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FGF and VEGF function in angiogenesis: signalling pathways, biological responses and therapeutic inhibition

Michael J. Cross and Lena Claesson-Welsh

Angiogenic growth factors such as fibroblast growth factors (FGFs) and vascular endothelial growth factors (VEGFs) are currently targets of intense efforts to inhibit deregulated blood vessel formation in diseases such as cancer. FGFs and VEGFs exert their effects via specific binding to cell surface-expressed receptors equipped with tyrosine kinase activity. Activation of the receptor kinase activity allows coupling to downstream signal transduction pathways that regulate proliferation, migration and differentiation of endothelial cells. Inhibitors of FGF and VEGF signalling are currently in clinical trials. In this article, the current knowledge of FGF- and VEGF-induced signal transduction that leads to specific biological responses will be summarized. Furthermore, the manner in which this knowledge is being exploited to regulate angiogenesis will be discussed.

Angiogenesis denotes the formation of new blood vessels from pre-existing vessels. Physiological angiogenesis, which is required for embryonic development, wound healing and the menstrual cycle, is characterized by tight regulation both spatially and temporally. Angiogenic factors, such as fibroblast growth factors (FGFs) and vascular endothelial growth factors (VEGFs), stimulate endothelial cells to secrete several proteases and plasminogen activators, resulting in the degradation of the vessel basement membrane, which in turn allows cells to invade the surrounding matrix. The cells migrate, proliferate and eventually differentiate to form a new, lumen-containing vessel. Finally, the endothelial cells deposit a new basement membrane and secrete growth factors, such as platelet-derived growth factor (PDGF), which attract supporting cells such as pericytes, ensuring the stability of the new vessel[1]. This is a complex process that involves the concerted action of several other factors, such as the angiopoietins and ephrins, that act on specific receptors to regulate vessel stability[2].

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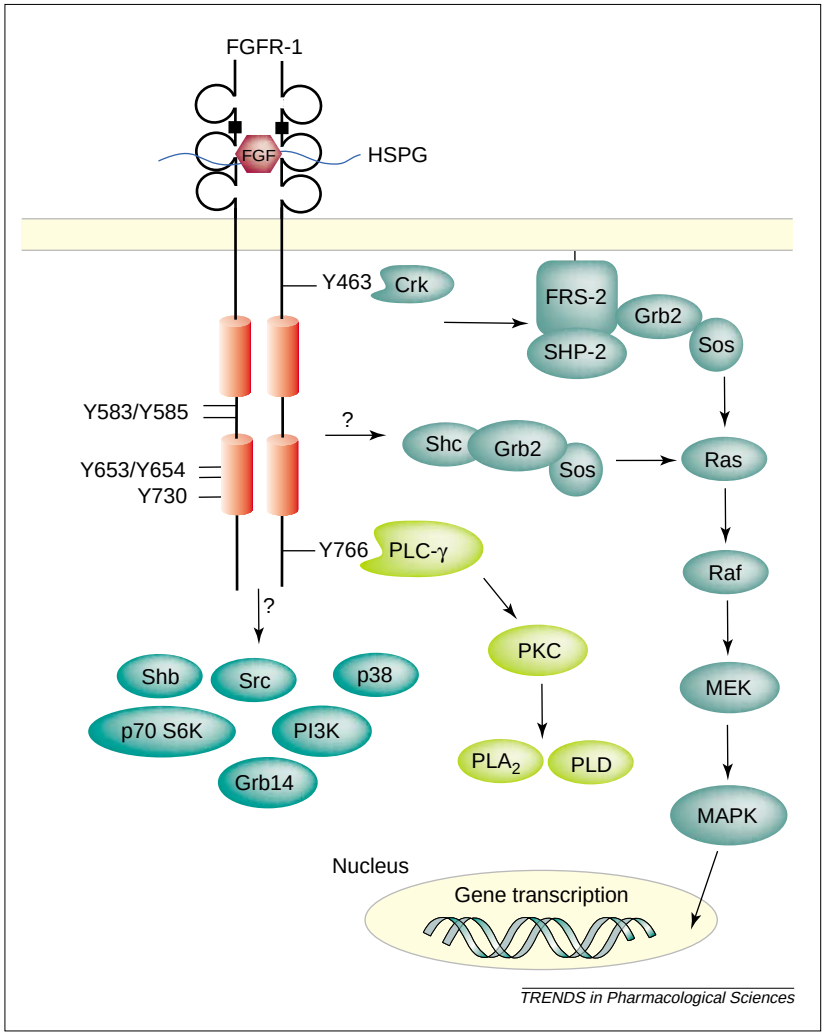

Fig. 1. Overview of FGFR-1 signalling. Binding of FGF results in receptor dimerization and the phosphorylation of specific tyrosine residues within the intracellular domain of the receptor (the positions of phosphotyrosine residues in the receptor amino acid sequence are shown). Several intracellular signalling proteins are activated either directly via receptor binding, such as Crk and PLC-γ, or via indirect mechanisms, such as Shc and FRS-2. Several other proteins, such as Src, Shb, p38 MAPK, PI3K, p70 S6K and Grb14 are also activated via FGFR-1, although their exact mechanism of activation has not been determined (indicated by ?). Abbreviations: FGF, fibroblast growth factor; FGFR-1, fibroblast growth factor receptor 1; FRS-2, fibroblast growth factor receptor substrate 2; Grb2, growth factor receptor-bound 2; Grb14, growth factor receptor-bound 14; HSPG, heparan sulfate proteoglycan; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; p70 S6K, p70 ribosomal S6 kinase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC-γ, phospholipase C-γ; PLD, phospholipase D; Shc, Src homology and collagen; SHP-2, SH2 phosphatase 2.

Several pathological conditions, such as tumour progression, rheumatoid arthritis and diabetes, are characterized by excessive angiogenesis where vessels develop in an uncontrolled or disorganized manner. The now generally accepted concept that growth of most types of tumours requires angiogenesis was put forward by Judah Folkman in the early 1970s. Thus, the dormant cancer *in situ* can expand once it has acquired the ability to disturb the balance between the production of stimulatory factors and the production of inhibitory factors, thereby promoting the angiogenic switch[3]. Many different tumour cells secrete VEGF, the expression of which is regulated by hypoxia. However, in general, tumours appear to produce more than one type of endothelial growth stimulus (e.g. FGF and VEGF) or might, by genetic drifting, produce different stimulators over time[4]. Recently, novel anti-angiogenic agents have been developed that inhibit the action of FGF and VEGF. Several of these compounds are now in clinical trials and might offer hope in the treatment and management of several diseases.

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FGF and VEGF: the angiogenic factors

FGF ligands and receptors

Basic fibroblast growth factor [bFGF (also known as FGF-2)] was the first pro-angiogenic molecule to be identified[5]. At present, the FGF family is known to contain at least 20 factors, which are ~30–70% identical in their primary amino acid sequences. The classical FGFs, FGF-1 and FGF-2, lack cytoplasmic sequences for extracellular export, in contrast to most growth factors that are secreted from their producer cells. The apparent lack of regulated FGF export has been an obstacle in the wide acceptance of a crucial role for FGF in angiogenesis; however, there are several working models for alternative modes of transport out of the cell rather than via the classical secretory apparatus[6]. FGFs bind with high affinity to heparan sulfate proteoglycans (HSPGs), which are located on the surface of most cells and within the extracellular matrix[7]. This pool of FGFs constitutes a reservoir of the growth factor that can be released in a regulated manner (e.g. by the action of heparanases). HSPGs also serve as co-receptors for FGF and modulate the effects of FGF both *in vitro* and *in vivo*[8].

The biological effects of FGFs are mediated by four structurally related receptor tyrosine kinases, denoted FGFR-1, -2, -3 and -4, which display broad expression patterns. Alternative splicing of the FGFR mRNA generates receptor variants[9,10], which display a range of receptor–ligand interactions[11]. Disruption of the genes encoding FGFR-1 or FGFR-2 leads to embryonal death before gastrulation[12,13]. This early lethality has made it impossible to define specifically the role of these FGF receptors in the later stages of development and in angiogenesis. However, recent work using adenovirus-mediated expression of dominant-negative FGFR-1 in mouse embryos has shown that FGFR-1 is required for the development and maintenance of the vasculature in the embryo[14]. By contrast, inactivation of the gene encoding FGF-2 results in mice that are morphologically normal but display decreased vascular tone and low blood pressure[15]. When reconciled with the receptor knockout data, this suggests redundancy in the FGF family. Disruption of the gene encoding FGFR-3 results in mice with skeletal abnormalities[16], whereas the result of inactivation of the gene encoding FGFR-4 has not been reported. Recent crystallography studies have revealed that although monomeric FGF-2 binds with low affinity to the FGFR-1 in the absence of heparin, the binding is stabilized by the presence of heparin or heparan sulfates, resulting in the formation of a

Table 1. FGFR-1 signalling mechanisms[a]

Signalling molecule	Receptor binding site	Activated via	Signalling cascade regulated	Physiological role	Refs
Crk	Y463	FGFR-1 binding	Activation of MAPK, Jun kinase	Proliferation	19
PLC-γ	Y766	FGFR-1 binding	Ins(1,4,5)P_3-mediated Ca^{2+} release and DAG generation	Cytoskeletal reorganization, receptor endocytosis	20,52,53
Unknown	759–774	Unknown mechanism	Unknown	Migration	54
PKC	–	PLC-γ-mediated DAG	Protein phosphorylation, regulation of PLA_2, PLD	Unknown	53
FRS-2	–	Crk/JM region of FGFR-1	Activation of p42/44 MAPK cascade	Proliferation	19,55,56
SHP-2	–	Binding to FRS-2	Sustained p42/44 MAPK activation	Differentiation	57
Src	–	FGFR-1 activation	Unknown	Differentiation	58
Shc	–	FGFR-1 activation	p42/44 MAPK	Proliferation, differentiation	58
Grb14	–	FGFR-1 binding	p42/44 MAPK?	Proliferation?	59
PI3K	–	Y766F, downstream of PLC-γ?	Rac, Akt activation?	Migration, survival?	53
p70 S6K	–	Unknown mechanism	Unknown	Proliferation	60
p38 MAPK	–	Unknown mechanism	Unknown	Proliferation, survival	61
Shb	–	FGFR-1 activation	Unknown	Differentiation, apoptosis	62

[a]Abbreviations: DAG, *sn*-1,2-diacylglycerol; FGFR-1, fibroblast growth factor receptor 1; FRS-2, FGF receptor substrate 2; Grb14, growth factor receptor-bound 14; Ins(1,4,5)P_3, inositol (1,4,5)-trisphosphate; JM, juxtamembrane; MAPK, mitogen-activated protein kinase; p70 S6K, p70 ribosomal S6 kinase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC-γ, phospholipase C-γ; PLD, phospholipase D; Shc, Src homology and collagen; SHP-2, SH2 phosphatase 2.

complex of two FGF molecules and two FGFRs (Ref. 17). Receptor dimerization results in the intermolecular autophosphorylation of specific tyrosine residues within the dimeric complex. Several autophosphorylation sites have been identified in FGFR-1 (Ref. 18) (Fig. 1). Some of these sites have been assigned a particular function in FGFR-1 signal transduction: Y463 in the juxtamembrane region is responsible for binding the small adaptor molecule Crk (Ref. 19); Y653 and Y654 in the second kinase domain are crucial for kinase activity; and Y766 is the binding site for phospholipase C-γ (PLC-γ)[20]. Several intracellular signalling cascades are known to be activated by FGFR-1-mediated signalling, including the Ras pathway, Src family tyrosine kinases, phosphoinositide 3-kinase (PI3K) and the PLC pathway (Table 1).

VEGF ligands and receptors

VEGF was initially termed vascular permeability factor (VPF) because of its ability to induce vascular leakage[21]. The VEGF family currently comprises six members: VEGF-A (which denotes the originally identified VEGF), placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and the orf parapox virus VEGF, referred to as VEGF-E. Alternative exon splicing of the gene encoding VEGF-A results in the generation of at least five molecular variants that differ in total amino acid number. In humans, these correspond to VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{165}, VEGF-A_{189} and VEGF-A_{206}, of which VEGF-A_{165} is the predominant form. This variant is secreted by a broad variety of cells and is a heparin-binding disulfide-linked homodimeric molecule, although heterodimers of VEGF-A_{165} and PlGF have also been identified[22]. VEGF expression is transcriptionally regulated by hypoxia[23,24], which occurs during tumour expansion and ischaemia. The importance of VEGF in vascular development is highlighted by the fact that loss of a single VEGF-A allele results in abnormal blood vessel development and embryonal death[25,26].

The biological effects of VEGFs are mediated via three specific cell surface-expressed receptors, VEGFR-1 (Flt-1), VEGFR-2 (KDR or Flk-1) and VEGFR-3 (Flt-4). All three consist of an extracellular domain comprising seven Ig-like domains, a transmembrane domain, followed by a kinase domain that is divided in two parts by the insertion of a non-catalytic 100-amino-acid residue sequence, and a C-terminal tail (Fig. 2). VEGFR-1 and VEGFR-2 are mainly expressed on endothelial cells, although other cell types of haemopoietic or other origins can also express these receptors[27]. VEGFR-3 is found mainly in the lymphatic endothelium; moreover, this is not a receptor for VEGF-A (Ref. 28). Gene knockout studies have revealed that both VEGFR-1 and VEGFR-2 are essential for the development of the vasculature in mouse embryos[29,30]. In the absence of VEGFR-2, haemangioblasts fail to differentiate into endothelial cells. However, in the absence of VEGFR-1, the vascular defect is in fact due to an increase in the number of haemangioblasts, the endothelial cell progenitors[31]. This suggests that in the embryo, VEGFR-1 is required to suppress excessive haemangioblast development, possibly by sequestering VEGF. This is also supported by the finding that the cytoplasmic domain of the VEGFR-1 is not required for vascular development[32]. As for VEGF, hypoxia has been shown to regulate both VEGFR-1 and VEGFR-2 expression[33,34], although

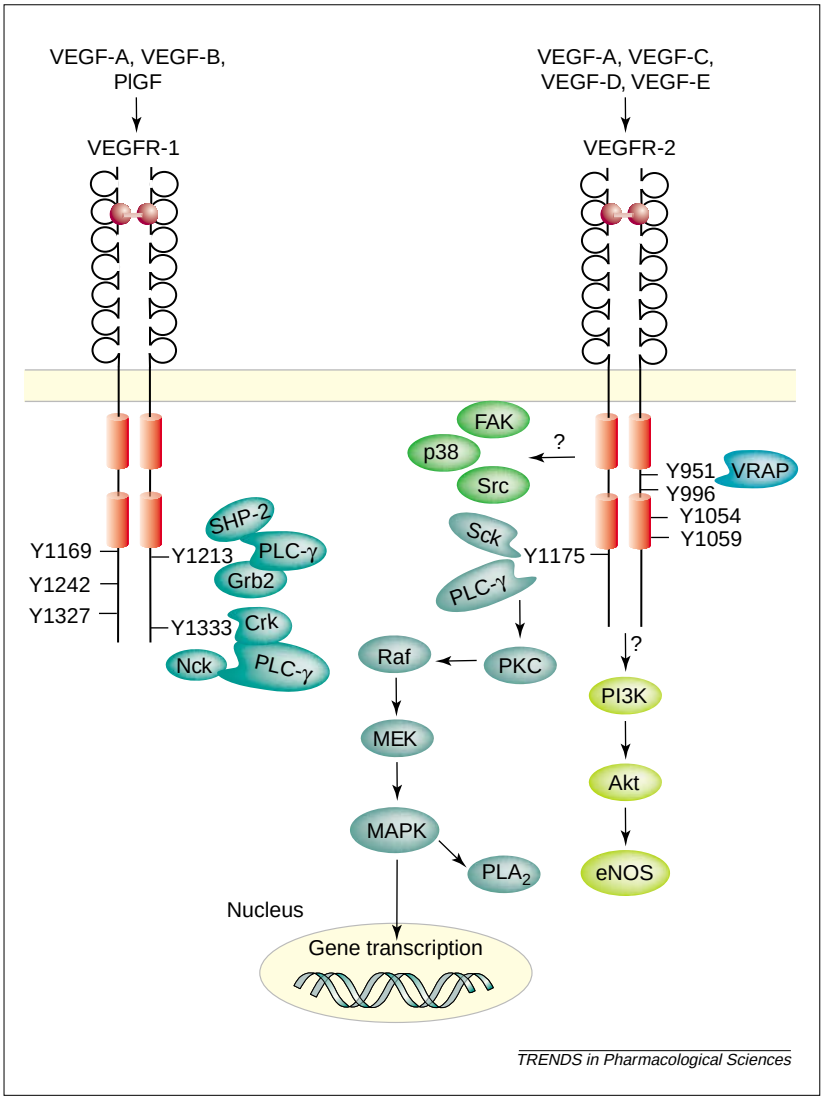

Fig. 2. Overview of VEGFR-1 and VEGFR-2 signalling. Ligand binding results in receptor dimerization and the phosphorylation of specific tyrosine residues within the intracellular domain of each receptor (the positions of phosphotyrosine residues in the receptor amino acid sequence are shown). In VEGFR-1, several signalling molecules can interact with Y1213 and Y1333 based on their SH2-domain-binding specificity. In VEGFR-2, several intracellular signalling proteins are activated directly via receptor binding, such as Sck, PLC-γ and VRAP. Several other proteins, such as Akt (protein kinase B), FAK, p38 MAPK, eNOS, Src and PI3K are also activated via VEGFR-2, although their exact mechanism of activation has not been determined (indicated by ?). Although Y1175 has not yet been identified as an autophosphorylation site, it has been shown to bind PLC-γ and Sck. Abbreviations: eNOS, endothelial nitric oxide synthase; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PI3K, phosphoinositide 3-kinase; PLA_2, phospholipase A_2; PLC-γ, phospholipase C-γ; PKC, protein kinase C; Sck, Shc-like protein; SHP-2, SH2 phosphatase 2; VEGFR-1, vascular endothelial growth factor receptor 1; VEGFR-2, vascular endothelial growth factor receptor 2; VRAP, VEGF receptor-associated protein.

there are hypoxia-responsive elements in only the VEGFR-1 promoter[35]. Recently, neuropilin-1 (NP-1), a receptor for the collapsin–semaphorin family, was found to bind VEGF-A_{165} (Ref. 36). NP-1 efficiently potentiates VEGF-induced endothelial cell migration, through an as yet unidentified mechanism.

VEGF receptor signal transduction remains poorly understood. VEGFR-1 is a weak kinase, at least in tissue culture cell lines. Several phosphorylation sites and potential binding molecules have been identified[27] (Fig. 2), although their roles in VEGF-stimulated cellular responses remain to be determined. VEGFR-2 exhibits a strong induction in kinase activity in response to VEGF-A; however, the position of all the autophosphorylated tyrosine residues have not been fully identified[37]. VEGFR-2 has been found in complex with the integrin $\alpha_v\beta_3$, which is specifically expressed on angiogenic endothelium. Activation of $\alpha_v\beta_3$ by plating cells on vitronectin resulted in increased VEGFR-2 kinase activity and augmented VEGF-mediated mitogenicity[38]. This interaction might allow direct transduction of VEGF effects on cell-matrix interaction.

Specific activation of VEGFR-1 with the ligand PlGF and VEGFR-2 with the ligand VEGF-E, and the use of cells transfected with each receptor has identified several signalling molecules downstream of each receptor (Fig. 2).

Signal transduction induced by FGF and VEGF in different model assays

FGF, as well as VEGF, stimulate survival, proliferation, migration and differentiation of primary and stable endothelial cells, although the efficiencies of transduction of these responses are dependent on the type of endothelial cell line. The signalling pathways activated by FGFR-1 have been the most extensively studied and are summarized in Table 1. Analysis of VEGFR signalling has led to the conclusion that, although the affinity for VEGF binding is approximately tenfold higher for VEGFR-1 than for VEGFR-2, it is the activation of the latter that is responsible for conveying the VEGF-mediated effects in endothelial cells. Thus, although VEGFR-1 has been shown to mediate chemotaxis in monocytes[39], in endothelial cells it is thought to sequester VEGF, thus regulating VEGFR-2 activation; such a mechanism is supported by the VEGFR-1 knockout phenotype discussed previously. The signalling molecules activated by VEGFR-2 and their physiological role are summarized in Table 2.

FGF and VEGF induce angiogenesis in the chicken chorioallantoic membrane (CAM). Recently, Eliceiri and co-workers reported that in the CAM, FGF-mediated angiogenesis was blocked following treatment with the specific mitogen-activated protein kinase kinase (MEK) inhibitor PD98059, indicating the importance of the Ras–MEK–MAPK (mitogen-activated protein kinase) pathway for FGF-stimulated angiogenesis[40]. Furthermore, in a report from the same group, introduction of dominant-negative Src cytoplasmic tyrosine kinase was shown to inhibit VEGF, but not FGF-induced angiogenesis in the CAM (Ref. 41). Recently, the effect of PI3K on CAM angiogenesis was tested by avian retroviral-mediated gene transfer of dominant-negative and constitutively active PI3K, the activity of which was found to be required for CAM angiogenesis[42].

In recent years, rapid progress has been made in our understanding of the developmental regulation of

Table 2. VEGFR-2 signalling mechanisms[a]

Signalling molecule	Receptor binding site	Activated via	Signalling cascade regulated	Physiological role	Refs
PLC-γ	Y801? Y1175?	VEGFR-2 binding	Ins(1,4,5)P_3-mediated Ca^{2+} release and DAG generation	Unknown	63,64
VRAP	Y951	VEGFR-2 binding	Possible adaptor protein	Unknown	65
Sck	Y1175	VEGFR-2 binding	Grb2–Ras–Raf–p42/44 MAPK?	Proliferation?	66
PLA_2		Ca^{2+}/p42/44 MAPK	Prostacyclin production	Permeability	67,68
PKC		PLC-γ-mediated DAG	Raf–p42/44 MAPK eNOS	Proliferation	69,70
PI3K		Unknown adaptor protein	Rac, Akt activation	Migration, survival	71
Akt		PI3K–PDK	Survival pathways (BAD/caspase), phosphorylation of eNOS	Cell survival (anti-apoptotic), permeability, migration	71 72–74
Src		VEGFR-2 binding	PLC-γ? NO?	Permeability?	75
p38 MAPK		Unknown mechanism	Actin polymerization	Migration?	76
FAK		Unknown mechanism	Focal adhesion formation	Migration?	77
eNOS		Akt, Ca^{2+}	NO-mediated cGMP generation, leading to PKG activation, Raf–p42/44 MAPK activation	Proliferation, permeability, migration	78,79 68,74

[a]Abbreviations: DAG, *sn*-1,2-diacylglycerol; Ins(1,4,5)P_3, inositol (1,4,5)-trisphosphate; FAK, focal adhesion kinase; Grb2, growth factor receptor-bound 2; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PKG, protein kinase G; PLA_2, phospholipase A_2; PLC-γ, phospholipase C-γ; Sck, Shc-like protein; VEGFR-2, vascular endothelial growth factor receptor 2; VRAP, VEGF receptor-associated protein.

the vasculature, through gene inactivation in mice. Targeted gene inactivation of several signal transduction molecules results in defective vascular development, which possibly implies that these molecules are downstream *in vivo* effectors of either FGFR or VEGFR activation. Notably, several of these molecules are components of the Ras–MAPK pathway. These include ShcA (Ref. 43), Ras-GAP (GTPase-activating protein)[44], B-Raf (Ref. 45) and MEK kinase 3 (MEKK3)[46]. Furthermore, although $Src^{-/-}$ mice show normal angiogenesis, VEGF-induced vascular permeability is impaired in both $Src^{-/-}$ or $Yes^{-/-}$ mice, but not in $Fyn^{-/-}$ mice[41] (Yes and Fyn are Src-family kinases).

Therapeutic modulation of angiogenesis

It is now well established that tumour progression is angiogenesis dependent. Many tumour cell lines secrete VEGF *in vitro* and VEGF mRNA levels are increased in most human tumours. Furthermore, both FGF-2 and VEGF are elevated in the serum of individuals with a variety of tumours[47]. Other diseases characterized by excessive angiogenesis include diabetic retinopathy and rheumatoid arthritis. Intense research in the past few years has been focused on developing inhibitors of FGF and VEGF action[47,48]. These angiogenesis inhibitors are directed towards a particular growth factor, growth factor receptor or an intracellular substrate for the receptor. Furthermore, endogenous angiogenesis inhibitors, which can act by preventing growth factor function, have also been described. Table 3 summarizes current efforts to develop drugs that specifically inhibit angiogenesis by targeting growth factor function. It is noteworthy that other inhibitors of endothelial cells are being developed, but are not listed because their mode of action is not directed against FGF or VEGF function, or their mode of action is unknown. (For a complete list of angiogenesis inhibitors in clinical trials, see http://cancertrials.nci.nih.gov/news/angio/)

Interest has also been focused on the potential administration of FGF-2 and VEGF to alleviate conditions characterized by insufficient blood supply, such as limb and myocardial ischaemia[49]. A recent study by Isner and colleagues has shown that intramyocardial administration of a plasmid encoding $VEGF_{165}$ improved myocardial infusion in a group of individuals with myocardial ischaemia[50]. Although such gene therapy approaches are still progressing, it is now clear that a localized increase in angiogenesis is of clinical benefit in some conditions.

Conclusion and perspectives

One of the major challenges to researchers in the angiogenesis field has been to identify the crucial signal transduction pathway by which FGF and VEGF modulate angiogenesis. Cell culture models have provided a plethora of data regarding FGF and VEGF signal transduction pathways and their physiological role; however, it is also apparent that most of these pathways are also used by growth factors that are not angiogenic. The future lies in identifying the crucial genes activated by the FGF and VEGF signalling pathways that are responsible for angiogenesis. Recently, St Croix and colleagues compared the pattern of gene expression in endothelial cells derived from the blood vessels of normal and malignant colorectal tissue[51]. Overall gene expression was similar between normal endothelial cells and tumour vasculature, indicating

Table 3. Inhibitors of FGF and VEGF function in anti-angiogenic treatment

Compound	Mechanism of action	Company	Clinical phase	Refs
Anti-VEGF humanized mAb	Sequestration of VEGF	Genentech	Phase II/III	80
Anti-VEGFR-2 Ab	Inhibition of VEGFR-2 activation	Imclone	Phase I	80
Angiozyme	Ribozymes that target VEGFR mRNA	Ribozyme pharmaceuticals	Phase I/II	80
Soluble VEGFR-1	Sequestration of VEGF	Genentech	Preclinical	80
SU5416	Inhibition of VEGFR-2 kinase activity	Sugen	Phase I/II/III	48
SU6668	Inhibition of VEGFR, PDGFR and FGFR kinase activity	Sugen	Phase I	47
ZD4190	Inhibition of VEGFR kinase activity	AstraZeneca	Phase I	80
CGP41251	Inhibition of VEGFR kinase activity	Novartis	Phase I	80
PTK787/ZK22584	Inhibition of VEGFR kinase activity	Novartis	Phase I/II	80
Interferon α	Inhibition of FGF-2 production	–	Phase II/III	47
Thalidomide	Inhibition of FGF-2-mediated angiogenesis	Celgene	Phase II	48
Platelet factor 4	Interaction with heparin binding to FGF-2 and $VEGF_{165}$			47

[a]Abbreviations: Ab, antibody; mAb, monoclonal antibody; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; PDGFR, platelet-derived growth factor receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

that normal and tumour endothelium are genetically very similar. However, 79 genes were differentially expressed with the levels of 46 being specifically elevated in tumour-associated endothelium. These important data identify, for the first time, a genetic angiogenic phenotype.

The advent of microarray technology and serial analysis of gene expression (SAGE), where gene profiles for specific growth factors can be studied, will enable the identification of the crucial angiogenic genes whose expression are regulated by FGF and VEGF, and the signalling pathways involved. Such knowledge will herald a new era in angiogenic signalling and facilitate the generation of angiogenic inhibitors that can specifically target the tumour vasculature.

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Chemical names

CGP41251: *N*-benzoylstaurosporine
PD98059: 2′-amino-3′-methoxyflavone
PTK787/ZK22584: 1-[4-chloroanilino]-4-[4-pyridylmethyl]phthalazine succinate
SU5416: 3-[(2,4-dimethylpyrrol-5-yl)-methylidene]-indolin-2-one
SU6668: (Z)-3-[2,4-dimethyl-5-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-1*H*-pyrrol-3-yl]-propionic acid
ZD4190: *N*-(4-bromo-2-fluorophenyl)-6-methoxy-7-[2-(1*H*-1,2,3-triazol-1-yl)-ethoxy]quinazolin-4-amine