Vascular endothelial growth factors (VEGFs) belong to the platelet-derived growth factor supergene family, and they play central roles in the regulation of angiogenesis and lymphangiogenesis. VEGF-A, the major factor for angiogenesis, binds to two tyrosine kinase (TK) receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), and regulates endothelial cell proliferation, migration, vascular permeability, secretion and other endothelial functions. VEGFR-2 exhibits a strong TK activity towards pro-angiogenic signals, whereas the soluble VEGFR-1 (sFlt-1) functions as an endogenous VEGF inhibitor. sFlt-1 is abnormally overexpressed in the placenta of preeclampsia patients, resulting in the major symptoms of the disease due to abnormal trapping of VEGFs. The VEGF-VEGFR system is crucial for tumour angiogenesis, and anti-VEGF-VEGFR molecules are now widely used in the clinical field to treat cancer patients. The efficacy of these molecules in prolonging the overall survival of patients has been established; however, some cancers do not respond well and reduced tumour sensitivity to anti-VEGF signals may occur after long-term treatment. The molecular basis of tumour refractoriness should be determined to improve anti-angiogenic therapy.

Keywords: hypoxia/preeclampsia/tumor angiogenesis/VEGF/VEGFR.

Abbreviations: AMD, age-related macular degeneration; Flt-1, Fms-like TK-1; lg, immunoglobulin; OS, overall survival; Nrpl, neuropilin-1; PIGF, placenta growth factor; PDGF, platelet-derived growth factor; PE, preeclampsia; PFS, progression-free survival; TK, tyrosine kinase; VEGFs, vascular endothelial growth factors.

The closed circulatory system of vertebrates is essential for supplying oxygen and nutrients to the tissues in the body. In the recent decades, the molecular basis of angiogenesis, a new blood vessel formation from pre-existing blood vessels, has been extensively studied and a variety of signaling systems, such as vascular endothelial growth factor (VEGF)-VEGFR, Angiopoietin-Tie, Ephrin-Eph and Delta-Notch, were found to play important roles in angiogenesis. Among them, VEGF-VEGFR is crucial not only for physiological angiogenesis from early embryonic to adult stages but also for pathological angiogenesis, such as in cancer (1–5).

Phylogenetically, non-vertebrates such as Drosophila possess a single VEGF-like receptor gene, which regulates cell migration and morphogenesis (6). However, this receptor in non-vertebrates does not have cell proliferation-stimulatory activity. In the fish, which are the earliest vertebrates, the VEGF gene was duplicated to produce four genes and obtained cell proliferation-stimulatory activity to regulate angiogenesis and lymphangiogenesis (7). In parallel, the structurally related PDGFR gene family members were generated, and a strong cell proliferation signalling system was acquired to regulate smooth muscle cells and haematopoiesis. The number of VEGF genes is conserved from amphibians to mammals, with three genes encoding three full-length receptors and one soluble molecule (sFlt-1/sVEGFR-1) (Fig. 1), which positively and negatively regulate the formation of blood vessels as well as lymph vessels.

This review briefly describes the structure and function of VEGF-VEGFR, the intimate relationship of VEGFR with preeclampsia (PE) and the development of anti-cancer drugs targeting VEGF-VEGFR signals as well as the questions that remain to be addressed.

Structure and Function of VEGFs

The mammalian genome encodes five VEGF family members, VEGF-A (also known as VEGF), placenta growth factor (PIGF), VEGF-B, VEGF-C and VEGF-D, which regulate vasculogenesis, angiogenesis and lymphangiogenesis (1–4). Particularly, VEGF-A is crucial for blood vessel formation during early embryogenesis. Not only the VEGF-A homozygous knockout mice but also its heterozygous mice (VEGF-A +/−) exhibit an embryonic lethal phenotype due to immature blood vessel formation, indicating that the local concentration of VEGF-A in the embryos has to be tightly regulated for proper angiogenesis (8, 9). Several VEGF-A subtypes are generated by alternative splicing. Among these, VEGF-A165 has the highest biological activity, with binding affinity for a co-receptor, neuropilin-1 (Nrp1). Recently, another alternatively spliced form of VEGF-A, VEGFxxb, was reported. VEGFxxb has a lower affinity for the...
receptor and competes with VEGF-A to negatively regulate angiogenesis (10).

VEGF-C and VEGF-D acquire a high affinity for VEGFR-3 after processing and stimulate lymphangiogenesis. VEGF-C, but not VEGF-D, is expressed during embryogenesis, and VEGF-C null mice (VEGF-C −/−) exhibit a lethal phenotype at the pre-natal stage due to fluid accumulation in tissues because of poor lymphangiogenesis (11). PI GF and VEGF-B bind to and activate only VEGFR-1. Although the kinase activity of VEGFR-1 is relatively weak compared with that of VEGFR-2, PI GF stimulates survival signals in endothelial cells and promotes angiogenesis without a severe inflammatory response (12). VEGF-B was reported to be important for the stimulation and maintenance of the coronary artery system (13).

**Dual Roles of VEGFRs in Angiogenesis**

In 1990, we isolated a gene encoding a novel tyrosine kinase (TK) receptor from the human placenta. The TK receptor had seven immunoglobulin (Ig)-like domains in the extracellular region and a TK domain with a 60-amino acid-long kinase insert sequence (14). Based on the structural similarity, we named it Fms-like TK-1 (Flt-1) (Fig. 1). In 1992, Flt-1 was shown to bind VEGF/vascular permeability factor, now known as VEGFR-1 (15).

VEGFRs are structurally related to the platelet-derived growth factor (PDGF) receptor family, PDGFR/Fms/Kit/FLT3, with five Ig domains in the extracellular region. PDGFR family members also have a TK domain with a long kinase insert sequence. However, the intracellular signalling of VEGFRs is quite different from that of PDGFRs/Fms/Kit. It is well established that the PDGFR/Fms/Kit family has one or more tyr (Y)–x–x–met (M) motifs in the kinase insert and following autophosphorylation at this tyrosine, the motif binds to the SH2 domain in the p85 subunit of PI3K and significantly stimulates the PI3K-Akt and Ras pathways for cell proliferation and transformation (16). On the other hand, none of the kinase insert sequences of VEGFRs has the Y–x–x–M motif, and the PI3K pathway is usually not highly activated after stimulation with VEGF (3).

We have previously shown that of the two major autophosphorylation sites in VEGFR-2, 1175-PY but not 1214-PY is essential for the VEGF-A-induced proliferation in endothelial cells (17). The 1175-phenylalanine (F) mutation of VEGFR-2 or the intracellular injection of an anti-1175-PY-specific blocking antibody significantly suppressed VEGF-dependent cell proliferation. Furthermore, 1175-PY was found to be the binding and activation site for phospholipase C (PLC)-γ, which activates the PKC-Ca2+-c-Raf-MEK-MAPK pathway (18). Mice with the 1173-F but not the 1212-F single amino acid mutation in VEGFR-2 (flk-1 1173F/F mice; two amino acids shorter in mice compared with that in humans) exhibit an embryonic lethal phenotype at E8.5–9.0 due to a lack of blood vessel formation, very similar to VEGFR2 (flk-1) −/−.
mice (19, 20). This indicates that the 1175-PY-PLCγ pathway downstream from VEGFR-2 is essential for vasculogenesis during early embryogenesis.

Furthermore, Sase et al. (21) reported that in an in vitro embryonic stem cell–endothelial cell differentiation system, the VEGFR-2 1175-PY-PLCγ pathway is required for the formation of vascular endothelial cells from progenitor cells. In addition, Xiong et al. (22) have recently shown that this VEGFR-2 1175-PY-PLCγ-Ca<sup>2+</sup> pathway together with the protein kinase-A pathway is important for the secretion of von Willebrand factor from vascular endothelial cells, which then triggers the inflammatory response in blood vessels.

Unlike VEGFR-2, VEGFR-1 has a very high affinity for its ligand, VEGF, with the affinity being about one order of magnitude higher than that of VEGFR-2 (23). However, the kinase activity of VEGFR-1 is lower, about one-tenth that of VEGFR-2. The VEGFR-1 gene produces two major proteins: a full-length receptor and sFlt-1 (14, 24). These facts suggest that VEGFR-1 may negatively regulate angiogenesis under certain conditions. Indeed, VEGFR-2 (flk-1) knockout mice (flk-1−/− mice) exhibit a lethal phenotype at E8.5–9.0 due to a lack of vasculogenesis, whereas VEGFR-1 (flt-1) knockout mice (flt-1−/− mice) die at almost the same stage, E8.5–9.0, due to overgrowth of endothelial cells and disorganization of blood vessels in the embryo (25). This indicates that during early embryogenesis, the two VEGFRs have opposite roles in angiogenesis: VEGFR-2 is a positive signal transducer, whereas VEGFR-1 is a suppressor of VEGFR-2 signalling.

To distinguish between the two possibilities that the VEGFR-1, TK generates a negative signal against angiogenesis or the ligand-binding site of VEGFR-1 blocks VEGF activity by trapping VEGF, we generated flt-1 TK-deficient (TK−/−) mice. Surprisingly, angiogenesis was almost normal in the flt-1 TK−/− mice, and these mice were basically healthy (26). This demonstrates that VEGFR-1 negatively regulates angiogenesis during early embryogenesis by trapping VEGF and decreasing the pro-angiogenic signals from VEGFR-2. Using the flt-1 TK−/− mice, our study as well as recent studies by others have shown that VEGFR-1 signalling is important for tumour growth, metastasis, and chronic arthritis in mice (27–31). Furthermore, flt-1 TK−/− mice with an op/op genetic background showed a significant suppression of bone marrow reconstruction, indicating that VEGFR-1 signalling is partly involved in bone marrow formation (32).

In addition, recent reports suggest that VEGFR signalling plays a role in the neuronal system, in both sensory and motor neurons. Thus, the VEGF-VEGFR system could be an attractive target for the treatment of neuronal disorders such as amyotrophic lateral sclerosis (33, 34).

**Importance of sFlt-1 (Soluble VEGFR-1) Upregulation in PE**

sFlt-1 is encoded by a short mRNA of the VEGFR-1 gene, and its structure, i.e. six Ig domains with a 31-amino acid tail, is highly conserved in mammals, birds and amphibians (35, 36). sFlt-1 traps VEGF and may thus play an important role in the negative regulation of angiogenesis in animals. Indeed, Ambati et al. (37) reported that sFlt-1 is expressed in the eye to maintain avascularity in the cornea.

In 2003–2004, several researchers reported that an abnormal increase in the level of serum sFlt-1 in pregnant mothers is well correlated with the degree of PE (38–40) (Fig. 1). In the placenta, sFlt-1 is expressed in the trophoblast cells. The trophoblast layer is located between the umbilical capillaries on the foetal side and the maternal blood vessels, suggesting that sFlt-1 forms a molecular barrier against abnormal vascular permeability and abnormal angiogenesis, such as the fusion of fetal capillaries with maternal blood vessels, by trapping VEGF and PlGF. The two major symptoms of PE are hypertension and proteinuria. As discussed later, hypertension and proteinuria are also the most common adverse effects encountered in cancer patients treated with VEGF-neutralizing antibody (41). In addition, artificial expression of sFlt-1 using a vector in a pregnant rat model induced hypertension and proteinuria (38). These facts strongly suggest that sFlt-1 overexpression in PE is a crucial cause of PE symptoms.

Podocytes in the glomeruli of the kidney secrete VEGF at physiological levels and maintain the glomerular endothelial cells in a healthy state, producing primary urine without leakage of serum proteins. Suppression of VEGF secretion from podocytes results in severe proteinuria (42, 43). Thus, overexpression of the VEGF-trapping molecule, sFlt-1, may block podocyte-derived VEGF and induce glomerular endothelial damage, resulting in proteinuria in PE.

The molecular basis of hypertension under VEGF blockade remains to be clarified. VEGF signals stimulate eNOS and increase the production of NO, a vasodilator, in endothelial cells. Furthermore, VEGF is a strong vascular permeability factor (44). Thus, a decrease in both NO and permeability via VEGF trapping by sFlt-1 may cause hypertension.

Given that trophoblasts are the main source of sFlt-1 in PE, viral or bacterial infection or abnormal stress, such as hypoxia in the placenta, may induce these cells to overexpress sFlt-1 (45). In addition to sFlt-1, other factors such as sEndoglin, a TGFB family member, promote the degree of PE (46). An sFlt-1-blocking agent that is safer to both foetus and mother can be a useful tool to control PE.

**Anti-angiogenic Therapy in Cancer via Suppression of the VEGF-VEGFR System**

In the 1970s, Folkman and coworkers (47) proposed that tumour angiogenesis is an attractive target for the treatment of cancer. From the 1980s to the present, the VEGF-VEGFR system has been demonstrated to be the major regulator of tumour angiogenesis (2–4) (Fig. 2). Solid tumours often become hypoxic due to a rapid growth of tumour cells. Hypoxic stress is a pivotal inducer of the VEGF gene via stabilization
and activation of the HIF transcription factor: the S'-upstream sequence of the VEGF gene has a HIF-response element motif, resulting in high gene expression. The VEGFRs, particularly VEGFR-2, are also upregulated to some extent under hypoxia. In 1993, Kim et al. (48) showed that an anti-human VEGF antibody efficiently suppressed the growth of human tumour xenografts transplanted in immunodeficient mice. This antibody can block only the human-type VEGF derived from tumour cells and not the mouse VEGF derived from the cells surrounding the tumours; however, tumour growth was significantly suppressed. These results suggest that tumour-derived VEGF plays a central role in tumour angiogenesis.

Till date, a VEGF-neutralizing antibody (bevacizumab) and small molecule kinase inhibitors (the multi-kinase inhibitors sunitinib and sorafenib) have been successfully developed for clinical use, although some adverse effects such as hypertension, proteinuria, thrombosis and bleeding have been observed. Bevacizumab was approved for the treatment of solid tumours, including colorectal, lung (non-epithelial, NSCLC) and breast cancers as well as glioblastomas. Kinase inhibitors are used for the treatment of renal and hepatocellular carcinomas as well as GIST. In the USA, the FDA approved bevacizumab in 2008 for the treatment of breast cancer because of a statistically significant efficacy in improving overall survival (OS); however, it was withdrawn in late 2011 due to a lack of clear efficacy data on OS in recent large-scale phase III studies, such as the E2100, AVADO and RIBBON1 clinical trials. On the other hand, other countries such as EU and Japan approved bevacizumab for breast cancer treatment based on a statistically significant improvement in progression-free survival (PFS).

These antiangiogenic drugs targeting the VEGF-VEGFR signal may induce tumour-suppressive effects through several pathways: (i) direct suppression of angiogenesis, (ii) promotion of the pre-existing endothelial cell apoptosis within tumour tissues, (iii) vascular normalization which decreases abnormal vascular permeability and intratumoural pressure, resulting in an increase in the efficacy of chemotherapy and (iv) suppression of pro-angiogenic inflammatory reaction.

Other anti-VEGF-VEGFR drugs, such as VEGF-Trap (a VEGFR1-VEGFR2 fusion protein), VEGFR-neutralizing antibodies and VEGFR-derived peptides for immunotherapy, have been developed and are now under clinical trials.

**Anti-angiogenic Therapy in the Treatment of Other (Non-cancer) Diseases**

Age-related macular degeneration (AMD) is caused by abnormal angiogenesis with oedema in the retina and patients gradually lose visual activity. Abnormal activation of the VEGF-VEGFR system is intimately involved in the progression of this disease. Therefore, an aptamer against VEGF-A_165_ a VEGF-neutralizing antibody (Fab type) and VEGF-Trap are now approved for AMD treatment. Particularly, the neutralizing antibody not only slows down disease progression but also promotes partial recovery of visual activity and is thus widely used in the clinical field.

**Unanswered Questions in the Current Anti-Angiogenic Therapy against Cancer**

Essentially, all the current anti-angiogenic therapies in cancer treatment mainly target the VEGF-VEGFR system. Although their efficacies in improving OS and/or PFS were statistically significant or strongly suggested in glioblastoma (49), they were still insufficient. Until now, pancreatic cancer and gastric cancer have not shown a clear response to this therapy. Furthermore, breast cancer did not show a response with respect to OS in a large-scale phase III trial.

One important question is whether patients should continue anti-VEGF-VEGFR drugs, such as bevacizumab, when the cancer recurs. A recent phase III study in colorectal cancer, TML (ML18147), which was reported in the ASCO Annual Meeting 2012, indicates that bevacizumab should be continued because a combination of renewed chemotherapy with bevacizumab showed a better OS than that without bevacizumab. This suggests that the dependency of tumour angiogenesis on the VEGF-VEGFR system continues, at least partly, even after the recurrence of colorectal cancer.

On the other hand, breast cancer patients showed a better PFS but not OS in a large-scale phase III trial. This suggests that at a later stage of anti-VEGF-VEGFR treatment, tumour cells might acquire a more aggressive phenotype, resulting in an acceleration of tumour growth/progression (Fig. 2). These accelerated tumour phenotypes may be acquired via several different mechanisms: (i) dependency of tumour angiogenesis on the VEGF-VEGFR system decreases, and other signalling systems such as FGF-FGFRs and...
HGF-c-Met stimulate tumour angiogenesis (50, 51); (ii) dependency of tumour growth on the blood vessels/angiogenesis decreases and tumour cells become more invasive (52) and (iii) tumour cells become more resistant to hypoxia and/or low nutrient conditions that could occur during anti-angiogenic therapy (53, 54). These secondary responses may occur in most tumours, including breast cancer. Thus, the molecular basis of these potential responses in tumours and the tumour microenvironment should be clarified to overcome the problems.

**Conclusion**

Recent studies on angiogenesis have demonstrated that several signalling systems, including VEGF-VEGFRs, play crucial roles under both physiological and pathological conditions and have heralded a new era in cancer treatment, introducing anti-angiogenic therapy. This new therapy has significantly improved the quality of life of patients with various solid tumours, but the efficacy is still insufficient. Signalling networks in angiogenesis as well as their modulation during anti-angiogenic therapy need to be extensively characterized.

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**Conflict of interest**

None declared.

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