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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. This is a complex process that involves the concerted action of several other factors, such as the angiopeptins and ephrins, that act on specific receptors to regulate vessel stability.
**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 protein 2; Grb14, growth factor receptor-bound protein 14; HSPG, heparin sulfate proteoglycan; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLA2, phospholipase A2; 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 cell 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. 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 drift, produce different stimulators over time. 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.

**FGF and VEGF: the angiogenic factors**

Basic fibroblast growth factor [bFGF (also known as FGF-2)] was the first pro-angiogenic molecule to be identified. 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. FGFs bind with high affinity to heparan sulfate proteoglycans (HSPGs), which are located on the surface of most cells and within the extracellular matrix. 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. 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, which display a range of receptor-ligand interactions. Disruption of the genes encoding FGFR-1 or FGFR-2 leads to embryonal death before gastrulation. 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. 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. When reconciled with the receptor knockout data, this suggests redundancy in the FGF family. Disruption of the gene encoding FGF-R-3 results in mice with skeletal abnormalities, whereas the result of inactivation of the gene encoding FGF-R-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 monomeric FGF-2-heparin complex. By contrast, the binding of FGF-2 to the FGFR-2 is much more sensitive to the presence of heparin or heparan sulfates, leading to the formation of a heterotetrameric FGF-2–heparin complex.
Table 1. FGFR-1 signalling mechanisms

<table>
<thead>
<tr>
<th>Signalling molecule</th>
<th>Receptor binding site</th>
<th>Activated via</th>
<th>Signalling cascade regulated</th>
<th>Physiological role</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crk</td>
<td>Y463</td>
<td>FGFR-1 binding</td>
<td>Activation of MAPK, Jun kinase</td>
<td>Proliferation</td>
<td>19</td>
</tr>
<tr>
<td>PLC-γ</td>
<td>Y766</td>
<td>FGFR-1 binding</td>
<td>Ins(1,4,5)P₃-mediated Ca²⁺ release and DAG generation</td>
<td>Cytoskeletal reorganization, receptor endocytosis</td>
<td>20,52,53</td>
</tr>
<tr>
<td>Unknown</td>
<td>759–774</td>
<td>Unknown mechanism</td>
<td>Unknown</td>
<td>Migration</td>
<td>54</td>
</tr>
<tr>
<td>PKC</td>
<td>Unknown</td>
<td>PLC-γ-mediated DAG</td>
<td>Protein phosphorylation, regulation of PLA₂, PLD</td>
<td>Unknown</td>
<td>53</td>
</tr>
<tr>
<td>FRS-2</td>
<td>Crk/J M region of FGFR-1</td>
<td>Activation of p42/44 MAPK cascade</td>
<td>Proliferation</td>
<td>19,55,56</td>
<td></td>
</tr>
<tr>
<td>SHP-2</td>
<td>Binding to FRS-2</td>
<td>Sustained p42/44 MAPK activation</td>
<td>Differentiation</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Src</td>
<td>FGFR-1 activation</td>
<td>Unknown</td>
<td>Differentiation</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>Shc</td>
<td>FGFR-1 activation</td>
<td>p42/44 MAPK</td>
<td>Proliferation, differentiation</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>Grb14</td>
<td>FGFR-1 binding</td>
<td>p42/44 MAPK?</td>
<td>Proliferation?</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>PI3K</td>
<td>Y766F, downstream of PLC-γ</td>
<td>Rac, Akt activation?</td>
<td>Migration, survival?</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>p70 S6K</td>
<td>Unknown mechanism</td>
<td>Unknown</td>
<td>Proliferation</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>p38 MAPK</td>
<td>Unknown mechanism</td>
<td>Unknown</td>
<td>Proliferation, survival</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>Shb</td>
<td>FGFR-1 activation</td>
<td>Unknown</td>
<td>Differentiation, apoptosis</td>
<td>62</td>
<td></td>
</tr>
</tbody>
</table>

*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)P₃, 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; PLA₂, phospholipase A₂; PLC-γ, phospholipase C-γ; Rac, Akt activation; SHP-2, SH2 phosphatase 2.

VEGF ligands and receptors

VEGF was initially termed vascular permeability factor (VPF) because of its ability to induce vascular leakage [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) [Ref. 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 is a heparin-binding molecule that is secreted by a wide variety of cell types, including vascular endothelial cells, fibroblasts, smooth muscle cells, and leukocytes. It is mainly expressed in the endothelium of blood vessels and lymphatics, and in the placenta. VEGF is a potent angiogenic factor that promotes the proliferation, survival, and migration of endothelial cells. It also induces the proliferation of pericytes and smooth muscle cells, and promotes the migration of fibroblasts and pericytes. VEGF has been shown to play a crucial role in angiogenesis, embryonic development, and tumor growth.

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 [Ref. 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 [Ref. 29]. 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 [Ref. 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 VEGF-R1 is not required for vascular development [Ref. 32]. As for VEGF, hypoxia has been shown to regulate both VEGFR-1 and VEGFR-2 expression [Ref. 33], although hypoxia may also contribute to angiogenesis through other mechanisms. VEGF expression is transcriptionally regulated by hypoxia [Ref. 33], 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 [Ref. 25].
there are hypoxia-responsive elements in only the VEGFR-1 promoter\textsuperscript{35}. Recently, neuropilin-1 (NP-1), a receptor for the collapsin–semaphorin family, was found to bind VEGF-A\textsubscript{165} (Ref. 36). NP-1 efficiently mediates angiogenesis in the chicken chorioallantoic membrane (CAM). Recently, Elicieri and co-workers reported that in the CAM, VEGF-induced angiogenesis was blocked following treatment with the specific mitogen-activated protein kinase (MEK) inhibitor PD98059, indicating the importance of the Ras–MEK–MAPK (mitogen-activated protein kinase) pathway for VEGF-induced angiogenesis\textsuperscript{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\textsuperscript{42}.

In recent years, rapid progress has been made in our understanding of the developmental regulation of...
Table 2. VEGFR-2 signalling mechanisms

<table>
<thead>
<tr>
<th>Signalling molecule</th>
<th>Receptor binding site</th>
<th>Activated via</th>
<th>Signalling cascade regulated</th>
<th>Physiological role</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLC-γ</td>
<td>Y801? Y1175?</td>
<td>VEGFR-2 binding</td>
<td>Ins(1,4,5)P3-mediated Ca2+ release and DAG generation</td>
<td>Unknown</td>
<td>63,64</td>
</tr>
<tr>
<td>VRAP</td>
<td>Y951</td>
<td>VEGFR-2 binding</td>
<td>Possible adaptor protein</td>
<td>Unknown</td>
<td>65</td>
</tr>
<tr>
<td>PLA2γ</td>
<td>Ca2+/p42/44 MAPK</td>
<td>Prostacyclin production</td>
<td>Permeability</td>
<td>67,68</td>
<td></td>
</tr>
<tr>
<td>PKC</td>
<td>PLC-γ-mediated DAG</td>
<td>Raf–p42/44 MAPK eNOS</td>
<td>Proliferation</td>
<td>69,70</td>
<td></td>
</tr>
<tr>
<td>PI3K</td>
<td>Unknown adaptor protein</td>
<td>Rac, Akt activation</td>
<td>Migration, survival</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>Akt</td>
<td>PI3K–PDK</td>
<td>Survival pathways (BAD/caspase), phosphorylation of eNOS</td>
<td>Cell survival (anti-apoptotic), permeability, migration</td>
<td>72, 74</td>
<td></td>
</tr>
<tr>
<td>Src</td>
<td>VEGFR-2 binding</td>
<td>PLC-γ NO?</td>
<td>Permeability</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>p38 MAPK</td>
<td>Unknown mechanism</td>
<td>Actin polymerization</td>
<td>Migration?</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>FAK</td>
<td>Unknown mechanism</td>
<td>Focal adhesion formation</td>
<td>Migration?</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>Akt, Ca2+</td>
<td>NO-mediated cGMP generation, leading to PKG activation, Raf–p42/44 MAPK activation</td>
<td>Proliferation, permeability, migration</td>
<td>78,79</td>
<td></td>
</tr>
</tbody>
</table>

*Abbreviations: DAG, sn-1,2-diacylglycerol; Ins(1,4,5)P3, 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; PLA2γ, phospholipase A2γ; PLC-γ, phospholipase C-γ; Src, Src-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−/− mice41 (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 tumours47. 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 action47,48. 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://cancertreatments.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 ischaemia49. A recent study by Isner and colleagues has shown that intramyocardial administration of a plasmid encoding VEGF improved myocardial infarction in a group of individuals with myocardial ischaemia50. 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 tissue51. Overall gene expression was similar between normal endothelial cells and tumour vasculature, indicating...
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 FGFR 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 endothelium.

References

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

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

*Table 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.

Acknowledgements

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**Chemical names**

- CGP41251: N-benzylstaurosporine
- PD98059: 2′-amino-3′-methoxyflavone
- PTK787/ZK22854: 1-(4-chloroanilino)-4-(4-pyridylmethyl)phenothiazine succinate
- SU5416: 3-[2-(4-methyl-5-oxo-1,2-dihydro-indol-3-yl)-3-ylidene]-5-(3-ylidene)-2H-pyrrol-1-yl-propanoic acid
- ZD1830: N-(4-bromo-2-fluorophenyl)-6-methoxy-7-[2-(1H-1,2,3-triazol-1-yl)-1H-indolin-2-one]