified on neuronal cells as a receptor for the class 3 semaphorins/collapsins family of neuronal guidance mediators [37]. Endothelial cells also express NRP-1 where it acts as an isoform specific receptor for VEGF [38]. NRP-1 lacks an intracellular tyrosine kinase domain and therefore must act in conjunction with other receptors to mediate VEGF signaling. NRP-1 can associate with both Flt-1 [39] and Flk-1/KDR to transduce a signal [40]. Indeed Flk-1/KDR interacts with NRP-1 to form a receptor complex that can enhance binding of $VEGF_{165}$ [41]. NRP-1 was shown to bind $VEGF_{165}$ *via* the exon 7 domain. Interestingly, VEGF was found to bind to tumour cells which did not express VEGFR-1 or VEGFR-2 [38] and it was subsequently found that these cells express NRPs. NRP-1 expression was observed in some tumours and originally thought to be exclusively expressed by the tumour endothelium but more recently NRP-1 expression was identified on tumour cells such as the MDA-MB-231 breast tumour cells, human astrocytomas, neuroblastoma, lung, pancreatic, gastric tumour cells and colon cancer cells [42–49]. Neuropilin-2 (NRP-2) can also bind VEGF, but like NRP-1 lacks a cytoplasmic signaling domain. It can bind $VEGF_{165}$ but not $VEGF_{121}$ and unlike NRP-1 it can also bind $VEGF_{145}$ [50]. NRP-2 can also bind the heparin binding form of placental growth factor (PLGF) and can also interact with VEGFR-1 [51].

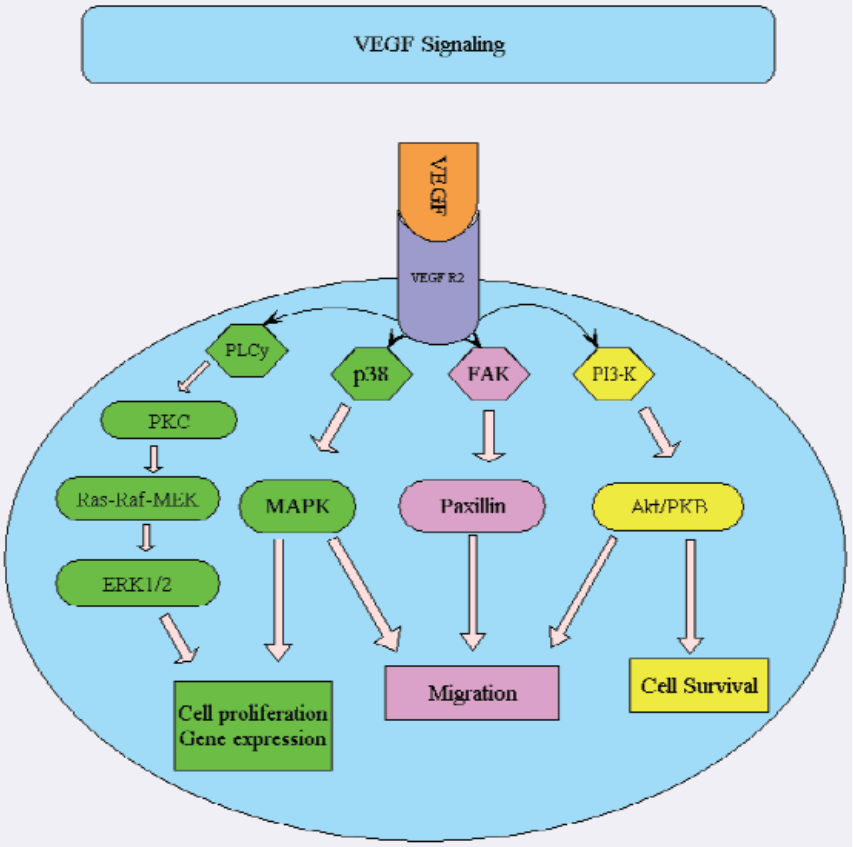

Fig. 3 VEGF signaling *via* VEGFR-2. VEGF plays a role in cell survival, migration, and proliferation of endothelial cells. VEGF binding to VEGFR-2 initiates a number of signaling cascades. PLCγ; PKC: protein kinase C; ERK: extracellular regulated kinase; MAPK: mitogen activated protein kinase; FAK: focal adhesion kinase; PI3-K: phosphatidyl inositol 3' kinase; Akt/PKB: protein kinase B.

VEGF signaling

The main functions of VEGF are to promote survival, induce proliferation and enhance migration and invasion of endothelial cells, which contribute to angiogenesis. It regulates these functions by interacting with its tyrosine kinase receptors and transmitting signals to various down stream proteins.

Cell proliferation and gene expression

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 (Fig. 3) [52, 53]. The mitogen activated protein kinase (MAPK) pathway is also implicated in cell proliferation in response to VEGF. Evidence suggests that VEGFR-2 is the receptor which mediates this, as VEGF can activate MAPK in pancreatic aortic endothelial (PAE) cells expressing VEGFR-2 where as cells expressing VEGFR-1 are unable to activate MAPK [54].

Cell survival

Under stress conditions such as serum depletion, VEGF binds to VEGFR-2, which activates the phosphatidyl inositol kinase (PI3-Kinase) pathway and Akt/protein kinase B (PKB) phosphorylation. Akt is a serine kinase involved in anti-apoptotic signaling and it is sufficient to promote survival of serum starved HUVEC. Using a PI3-K inhibitor, wortmannin, abolishes Akt activation and completely blocks VEGF mediated survival. VEGF binding to VEGFR-1 does not activate this pathway and is not involved in VEGF mediated cell survival [55].

Integrins/cell adhesion receptors such as the endothelium specific adhesion molecule αvβ3 also play a role in VEGF signal transduction. The cell adhesion molecule VE-Cadherin interacts with VEGFR-2 forming a complex with β–catenin and PI3-Kinase to promote cell survival. Disruption of the VE-Cad gene in mice prevents endothelial cells from responding to survival signals. VEGFR-1 has no association with the VE-Cad complex [56].

Migration

The fact that VEGF acts as a chemo-attractant for endothelial cells suggests it plays a role in migration and invasion. In addition to endothelial cells, VEGF also stimulates migration of vascular smooth muscle cells, monocytes, mononuclear phagocytes and polymorphonuclear cells [8, 26] and migration and invasion of some tumour cells such as breast and leukemia. The VEGF receptors VEGFR-1 and VEGFR-2 and the NRPs have all been implicated in VEGF-mediated cell migration and invasion [32, 57–59]. In tumours, increased tumour cell migration and invasion facilitates tumour cell dissemination to secondary organs - metastasis.

VEGF induces cell migration by activating factors such as focal adhesion kinase (FAK) and Paxillin and also *via* the PI3 Kinase/Akt pathway (Fig. 3). FAK activation is mediated by the c-terminal region of VEGF-R2 [60]. VEGF activation of the p38/MAPK stress pathway is also implicated in cell migration and p38 inhibitors decrease cell migration [61]. Using VEGF mutants it was determined that only VEGFR-2 and not VEGFR-1 resulted in p38 phosphorylation suggesting that VEGFR-2 is the main mediator of cell migration in endothelial cells (HUVEC) [31]. Similarly, VEGFR-1 activation had no effect on migration of bovine aortic endothelial cells [31, 62].

Receptor interaction

Along with initiating downstream effects, VEGF receptors have been shown to interact with each other in order to enhance signaling. Of special interest is the signaling of the more novel VEGF receptors, the neuropilins, which lack tyrosine kinase activity and must therefore interact with other receptors to transduce signaling. NRP-1 has been shown to interact with both VEGFR-1 and VEGFR-2, and NRP-2 forms complexes with VEGFR-1 [39, 40, 51]. NRP-1 can also act as a survival factor for tumour cells that don't express the other classic VEGF receptors [42].

In neuronal cells, Neuropilins can form complexes with the Plexin family to form functional semaphorin 3A (Sema3A) receptors which can transduce biological signals [63]. Plexins may also be important in VEGF signaling in tumour cells, which express NRPs but no VEGF receptor tyrosine kinases. Bachelder *et al.* saw expression of Plexin-A1 and Sema3A on breast tumour cell lines where NRP-1 was the only VEGF receptor and identified a competitive relationship between VEGF and Sema3A with respect to tumour cell migration [64].

Gray *et al*. (2005) [65, 66] showed that over-expressing NRP-1 in a pancreatic tumour cells line (Panc-1) inhibited *in vivo* tumour growth and decreased cell migration. They used a construct of NRP-1, which was missing the VEGF/Sema3a interacting domain as well as a full-length NRP-1 construct. Since they found the same results for both constructs they established that these signals are independent of VEGF and Sema3A. These cells do not express the other classical VEGF receptors or the NRP-1 co-receptor Plexin-A1. Decreasing NRP-1 expression using siRNA, increased tumour cell migration and in vivo tumourgenicity. However they also showed in a separate study that over-expression of NRP-1 increased chemo-resistance of the same cell line where as down regulation of NRP-1 with siRNA increased chemosensitivity [65, 66].

VEGF signaling mechanisms in tumour cells with NRP as their sole VEGF receptor are not fully understood. Different effects have been shown in relation to NRP-1 expression and tumour outcome, which may depend on the expression of its co-factors. Elucidation of the overlapping signaling pathways between neuronal and endothelial cells may help to identify VEGF signaling mechanisms *via* the neuropilins.

VEGF in embryogenesis

VEGF levels are critical in establishing a vascular network during embryo development. If a single VEGF allele is missing embryonic lethality in mice occurs between day 11 and 12 most likely as a consequence of defective vascularisation. A reduced number of nucleated red blood cells within blood islands and in the yolk sac were observed in these VEGF mutant embryos [67].

The VEGF receptors also play a crucial role in early development as targeted deletion of VEGFR-1 in mice results in death at embryo day 8.5 due to

a lack of the functional blood vessels [68]. Interestingly, when only the tyrosine kinase domain was deleted leaving the transmembrane and extracellular domains intact, the embryos developed normal blood vessels and survived. One explanation might be that VEGFR-1 is an antagonist of VEGF signaling rather than a signal transducer in embryogenesis [69]. A soluble form of VEGFR-1 (sVEGFR-1) can form a heterodimeric complex with VEGFR-2 preventing VEGF binding o the more mitogenic VEGFR-2 thus inhibiting VEGF signalling [70].

VEGFR-2 also plays a critical role in development where it is expressed in embryonic angioblasts, blood islands and angiogenic blood vessels. VEGFR-2 null mouse embryos die at day 8.5–9.5 due to impaired development of both endothelial and hematopoietic cells [68].

VEGF-C/VEGF-D signaling *via* VEGFR-3 is crucial for the development of the embryonic vascular system and also for the maintenance of the lymphatic system. Cardiovascular failure and defective remodeling of the primary vascular plexus occurs after embryonic day 9.5 if VEGFR-3 is knocked out, but vascular sprouting and network formation occurs normally [71]. Neuropilin-1 is involved in the developing neural system where it is expressed by neurons and acts as a receptor for the class 3 semaphorins, which are involved in chemorepulsive axonal guidance. It plays an essential role in the directional guidance of nerve fibers as was deduced from studies where NRP-1 null mutant mice displayed disorganized nerve pathways [72]. NRP-1 also plays a role in vasculogenesis during development. Deletion of NRP-1 in the mouse embryo results in defects in embryonic vascularisation and cell death at day 12–13 [73]. Vascularisation of both the central nervous system and peripheral nervous system is also affected. Vascular regression is observed and conversely, mice over-expressing NRP-1 have excessive blood vessel formation [74].

NRP-2 knock-out is not embryonic lethal and does not lead to an abnormal vascular phenotype but double knockout of NRP-1 and NRP-2 leads to a more severely abnormal vascular phenotype than NRP-1 knockout alone, with mouse embryos dying earlier at day 8.5. The abnormal vascular phenotype in NRP1 and 2 knockout mice is similar to VEGFR-2 deficient mice; blood islands are absent and embryonic and yolk sac blood vessels are not observed [73]. In the early developmental stages of chick embryos, NRP-1 expression is restricted to arteries whereas NRP-2 is primarily found on veins of the developing vascular system, suggesting that the neuropilins play a role in arterial/venous differentiation [75]. NRP-2 has been implicated in lymphatic vessel formation and is co-expressed in lymphatic endothelial cells with VEGFR-3. NRP-2 null mice display an absence or severe reduction in lymphatic vessels and capillaries in most tissues [76].

In summary, most of the VEGF receptors are required to mediate normal embryonic angiogenesis and development and coordinated controlled expression of VEGF and its receptors is necessary for normal growth and development.

VEGF in normal adult physiology

In normal healthy adults VEGF signaling is largely restricted to wound healing and the female reproductive cycle.

Wound healing

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 [8]. VEGF also increases permeability by affecting the endothelial cell junction proteins, which could enhance the formation of granulation tissue [77]. Receptor activation by VEGF then results in endothelial cell proliferation and migration. In addition to stimulating angiogenesis (sprouting of new vessels from pre-existing vasculature), VEGF also plays a role in vasculogenesis by recruiting endothelial progenitor cells from the bone marrow for endothelial vessel formation [78]. Finally, VEGF stimulates pericytes to coat and stabilize the vasculature [8].

Reproductive cycle

VEGF plays an important role in the female reproductive cycle as one of the primary angiogenic fac-

tors regulating follicular and luteal vascular development. It is present exclusively in the luteal connective tissue and perivascular cells. In contrast to pathological conditions, growth and angiogenesis in the female reproductive cycle is highly regulated and well coordinated [79]. 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 [80].

VEGF in non-malignant disease

Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is an autoimmune disease, which affects the peripheral and synovial joints. The synovium or lining becomes inflamed and increases in mass. Cells within the synovium invade and digest bone and cartilage, which leads to the destruction of the joints. Angiogenesis is recognized as playing an important role in the maintenance and progression of RA. VEGF is important in this disease by stimulating vascular permeability and angiogenesis. Endothelial proliferation is higher in synovial fluid in RA patients compared to normal controls [81]. The increase in the volume of synovial fluid induces a hypoxic state by increasing the distance between proliferating cells and the nearest blood vessels. Increased proliferation of the synovial cells increases the demand for oxygen and nutrients thus increasing the hypoxic environment which then stimulates angiogenesis [82]. Serum VEGF levels are elevated in RA patients, the local hypoxia in the joints probably contributing to these elevated levels [83]. Radiography studies showed that the level of serum VEGF correlates to the level of joint damage [84]. Other pro-angiogenic factors are also elevated in the synovial fluid of RA patients such as Fibroblast Growth Factors (FGFs) and PDGF [85]. Both $VEGF_{121}$ and $VEGF_{165}$ are expressed in RA synovial fluid along with the VEGF receptors Flt-1, KDR and NRP-1, all of which are elevated compared to control patients [86].

The available evidence therefore suggests that angiogenesis plays a crucial role in RA and targeting VEGF may be of therapeutic benefit in this condition. Studies in animal models of RA showed that treatment with the soluble form of Flt-1 (sFlt-1), which inhibits VEGF, significantly reduced joint inflammation and reduced bone and cartilage destruction [87].

Diabetes and ischemic retinopathies

Plasma VEGF is elevated in diabetic patients. Elevated blood glucose exerts toxic effects on the endothelium. It can induce a hyperglycemic pseudo-hypoxic state which in turn induces VEGF production [88]. This has an effect on the endothelium and plasma VEGF levels have been shown to correlate to endothelial damage and dysfunction in diabetes [89]. More extensively studied is its role in diabetic retinopathy. Excessive secretion of VEGF in the retina leads to ocular neovascularisation, hemorrhages and it also promotes vascular permeability, which results in visual impairment/blindness. VEGF also contributes to high ocular fluid levels found in patients with this disease [8]. Elevated levels of VEGF are found in the aqueous humor of glaucoma patients providing further evidence that VEGF is an important factor in the pathology and intraoccular neovascularisation in patients with ocular disease [90].

Since VEGF is implicated in the pathological development of retinal neovascularisation in ischemic retinopathies, several strategies targeting VEGF have been undertaken. An animal model of ishemia-induced retinal neovascularisation was treated with a chimeric protein of the VEGF receptors VEGFR-1 and VEGFR-2. A single intravitreal injection of the chimeric protein into the eye decreased retinal neovascularisation by up to 100% compared to those treated with a control chimeric protein [91]. In another study using a mouse model of ischemic retinopathy, treatment by gavage with a drug (PTK787) that blocks phosphorylation by VEGF and PDGF receptors led to a complete inhibition of retinal neovascularisation [92]. Both of these results along with the elevated levels of VEGF found in these pathologies indicate that VEGF signaling plays an important role in ischemic retinopathies making VEGF an ideal target molecule in these diseases.

Psoriasis

Psoriasis is a chronic skin condition caused by inflammatory cell infiltrate and proliferation of blood vessels. The reddened appearance of the skin is caused by highly abnormal dermal blood vessels, which are highly permeable and lead to edema [93]. Other characteristics include dermal thickening and keratinocyte differentiation. The keratinocytes over-express VEGF and its receptors, which leads to neovascularisation [94]. In the psoriasis patient, the skin is predisposed to the initiation of angiogenesis if the correct stimuli are present. Excess VEGF in the skin can induce a vascular inflammatory response resulting in a more widespread tissue inflammation [93].

In mice chronic transgenic delivery of VEGF to the skin induces inflammation and all the characteristics of psoriasis suggesting a causative role for VEGF in this disease. Using a potent VEGF antagonist, the VEGF-Trap, reverses this phenotype [93]. Current treatment involves immunosuppressive and anti-inflammatory drugs which have toxic effects and are not always effective. Targeting VEGF may be a novel approach to the treatment of psoriasis. As a corollary, the treatment of other conditions with VEGF stimulants could possibly result in chronic skin inflammation as a side effect.

VEGF in malignant disease

Tumour angiogenesis

In order to grow beyond 2-3 mm^3 tumours require a vascular supply to provide nutrients and oxygen. They achieve this by secreting angiogenic growth factors such as VEGF in order to recruit a vasculature from pre-existing blood vessels (angiogenesis). VEGF is secreted by the tumour cells themselves and infiltrating immune cells such as monocytes [77]. VEGF binds to its receptors on the pre-existing endothelium, stimulating endothelial cell proliferation and migration into the tumour resulting in vascular sprouting. These sprouts ultimately form new blood vessels within the tumour. VEGF also plays a role in vasculogenesis by recruiting endothelial progenitor cells from the bone marrow for endothelial vessel formation [77]. The new vasculature also provides the tumour with a route by which tumour cells can get into the blood circulation and form distant metastases [95].

Elevated VEGF production by tumours is associated with increased tumour vascularity, metastasis, chemoresistance and poorer prognosis compared to VEGF-negative tumours. Circulating VEGF is elevated in breast, lung and gastrointestinal cancers [96]. In post operative samples, breast tumours over-expressing VEGF were associated with early relapse compared to tumour samples with low levels of VEGF [97]. VEGF expression therefore can act as a prognostic factor where high levels of VEGF in the circulation or tumour tissue is negatively correlated to relapse free and overall survival. It is unknown as of yet if this is solely due to increased angiogenesis in a tumour or if VEGF plays other roles in the progression of cancers.

Angiogenic switch

Angiogenesis depends on the balance of endogenous pro-angiogenic stimulators and anti-angiogenic inhibitors. Tumour growth creates an imbalance by secreting pro-angiogenic factors to initiate angiogenesis, the "angiogenic switch"[98]. It is not yet understood why some tumours remain dormant while others initiate angiogenesis and progress to a malignant phenotype. Nyberg *et al.* (2005) [99] suggested that the physiological balance between the endogenous inhibitors and stimulators could predict the individuals' predisposition to the switch in pathological conditions. If an individual has more endogenous stimulators than inhibitors the balance favors angiogenesis whereas if the individual has more endogenous angiogenesis inhibitors it is more difficult to switch to an angiogenic phenotype [99].

Autocrine effects of VEGF

It was originally thought that tumour cells secrete VEGF in a paracrine manner in order to attract and stimulate proliferation of endothelial cells. It has since been shown that VEGF can also act in an autocrine manner having a protective/survival effect on a number of cell types including endothelial cells, embryonic stem cells and hematopoietic stem cells [100–102].

We and others have also shown that VEGF can act as an autocrine survival factor for the tumour cells themselves [42, 103]. We showed that blocking VEGF with neutralising antibodies induced apoptosis of two cell lines, the 4T1 murine mammary adenocarcinoma and the human MDA-MB-231 [104]. Similarly, blocking VEGF binding to NRP-1 also induced apoptosis of these cells [43]. An elegant study by Dias *et al.* showed that inhibition of both the paracrine and autocrine VEGF signaling pathways was required to achieve complete remission of human leukemia xenografts [105].

Tumour cells have been shown to secrete elevated levels of VEGF under stressed conditions such as serum starvation (*in vitro*), hypoxia, radiation and chemotherapy [106–109]. Inadvertently, stresses such as radiotherapy or chemotherapy can therefore contribute to enhanced survival of tumour cells due to elevated VEGF and render them less sensitive to conventional chemotherapy and radiotherapy treatments [110, 111]. This chemoprotectant effect requires VEGF signaling *via* the PI3-Kinase pathway. More recently a member of the inhibitor of apoptosis family, survivin, has been implicated in VEGF-mediated chemo-resistance. Survivin levels increased 10–20 fold in endothelial cells stimulated with VEGF. PI3-Kinase inhibition down regulated VEGF-mediated survivin expression. Inhibition of survivin also decreased VEGF mediated chemoresistance of endothelial cells. VEGF signaling through survivin preserves cellular integrity in the presence of chemotherapy by stabilizing the microtubule network and maintaining their cytoskeletal integrity [112]. Tumour cell expression of survivin has also been shown and a correlation to its apoptotic function is linked to its ability to inhibit caspases, which could also contribute to chemoresistance [113].

Targeting VEGF should block both the pro-angiogenic (paracrine) and pro-survival (autocrine) effects of VEGF including survivin inhibition, and thereby sensitise tumour cells to conventional therapies both by reducing angiogenesis and blocking an autocrine survival effect [101].

Tumour lymphatics

The lymphatics in a tumour also play a role in its growth and metastases. Generally the lymphatics within the tumour are compressed and non functional, but they are enlarged at the periphery due to excess VEGF C expression. These lymphatics collect interstitial fluid and metastatic tumour cells resulting in lymphatic metastases [19].

VEGF and hypoxia

All cell types, including tumour cells, require oxygen for energy production and tissue function. Lack of an adequate blood supply and increasing distances from existing blood vessels means tumour cells are starved of oxygen. This leads to areas of hypoxia or even anoxia in the tumour. In tumours, hypoxia can lead to malignant progression by inducing adaptive changes in tumour cells to enable survival in this harsh environment. These include changes in gene expression, inactivation of tumour suppressor genes, activation of oncogenes, genomic instability and clonal selection. The harsh environment exerts a strong selective pressure on tumour cells selecting for the most malignant in a "survival of the fittest" mechanism. The cells adapted to hypoxia proliferate and survive better than the non-adaptive ones and become the predominant population in the tumour leading to a more aggressive phenotype. Down regulation of adhesion molecules in response to hypoxia increases tumour cell detachment and thus metastasis [114].

Other problems associated with tumour hypoxia are the direct and indirect effects it has on chemo-resistance. Some chemotherapy drugs such as alkylating agents are less effective in hypoxic conditions. In response to hypoxia, the cells cycle slows down or arrests in G1. Since some chemotherapies act at the S1 phase of the cell cycle inhibiting DNA synthesis they are thus less effective in a hypoxic environment [115]. Hypoxia selects for mutations in the p53 tumour suppressor gene. Cells with mutations in p53 are resistant to DNA damage induced apoptosis and are more likely to be chemoresistant than cells with wild type p53. p53 mutation results in decreased hypoxia mediated apoptosis and increased VEGF production [116]. Induction of the multi-drug resistant protein (MDR) gene expression has also been observed under hypoxia [117].

In radiotherapy hypoxia poses a problem also as radiotherapy acts on rapidly proliferating cells and depends on the presence of oxygen to increase the reactive oxygen species. Radio-sensitivity decreases at low tumour oxygen concentrations. Oxygen actually enhances radiation as it increases DNA damage by formation of oxygen-derived free hydroxyl radicals, which occurs directly after radiotherapy. In fact the dose of radiation required for tumour cell apoptosis is 2–3 times higher in hypoxic conditions than normoxic [118].

In the clinical setting tumour hypoxia correlates to shorter survival compared to patients with hypoxia free tumours. A correlation between tumour hypoxia and malignant progression in uterine cervical cancer has been established and it can act as a marker of poor outcome and overall survival in soft tissue and head and neck cancers [119].

In response to hypoxia, tumours secrete angiogenic growth factors to stimulate vessel growth and oxygen delivery. This is mediated by a hypoxia inducible factor (HIF-1), which modulates the expression of a number of hypoxia inducible genes and angiogenic factors such as VEGF. HIF-1 consists of 2 subunits HIF-1α and HIF-1β. HIF-1α is a cytoplasmic protein and is responsive to oxygen levels whereas HIF-1β is a nuclear protein expressed independently of oxygen tension. In normoxia, HIF1α is rapidly degraded. In response to hypoxia, HIF-1α is stabilized and translocates to the nucleus, heterodimerises with HIF-1β forming an active HIF-1 protein. This protein binds to specific hypoxia response elements (HREs) within the promoter of hypoxia-inducible genes and activates transcription [120]. Another protein involved in hypoxia-induced VEGF expression is HuR. This protein binds and stabilizes the VEGF mRNA message and promotes transfer to the cytoplasm [121].

VEGF is also up regulated in response to hypoxia in physiological situations including wound healing, ovulation and atherosclerosis. Up-regulation is also observed in patients with diabetic retinopathy. The presence of hypoxia in tumours reflects a poorer prognosis with increased treatment failure and decreased overall survival. Overcoming hypoxia in the tumour environment should lead to a better outcome.

Targeting VEGF as cancer therapy

Since VEGF plays such an important role in tumour progression and metastasis it is an attractive target in the treatment of cancer. In tumours, the vasculature is haphazard, disorganized and comprises of leaky blood vessels and excessive branching. It is structurally and functionally abnormal. This leads to poor drug delivery and hypoxic areas within the tumour. Interstitial fluid pressure is elevated in a tumour due to the hyperpermeable vessels, which also impairs drug penetration [122]. Targeting the tumour vasculature deprives it of the nutrients and oxygen necessary for growth. This approach should also inhibit metastasis. Unfortunately there are no tumour specific anti-angiogenic compounds and thus, to date, treatment with anti-angiogenics alone is unable to cause tumour regression. However, these agents are likely to prove useful in disease stabilization. Increasing the dose of these compounds can have an adverse effect on the normal vasculature thereby limiting its use. In combination with conventional chemotherapy, anti-angiogenic strategies have a synergistic effect.

Many anti-angiogenic strategies have been undertaken to date including anti-VEGF antibodies such as bevacizumab (Avastin, Genentech Inc), soluble VEGF receptor analogues such as the VEGF TRAP, small molecule VEGF inhibitors such as SU5416, SU11248, PTK787/ZK222584 and ribozymes such as angiozyme [123]. Targeting the more novel VEGF receptor NRP-1 with peptides has also shown some promising pre-clinical data [43, 124].

Normalisation theory

Although reducing tumour vasculature inhibits tumour growth and metastasis, it might also be expected that a decrease in tumour vasculature would decrease the efficacy of drug delivery. This however is not the case as anti-angiogenic therapy actually increases the efficacy of chemotherapy or radiotherapy [106, 125]. To explain this paradox, Rakesh Jain suggested that treating the tumour with an anti-angiogenic compound attacked the less stable, leaky blood vessels first. This in effect creates a more normal stable vasculature in the tumour environment, which can now deliver oxygen and nutrients more efficiently

[126]. One would expect however that the increase in nutrients and oxygen delivery to the tumour would increase tumour growth. Cytotoxic drug therapy during this normalization period is more efficiently delivered throughout the tumour, as chemotherapy delivery is usually impaired due to the lack of an efficient delivery system (vasculature). The increase in oxygen may increase proliferation of cells but chemotherapy and radiotherapy actually work better on rapidly proliferating cells [127]. In addition, as VEGF production is increased in response to cytotoxic therapy or radiotherapy, anti-angiogenic therapies that block VEGF will also block the autocrine pro-survival activity of VEGF rendering the cells more sensitive to chemotherapy and radiotherapy [103].

The more efficient oxygen delivery with the normalized vasculature decreases the hypoxic areas of the tumour. Hypoxia as discussed above can actually enhance tumour progression by increasing genetic instability and selecting for tumour cells with a more resistant phenotype capable of surviving in this environment. Hypoxic cells also have an increased metestatic potential [115]. Decreasing the hypoxic areas with this normalisation process should render the cells more sensitive to the chemotherapy that is now delivered more efficiently. The anti-angiogenic drugs may also target the tumour cells themselves most notably those expressing the VEGF receptors and where VEGF acts as a survival factor. The normalised vasculature is less likely to be permeable to shedded tumour cells, which form distant metastases [126, 127].

This combination therapy should enhance tumour treatment as it increases chemo and radio delivery, decreases hypoxia and sensitises tumour cells by overcoming the production of survival factors. It should also decrease the number of metastases. Doses and treatment schedules need to be carefully selected to achieve this normalization window where it is optimal to treat with chemotherapy. In support of the normalization hypothesis, a number of clinical trials have shown synergistic effects of anti-angiogenics in combination with chemotherapy [128, 129].

Metronomic therapy: chemotherapy as anti-angiogenic therapy

The initial approach to the treatment of tumours was to target the tumour cells with the maximum tolerated doses (MTD) of a cytotoxic agent. This unfortunately leads to many undesirable side effects and damage to normal cells such as bone marrow progenitors, hematopoietic stem cells and hair follicles. It can also lead to myelosuppression. To overcome these toxic effects a treatment free period in the chemotherapy schedule is required to allow normal cells to recover. Unfortunately during this period the tumour cells and tumour associated endothelial cells can also recover and the tumour cells may become resistant to further treatment [130].

A new schedule was proposed by Kerbel *et al.* (2004) [130] to decrease the dose of chemotherapy but to increase the frequency of administration. By doing this the intervals between doses can be decreased as the toxic side effects are reduced. This has been termed “metronomic dosing” [130]. The shorter interval time did not allow for the endothelial and tumour cells to recover and tumour cells were less likely to become resistant to the chemotherapy. By using this regimen, an increase in apoptosis of endothelial and tumour cells was observed and overall tumour growth was suppressed more efficiently than by using the conventional MTD dosing and schedules [131]. The reasons this therapy is more efficacious are not fully understood but there are a number of hypotheses put forward.

One of the main hypotheses is that the metronomic dosing acts as an anti-angiogenic therapy. Since the endothelial cells proliferate at a lower rate than the tumour cells, they are less affected by the chemotherapy drugs, which act more efficiently on rapidly dividing cells. The recovery of the endothelial cells during the drug free periods can support the growth and emergence of chemoresistant tumour cells. Using metronomic treatment targets these endothelial cells in the tumour bed as it is given in a continuous mode. This results in tumour cell apoptosis *via* an anti-angiogenic mechanism as opposed to a direct cytotoxic effect and is independent of the resistance to chemotherapy. This has also been termed the anti-angiogenic schedule [130].

Along with recruiting a blood supply from pre-existing blood vessels (angiogenesis) tumours can acquire a vasculature by recruiting progenitor cells from the bone marrow for the generation of blood vessels (vasculogenesis). This involves the

mobilization of viable circulating endothelial progenitors (CEPs) to the tumour. The induction of myelosuppression by the chemotherapy causes a response by the host, which is seen by the marked increase and mobilization of hematopoietic progenitors from the bone marrow to the peripheral blood stream. With treatment using the conventional MTD schedule a robust CEP mobilization is observed at the end of the drug cycle, during the drug free interval. The influx of CEPs to a tumour replaces damaged endothelial cells thereby promoting tumour growth. An increase in CEPs parallels tumour growth and a correlation between circulating endothelial cells (CECs) and VEGF in tumour bearing mice has been observed [132]. In contrast to the MTD schedule, CEPs were not mobilized in response to the metronomic schedule and CEP viability was decreased. An increase in CEP apoptosis was also observed. The anti-vasculogenesis effects of metronomic dosing support the hypothesis that it directly targets the endothelial cells and thereby indirectly targets the tumour cells [133].

A secondary effect observed by Boci *et al.* was an increase in the levels of endogenous angiogenesis inhibitor thrombospondin-1 (TSP-1) in the plasma of mice treated with metronomic chemotherapy [134]. TSP-1 is highly specific for endothelial cells where it inhibits proliferation and induces apoptosis. Since metronomic therapy induces TSP-1 it may be the reason why endothelial cells are specifically targeted. TSP-1 like other endogenous inhibitors may also decrease the CEPs which would also support the previous theory [134].

This novel approach to chemotherapy treatment suggests that anti-angiogenic scheduling of chemotherapy may improve outcome and reduce side effects seen with the conventional MTD chemotherapy regimes. Combining chemotherapy with a specific anti-angiogenic compound such as a neutralising antibody of VEGFR-2 (DC101) was shown to completely eradicate an orthotopically transplanted multi drug resistant human breast tumour (MDA-MB 231 and MDA-MB 435 variants) in SCID mice [135]. This metronomic therapy is actually an indirect (by increasing TSP-1 levels and effect on CEPs) as well as direct (cytotoxic to endothelial cells) attack on the tumour vasculature and provides a novel approach for using chemotherapy as an anti angiogenic as opposed to simply a cytotoxic agent in the treatment of tumours.

Conclusions

VEGF plays important roles in angiogenesis and cell survival pathways. In healthy adults its role is mainly limited to angiogenesis during wound healing and the menstrual cycle. It also plays a crucial role during embryogenesis. VEGF plays important roles in a number of human pathologies especially in cancer. Tumours secrete VEGF to stimulate new vessel formation, particularly in response to hypoxia. These new vessels provide oxygen and nutrients to the tumour but also allow tumour cells access to the circulation facilitating metastasis. In addition to stimulating angiogenesis, it appears that VEGF may also have autocrine functions acting as a survival factor for tumour cells protecting them from stresses such as hypoxia, chemotherapy and radiotherapy. Angiogenesis and VEGF in particular, are attractive targets for anti-cancer strategies and blocking VEGF has been shown to both block angiogenesis and improve the efficacy of chemotherapy and radiotherapy.

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