

in the preeclamptic placenta, and in the serum and urine of preeclamptic patients (17-24). It also caused a renewed interest in the role of hypoxia in preeclampsia and stimulated studies unraveling the link between angiogenesis and hypoxia in the placental trophoblasts (25-27).

This review will focus on recent advances in the emerging field of placental angiogenesis and its association with hypoxic conditions, as well as describe some of the potential directions of future research.

Key Players in Placental Angiogenesis in Health and Disease

Adequate placentation involves a two stage mechanism (28). Trophoblast invasion requires a process of vascularization to establish a fetoplacental vascular network, and finally, complete remodeling of the spiral arteries ensues in or-

der to form an uteroplacental circulation. The first chorionic villi vessels are a primitive vascular network that arises through a primary process termed vasculogenesis (29,30). Later on, the secondary stage of angiogenesis takes place, forming new vessels in a non-branching fashion. The process of trophoblast invasion and remodeling of the spiral arteries that has been studied extensively by Fisher et al (31-35) seems to have an utmost significance to the success of a pregnancy and when defective, preeclampsia most likely follows. During this process, a subset of the trophoblast cells, the extravillous trophoblasts, transform to endothelial phenotype cells, thus expressing typical endothelial markers such as vascular endothelial-cadherin (VE-cadherin), and $\alpha_v\beta_3$ integrin (33). Failure of this transformation most likely results in an inadequate blood supply to the growing placenta, hypoxia, and a shift in the secretion of angiogenic factors (Fig. 1). Preeclampsia has recently been de-

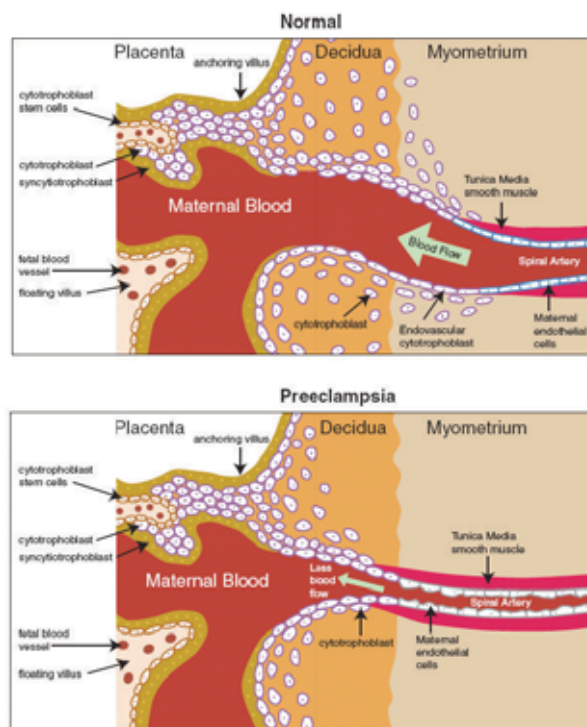


Figure 1. Abnormal placentation in preeclampsia. Exchange of oxygen, nutrients, and waste products between the fetus and the mother depends on adequate placental perfusion by maternal vessels. In normal placental development, invasive cytotrophoblasts of fetal origin invade the maternal spiral arteries, transforming them from small-caliber resistance vessels to high-caliber capacitance vessels capable of providing placental perfusion adequate to sustain the growing fetus. During the process of vascular invasion, the cytotrophoblasts differentiate from an epithelial phenotype to an endothelial phenotype, a process referred to as "pseudovasculogenesis." In preeclampsia, cytotrophoblasts fail to adopt an invasive endothelial phenotype. Instead, invasion of the spiral arteries is shallow and they remain small caliber, resistance vessels. This may result in the placental ischemia.

scribed as a state of imbalance between pro-angiogenic and anti-angiogenic factors (18). The main pro-angiogenic factors that promote angiogenesis in the placenta belong to the VEGF family. In addition to the VEGF family members, angiopoietins (Ang-1 and Ang-2) and their receptor Tie-2 are also expressed abundantly in the normal placenta (36-39) and act in the later stages of angiogenesis.

VEGF-A, the main family member of the VEGF family in the placenta, binds to two tyrosine kinase receptors, Flt-1 (also referred to as VEGFR-1) and kinase insert domain receptor (KDR) (human) /Flk-1 (murine), (also known as VEGFR-2) (40). Other than VEGF, the other major pro-angiogenic protein is placental growth factor (PlGF). VEGF-A and PlGF are produced by almost all types of trophoblasts (22). Both VEGF receptors Flt-1 and KDR are expressed on trophoblast cells in addition to endothelial cells.

sFlt-1 is the major endogenous inhibitor of angiogenesis found in the placenta. This potent antiangiogenic protein is encoded by alternative splicing of the Flt-1 gene, leading to a shorter extracellular domain that still retains the ability to bind to VEGF and PlGF. Thus, when circulating in the serum, sFlt-1 can bind to VEGF and PlGF and prevent them from binding to cell-surface receptors. Clark et al (41) have shown by *in situ* hybridization that trophoblasts express the sFlt-1 messenger RNA. Serum from pregnant women has been found to contain a VEGF-binding protein that later was confirmed to be sFlt-1 (42,43).

The hypothesis that the preeclamptic placenta elaborates soluble factors, which induce endothelial cell dysfunction was first suggested by Roberts et al (44,45). After sFlt-1 was suggested as a candidate molecule that induces maternal preeclampsia (16), several studies have confirmed high levels of circulating sFlt-1 in maternal serum (16,17,20-24).

sFlt-1 was initially identified as a potential soluble factor that mediates maternal endothelial dysfunction by gene expression profiling of placental tissue from women with and without preeclampsia. Using microarray chips mRNA for sFlt-1 was found to be dramatically up-regulated in preeclamptic placentas (16). In addition, sFlt-1 levels in patients with preeclampsia were found to fall to baseline 48 hours after delivery. Increased circulating sFlt-1 concentrations in patients with

preeclampsia were associated with decreased circulating levels of free VEGF and PlGF. Hence, a logical assumption was that excess circulating sFlt-1 may lead to an anti-angiogenic state and cause endothelial dysfunction and the clinical syndrome of preeclampsia.

A few additional experiments further established the role of sFlt-1 as a key anti-angiogenic molecule, predominantly involved in the pathophysiology of preeclampsia. First, we found that preeclamptic serum inhibited endothelial tube formation, an effect that disappeared once repeated with serum from 48 hours post-partum. These results could be reproduced when sFlt-1 was added to normotensive serum at concentrations noted in patients with preeclampsia, and could be restored by adding exogenous VEGF and PlGF (16). This study suggested to us that the anti-angiogenic properties of serum from preeclamptic patients were due to blockade of VEGF and PlGF by excess circulating sFlt-1. Finally, gene transfer of sFlt-1 into pregnant rats by an adenoviral vector produced hypertension, proteinuria, and glomerular endotheliosis, the classical pathological renal lesion of preeclampsia (16). Hence, we concluded that excess sFlt-1 made by preeclamptic placentas might be responsible for the hypertension and proteinuria of preeclampsia by inducing a deficiency of VEGF and PlGF.

Recently, a case-control study using blood samples from the Calcium for preeclampsia Prevention trial (CPEP) was performed, measuring circulating angiogenic markers in patients with preeclampsia and matched controls in order to determine if changes in their levels antedate the clinical symptoms and signs of preeclampsia (17). During the third trimester of pregnancy, the level of sFlt-1 increased and the level of PlGF decreased in healthy controls, an effect that was very pronounced in preeclampsia patients (17). The sFlt-1 level started increasing five weeks before the onset of preeclampsia, and was accompanied by decreases in both free PlGF and VEGF. Other groups have reported similar changes in PlGF during the second trimester in women destined to develop preeclampsia (23,46). The decreases in PlGF occurred even as early as the first trimester (although not as dramatically as in the second trimester), which has also been used in other studies as a possible prediction tool for the early diagnosis of preeclampsia (47-49).

A most recent study (19) measured urinary concentrations of PIGF in archived urine samples from the CPEP study. The same cohort of women used for the previous trial (17) was used in this study. The results suggested that urinary concentrations of PIGF were significantly lower at mid-gestation among women who subsequently developed preeclampsia. These results were reproduced in a further study (50). Together, all these data imply that sFlt-1 binds to PIGF and VEGF, thereby, causing a shift towards an anti-angiogenic state, which in turn causes maternal endothelial damage and clinical preeclampsia.

Placental Hypoxia: Cause or Effect?

It has been known for some years that in preeclampsia, endovascular invasion of cytotrophoblasts remains superficial and the uterine blood vessels do not undergo adequate vascular transformation. The spiral arteries fail to convert from small caliber vessels to large capacitance uteroplacental arteries (51). Furthermore, Zhou et al (32) have shown that the invasive trophoblasts fails to undergo the process of pseudo-vasculogenesis. The functional consequences of these abnormalities are still unknown, but it is likely that there is compromise of blood flow into the intervillous space leading to placental ischemia. Additionally, it has been claimed that in preeclampsia, placental ischemia is an early event (52), leading to placental production of soluble factors that cause maternal endothelial dysfunction, and resulting in the clinical disease. These hypotheses have led to a fundamental question: Is placental hypoxia in preeclampsia a cause or an effect of soluble factors, such as sFlt-1 (26)?

Reduction in the placental perfusion pressure has long been thought to underlie preeclampsia. Uteroplacental ischemia as an animal model of preeclampsia has been extensively studied, based on the fact that in women destined to develop preeclampsia, uteroplacental blood flow is reduced by 50-70% (53). As early as 1939, Ogden et al (54) tried to clamp the descending aorta of anesthetized dogs and induced about a 50% reduction in placental perfusion pressure. Pregnant dogs' blood pressure increased about 25 mm immediately. This effect could not be demonstrated in non-pregnant dogs.

Endothelial and tumor mammalian cells are known to express proteins essential for invasion in hypoxic conditions. These up-regulate the expression of heat shock and glucose-regulated proteins, as well as cytokines and growth factors (55-59). Such proteins are, for example, endothelin (60), vascular endothelial growth factor (VEGF) (61) and IL-1 α (62). Landmark studies showed that hypoxia could induce expression of platelet derived growth factor (PDGF) mRNA (63) and VEGF mRNA (61) in tissue culture, indicating that oxygen was an important regulator of angiogenesis. A large number of genes involved in different steps of angiogenesis such as angiopoietins, fibroblast growth factors and their various receptors, and genes involved in matrix metabolism are independently responsive to hypoxia in tissue culture.

Rajakumar and Conrad (64) investigated the expression of hypoxia-inducible factor (HIF) in normal human placentas and found HIF-1 α and -2 α mRNA present in placentas of all gestational ages. Both were expressed by the syncytiotrophoblast, villous cytotrophoblast, and fetoplacental vasculature. In two additional studies (65,66) they demonstrated that the protein expression of HIF-2 α , but not of HIF-1 α or -1 β , is selectively increased in the preeclamptic placenta and is not down-regulated upon oxygenation. As both Flt-1 and VEGF are hypoxia-inducible genes (67,68), responding to HIF and are induced in preeclamptic placentas, these data may support a different mechanism of induction, in the face of the unchanging HIF-1 α in preeclampsia. Furthermore, until recently it was unclear what the net effect of placental hypoxia would be on the balance of Flt-1 and VEGF expression (as both are hypoxia-inducible). Nagamatsu et al (25) have demonstrated that lowering the oxygen percentage from 20% O₂ to 8% O₂ and to 2% O₂ in a primary cytotrophoblast culture caused a rise in sFlt-1 concentration and sFlt-1mRNA was strikingly increased. Although total VEGF levels in these cells increased modestly with hypoxia, free VEGF levels were undetectable along with very low free PIGF concentrations in the media in the presence of stable PIGF mRNA levels. This study provided some evidence that excess sFlt-1 production seen in preeclampsia may be a consequence of placental hypoxia. However, it does not rule out the possibility that alterations in placental sFlt-1 in preeclampsia may be primary and directly lead to the abnormal placentation/pla-

cental hypoxia. Ahmad and Ahmed (27) have also shown that exposure of normal villous explants to hypoxia (1%O₂) caused a 2-fold elevation in the level of sFlt-1 secretion, as compared with 5% O₂ exposure. Nevertheless, when they pre-incubated human umbilical vein endothelial cells (HUVEC) with either conditioned media from preeclampsia placental explants or exogenous sFlt-1, cell migration was significantly reduced. The latter experiment suggests that sFlt-1 may also compromise endothelial trophoblast (which converts into an endothelial cell phenotype) migration and invasion, thus causing defective spiral artery transformation and in turn, hypoxia.

Earlier extensive studies were conducted by Susan Fisher's group. They suggested that oxygen tension could also regulate cytotrophoblasts ability to differentiate and, as a consequence, express proteins that are critical for placental invasion (52). Preeclampsia, on the other hand, is associated with failure of cytotrophoblasts to invade the spiral arterioles (69). Therefore, they hypothesized that in this disease the events that normally take place during the first trimester of pregnancy, which convert the maternal-fetal interface from a relatively hypoxic environment to one that is relatively well oxygenated, fail to occur. In two different invasion assays, 2% oxygen-exposed cytotrophoblasts showed markedly reduced invasion, similar to *in vivo* cytotrophoblast behavior, as seen in preeclamptic placental sections. Hypoxia was also shown to alter the normal pattern of cytotrophoblast integrin switching. Cytotrophoblasts maintained in 20% O₂ rapidly up-regulated their expression of integrins α_5 , a marker first expressed just after these cells leave their basement membrane, and α_1 , which is expressed later, as the cells invade the uterus (70,71). Cells exposed to 2% O₂ completed the initial stage of this process by expressing α_5 , but failed to express α_1 , suggesting that they can initiate, but not complete, the normal integrin switching program.

Fisher suggested that the effects of the relatively hypoxic environment on the proliferative capacity of cytotrophoblasts before 10 weeks of gestation could account for the discrepancy between the rapid increase in placental mass and the slower growth of the embryo proper. Relatively high oxygen tension promotes cytotrophoblast differentiation and explains these cells' extensive invasiveness of the arterial rather than the venous

side of the uterine circulation. Conversely, failure of cytotrophoblasts to gain access to an adequate supply of maternal arterial blood may impair their ability to differentiate into fully invasive cells. The latter scenario could be a contributing factor to preeclampsia.

The effects of hypoxia on the VEGF receptors were initially studied in endothelial cells. Gerber et al showed that hypoxia up-regulated the Flt-1 receptor expression in human umbilical vein endothelial cells (HUVEC), whereas Flk-1/KDR mRNA levels were unchanged or slightly repressed (68). They found an Flt-1 promoter region that included a sequence matching the hypoxia-inducible factor-1 (HIF) consensus binding site previously found in other hypoxia-inducible genes such as the VEGF gene and erythropoietin gene.

How should the algorithm of preeclampsia pathophysiology be? Placental hypoxia → sFlt-1 rise → maternal endothelial dysfunction → preeclampsia? Or maybe: sFlt-1 rise → Placental hypoxia → maternal endothelial dysfunction → preeclampsia? In other words, which is the chicken and which is the egg (26)?

What additional data supports hypoxia, as the primary placental insult, causing preeclampsia? Susan Fisher's group studied a primary culture of cytotrophoblasts isolated from placentas of 3rd trimester HELLP syndrome patients (72). As compared with controls, the sFlt-1 levels in the cytotrophoblast-conditioned medium released approximately twice the amount of the soluble receptor. Based on *in vitro* studies, connecting hypoxia to trophoblast changes characteristic of preeclamptic placentas it is likely that the severe preeclamptic placentas were hypoxic, and therefore sFlt-1 increased secretion is secondary to hypoxia. The study of Nagamatsu et al (25) supports such a conclusion. A negative correlation between oxygen tension and sFlt-1 concentration in the cytotrophoblast cell culture medium was observed. These data are based on *in vitro* experiments and, therefore, cannot be interpreted as the actual *in vivo* mechanism responsible for preeclampsia initiation.

What additional evidence strengthens the notion that an elevated level of sFlt-1 secreted by the placenta is the primary insult in preeclampsia, preceding hypoxia? Decreased serum levels of PlGF have been clearly shown to precede the clin-

ical signs of preeclampsia (17). First trimester reduction of serum PIGF concentration in patients destined to develop preeclampsia (47) may suggest that placental angiogenesis imbalance is the primary cause of abnormal placentation, which eventually leads to preeclampsia in the second or third trimester of pregnancy. Moreover, sFlt-1 has been shown *in vitro* to interfere with cytotrophoblast invasion and differentiation (72). Preliminary experiments in our laboratory with a rat choriocarcinoma cell line (Rcho-1) have demonstrated that sFlt-1 may suppress the differentiation process of rat cytotrophoblast cells into giant trophoblast cells, the rodent equivalent of the human endothelial invasive trophoblast (73). If these effects prove genuine in other trophoblast assays as well, we may be able to speculate that angiogenesis balance is crucial to the very first steps of differentiation and invasion of cytotrophoblasts and maternal vessel remodeling. Hypoxia may, in turn, result in more sFlt-1 production, thus leading to a vicious cycle of sFlt-1 production, eventually causing preeclampsia (Fig. 2). Put differently, the preeclampsia algorithm would look this way: sFlt-1 rise → angiogenesis imbalance → failure of trophoblast invasion and physiological remodeling of uterine spiral arteries → hypoxia → further sFlt-1

rise → maternal endothelial dysfunction → preeclampsia.

Conclusions, Future Research and Treatment Options

The maternal syndrome of preeclampsia is thought to be secondary to abnormal placentation and excess placental production of sFlt-1 (Fig. 1). It is still unclear whether placental hypoxia or excess sFlt-1 production is the trigger event in the pathogenesis of preeclampsia, though most current studies provide evidence that the massive sFlt-1 production noted during clinical preeclampsia may be secondary to placental hypoxia. Further studies in transgenic animals looking at local sFlt-1 over-expression in the placenta may shed light on the role of sFlt-1 during early placental development.

Despite extensive research, preeclampsia remains one of the leading causes of maternal mortality worldwide and yet, there is no reliable screening test (74) or effective treatment to cure this disease. A prospective, multi-center trial to establish the sensitivity, specificity, and predictive value of both serum sFlt-1/PIGF and urinary PIGF as screening tests for preeclampsia in various populations, including healthy women and in various preeclampsia risk groups, may commence soon. Results of such a study will have a tremendous effect on the delivery of optimal maternal and neonatal care. Furthermore, the identification of circulating antiangiogenic factors such as sFlt-1 may facilitate the development of new pharmacologic therapies that would be effective in treating or preventing this devastating disease. That could make a gigantic step towards better feto-maternal well-being.

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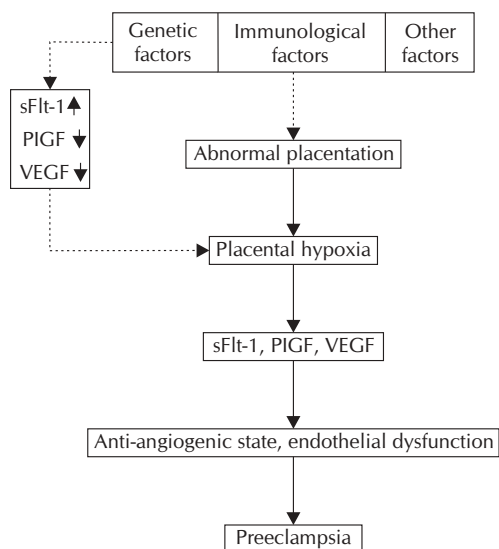


Figure 2. Hypothesis of the role of sFlt-1 and placental hypoxia in the pathogenesis of preeclampsia. Abbreviations: sFlt-1 – soluble Fms-like tyrosine kinase-1; PIGF – placental growth factor; VEGF – vascular endothelial growth factor; full lines – proven relationships; dashed lines – unproven relationship. Copyright 2004, The Endocrine Society (26).

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