

## Endothelial progenitor cells and preeclampsia

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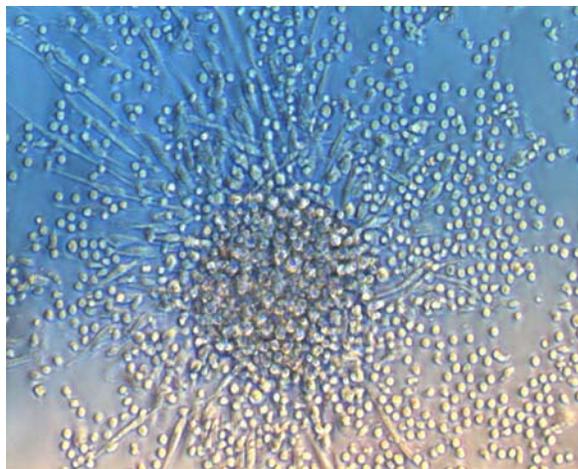
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### 1. ABSTRACT

The maternal cardiovascular adaptation to pregnancy involves a complex physiologic response to the presence of the growing conceptus, including alterations in maternal vascular endothelial cells that contribute to a profound fall in total systemic vascular resistance. There is a large body of evidence that adverse changes in the vascular endothelium underlie the multisystemic maternal manifestations of preeclampsia. Our knowledge is incomplete regarding the mechanisms of adaptive endothelial changes of normal pregnancy and why these changes are attenuated or fail in women who develop preeclampsia. Populations of bone-marrow derived endothelial progenitor cells (EPCs) exist in the adult that are mobilized into the circulation by stimuli such as estrogen and vascular endothelial growth factor. These EPCs can then differentiate into endothelial cells lining the

lumen of blood vessels and/or release growth factors that act in a paracrine fashion to support the endothelium. EPCs are thus thought to function as a cellular reservoir to replace dysfunctional or senescent endothelial cells, and therefore may be critical to the overall health of the vascular endothelium. Data are emerging to suggest that the number of EPCs in the maternal circulation increases with normal pregnancy and that this change fails to occur in women with preeclampsia. While speculative at this point, our overall hypothesis is that an excess of antiangiogenic factors [such as the soluble receptors, soluble fms-like tyrosine kinase (sFlt-1) and soluble endoglin] interfere with nitric oxide-driven mobilization or activity of EPCs in the maternal circulation, contributing to the widespread endothelial dysfunction underlying the clinical manifestations of preeclampsia.



**Figure 1.** A typical colony-forming-unit (CFU) of EPCs, characterized by a central cluster of cells surrounded by elongated cells radiating from the periphery of the cluster, observed 6 days after plating peripheral blood mononuclear cells on fibronectin-coated dishes. As described by Hill *et al* (45), the initially nonadherent cells are recollected after 48 hours and replated to eliminate mature circulating endothelial cells. Quantification of these CFUs provides an estimate of the number of circulating EPCs and their clonogenic potential.

## 2. ENDOTHELIAL PROGENITOR CELLS

### 2.1. Introduction

In its physiologic state, the vascular endothelium modulates vascular tone and acts in anti-inflammatory and anti-thrombotic capacities. Damage to the endothelium results in impairment of these functions and, ultimately, cardiovascular disease.

Until recently, it was thought that endothelial repair in adults was locally mediated (via angiogenesis) and that the process of endothelial differentiation from precursor populations (vasculogenesis) occurred strictly during fetal life. In the past decade, the comprehensiveness of this explanation has come into question. In 1997, Asahara and colleagues demonstrated that a subset of hematopoietic progenitor cells could differentiate into mature endothelial cells *ex vivo* (1). The following year, Shi and colleagues used a canine bone marrow transplantation model to demonstrate that a population of bone-marrow derived progenitor cells could be mobilized to colonize the endothelial surface of a synthetic vascular graft (2). This population of cells is now designated endothelial progenitor cells (EPCs). Investigation of the role of EPCs in endothelial health and cardiovascular disease has expanded remarkably since these findings.

The clinical manifestations of preeclampsia reflect a state of endothelial dysfunction unique to pregnancy. Preeclampsia is also associated with an increased risk of later-life cardiovascular disease (3). Our objective in this review is to discuss the role of EPCs in pregnancy and specifically in preeclampsia, within the context of emerging concepts of EPC physiology.

### 2.2. Characterization of EPCs

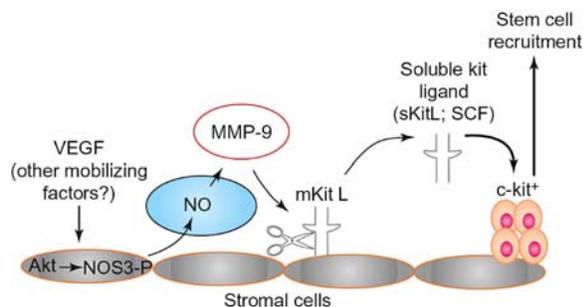
By definition, EPCs comprise a population of bone-marrow derived precursor cells that have the ability to differentiate into mature endothelium. However, such a classification encompasses a heterogeneous group of cell types. This heterogeneity confounds the formation of a consensus regarding optimal methodologies for EPC identification and enumeration.

Methods of EPC quantification include analysis of colony forming units (CFUs) in culture (see Figure 1) and flow cytometry. CFU methodologies assess clonogenic potential of a heterogeneous group of progenitor cells, as discussed below. Flow cytometric techniques provide precise quantification of specific cellular populations on the basis of cell surface antigens. Magnetic bead sorting techniques similarly facilitate the separation of specific populations for use in functional studies.

The specific antigens utilized in magnetic bead sorting and flow cytometry vary significantly. Stem cell antigens are typically used in combination with VEGFR-2 (vascular endothelial growth factor receptor 2, also known as KDR, kinase insert domain receptor) to ensure both the progenitor and endothelial nature of the cell. The most commonly used antigens include CD34 and CD133 (AC133). The rarity of CD133+ or CD34+ EPCs, which represent approximately 0.001 to 0.03% of total peripheral blood mononuclear cells, complicates flow cytometric techniques, because even a minimal degree of contamination or nonspecific binding can introduce error (4, 5). While combined CD34 and VEGFR-2 positivity identifies populations of cells that contain stem cells with the capacity to differentiate into endothelium, they may also identify a subpopulation of circulating mature endothelial cells. CD133 antigen expression does seem to be limited to primitive stem cells, and these cells have also been shown to have the capacity to differentiate into endothelium (6). It does seem to be generally agreed upon that the combination of CD133 and VEGFR-2 identifies an early lineage EPC population (4, 7).

With time, EPCs in peripheral blood appear to lose CD133 and acquire CD34, with CD34+/VEGFR-2+ cells becoming the main constituent of the EPC pool. One recent functional study suggested that CD133+/VEGFR-2+ EPCs represent a less mature (earlier lineage) variation of EPCs than CD34+/CD133+/VEGFR-2+ cells, and that the former population have more efficient abilities to migrate (home) to sites of endothelial injury and to promote regeneration than the latter. Overall, CD133+/VEGFR-2+ and CD34+/CD133+/VEGFR-2+ EPCs are considered less mature than CD34+/VEGFR-2+ EPCs, and may represent those recently mobilized from bone marrow (8).

In culture, cell surface marker expression changes, consistent with acquisition of mature endothelial markers. These changes have been found to occur within 2 days of initiation of culture (9). The CD34-/CD133+ cells differentiate *in vitro*, acquiring CD34 and mature endothelial markers as they downregulate CD133 (10).



**Figure 2.** Matrix metalloproteinase-9 (MMP-9) is regulated by nitric oxide synthase-3 (NOS-3)-derived nitric oxide (NO). Vascular endothelial growth factor (VEGF) and other mobilizing stimuli might activate the NOS3 within the bone marrow stromal cells. This stimulates and/or maintains MMP-9 activity resulting in cleavage of soluble kit ligand (sKitL) from membrane-bound kit ligand to mobilize c-kit<sup>+</sup> stem cells into the circulation. (Reprinted, with permission, from Aicher, A., C. Heeschen & S. Dimmeler: The role of NOS3 in stem cell mobilization. Reproduced with permission from 29).

CFU enumeration methodologies take advantage of the fact that in addition to cells coexpressing VEGFR-2 and CD133 or CD34, a much larger subset of primary adherent peripheral blood mononuclear cells can also form EPCs (11). Bone marrow-derived CD14<sup>+</sup> monocytes/macrophages are the major source of a type of EPC perhaps best described as "proangiogenic monocytes" or "circulating angiogenic cells (CACs)". These more plentiful EPC/CACs adhere to fibronectin-coated plates in endothelial growth-promoting conditions and can be identified by the uptake of DiI-Ac-LDL and surface binding of lectins such as *Ulex europaeus*, which are thought to be endothelial specific (10). Recent data suggest that most of these CD14<sup>+</sup> mononuclear cell-derived EPC/CACs actually express low levels of CD34, below the detection sensitivity of classic flow cytometry (12). There is some evidence that circulating endothelial cell precursors within the mononuclear cell fraction consist of two populations that can be separated on the basis of the timing of their outgrowth in culture. The later-outgrowth cell population may more specifically represent a true bone-marrow derived progenitor cell with a greater capacity for expansion (13).

One recent investigation comparing different methods of identifying EPCs found that these methods often correlated poorly, particularly when antigen-based methods were compared with assessments of clonogenic potential (CFU enumeration). Moreover, they found that CFU enumeration correlated well with adhesive properties of cells, whereas antigenic markers correlated well with angiogenic factors (14). Another complicating factor is the presence of secondary bone-marrow-derived populations of EPCs, including myeloid lines (15), as well as other peripheral populations that provide EPCs to some extent as well (reviewed in 7). As consensus regarding the use of cell-surface antigens has not yet been reached, rigorous

functional studies of these different cell populations will be crucial in standardization of these definitions (4).

Clarifying the differences between various EPC populations will be important, particularly as investigation moves into the therapeutic potential of these cells. The inclusion of multiple subgroups of putative EPCs may be beneficial, in that they may contribute to repair via several different mechanisms. On the other hand, detailed characterization of each of the cell types may enable specificity of therapy with minimization of side effects. Ultimately, further studies regarding each of these methods and cell populations will help to inform a consensus regarding the identification of EPCs. The currently available data suggest that several distinct populations of EPCs exist, each with different functional capabilities, and multiple approaches are warranted in investigations in this area.

### 2.3. Mechanisms of EPC function

The populations of EPCs act to maintain endothelial health. They play significant roles in both neovascularization of ischemic tissue and in the repair of injured endothelium. The ways in which they do so are incompletely elucidated, but they seem to function both to promote angiogenesis as well as to enable complete vasculogenesis in their capacity as angioblasts.

The function of EPCs has been described as a three-step process: 1) mobilization from bone marrow, 2) homing to sites of injury, and 3) adhesion/incorporation (16). Each of these phases is influenced by both endogenous factors and may also be modified by the administration of exogenous agents.

Multiple factors influence the mobilization of EPCs from bone marrow. Asahara initially demonstrated that vascular endothelial growth factor (VEGF) modulates EPC behavior and increases mobilization (17). This group also demonstrated that tissue ischemia directly mobilizes EPCs, and administration of exogenous granulocyte macrophage-colony stimulating factor (GM-CSF) further augments this response (18). Other factors stimulating these processes include placental growth factor (PlGF), stromal cell-derived factor-1 (SDF-1), erythropoietin (Epo), HMG-CoA reductase inhibitors (statins), peroxisome proliferator-activated receptor-gamma (PPAR-gamma) agonists, exercise, and estradiol (19-27).

Activation of endothelial nitric oxide synthase (eNOS, NOS3) in the bone marrow is thought to be the common pathway for mobilization of EPCs (see Figure 2) (28-30). Nitric oxide (NO), in turn, activates matrix metalloproteinase 9 (MMP-9), leading to shedding of c-kit from the cell surface of stem/progenitor cells, an essential step in release of EPCs from the bone marrow stroma (5). VEGF activates eNOS within vascular endothelial cells and EPCs through the induction of calcium flux and the phosphorylation of eNOS via the phosphatidylinositol-3-kinase Akt pathway (23, 29). Additional pathways may be involved; studies of endothelial cells have clearly shown that the ERK pathway is involved in the phosphorylation of eNOS as regulated by estrogen (31). The mechanism by

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which activated eNOS subsequently increases MMP-9 expression in EPCs remains undetermined. Neither VEGF, aerobic exercise or estrogen are able to mobilize EPCs into the circulation of eNOS (Nos3<sup>-/-</sup>) knockout mice, and these mice exhibit impaired neovascularization related to a deficit in EPCs (28, 29). Further, eNOS blockade with N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME) in mice blunts the exercise-induced mobilization of EPCs (26). Particularly relevant to pregnancy, estradiol also augments EPC mobilization and function via mechanisms involving nitric oxide (NO) and MMP-9 and additionally inhibits EPC apoptosis (27, 32).

Homing of EPCs to sites of endothelial injury or tissue hypoxia, inflammation, or necrosis remains incompletely understood. SDF-1 may facilitate this behavior (33), and there is some evidence that this mechanism involves upregulation of SDF-1 by hypoxia-inducible factor-1 (HIF-1) (34).

After homing to an area of injury, EPCs adhere to the vascular wall and become incorporated into the vascular architecture. There, they can become either direct endodonors, taking on an endothelial phenotype themselves, or they can invade beyond the endothelial monolayer, residing immediately behind the vessel wall and acquiring a more supportive or paracrine role (5). The process of adhesion relies upon ICAM-1 expression in ischemic tissue (35), and alpha 4, beta 1 integrins and beta 2 integrins play a role (36, 37). Invasion beyond the endothelial layer requires protease activity, notably cathepsin L (38).

EPCs may promote neovascularization by mobilizing and supporting local tissue-residing cells. Support for this mechanism comes from studies showing that conditioned media from these cells induce a strong migratory response of mature endothelial cells (39-41). The specific paracrine factors involved remain incompletely delineated; however the most comprehensive assessment of EPC cytokine expression implicates VEGF, SDF-1, insulin-like growth factor-1 (IGF-1), and hepatocyte growth factor (HGF) (39). There is also evidence that IL-8 may play a role in EPC signaling (42). Beyond secreted factors, there is also evidence that EPCs can promote phenotypic change locally via direct cell-to-cell contact (43).

### 3. CARDIOVASCULAR DISEASE AND EPCS

Despite our incomplete understanding of the identification and functionality of EPCs, strong evidence links EPC number and function to clinical cardiovascular outcomes. Patients with cardiovascular disease have been shown to have both decreased number and function of EPCs in comparison to healthy controls, and the degree of decrement correlates with the number of cardiovascular risk factors (44). Moreover, in a group of healthy volunteers, the number of circulating EPCs correlates inversely with the subjects' Framingham risk score as well as with flow-mediated brachial artery reactivity, a physiologic measure of endothelial function (45). In

patients with known cardiac disease, reduced levels of circulating EPCs are independently predictive of future cardiovascular events (46, 47). Thus far, these data clearly support a strong association; whether this represents a causal or mechanistic relationship remains undetermined.

Recently attention has focused on the potential therapeutic role for EPCs in cardiovascular disease states. Thus far, the goal has been to assess the role of EPCs in reducing tissue ischemia and repairing vascular injury in acute settings, primarily focusing on acute myocardial infarction and peripheral limb ischemia. Initial studies utilized animal models, with more recent investigations in humans. In animal studies, therapeutic efficacy of EPCs has been shown after local or systemic injection, with improvement in capillary density, blood flow, and cardiac function (reviewed in 48).

Several preliminary studies have investigated the feasibility, safety, and potential clinical efficacy of intracoronary infusion of EPCs or more broadly defined stem cells in human patients with acute myocardial infarction. Taken together, they demonstrate good safety and feasibility. Overall improvements in measures of cardiac function are persistent at up to one year of follow-up (49-52). These results have prompted initiation of larger-scale clinical trials.

An alternative approach to direct autologous cell transfer involving the use of G-CSF to stimulate endogenous mobilization of EPCs has been studied as well. Three small trials have suggested that this method successfully mobilizes EPCs and seems to confer some improvement in cardiac function parameters (53-55). However, one of these trials was stopped early due to safety concerns about stent restenosis in the G-CSF group (55). A larger trial compared G-CSF with placebo and reassuringly found no difference in restenosis rates; however, there was also no treatment benefit in terms of cardiac function (56). This indirect method of EPC stimulation remains a subject of cautious investigation.

Early trials of autologous intracardiac injection of stem cells in more chronic settings have yielded mixed results and overall appear less promising (reviewed in 16, 48). In peripheral vascular disease, one study suggests that stem cell injection may improve outcomes (57). Other areas of investigation include treatment of pulmonary hypertension, genetic engineering of EPCs in an attempt to enhance the adaptive qualities of these cells (48), and the use of EPCs to treat preclinical atherosclerotic disease before an ischemic event has occurred (58).

### 4. PREGNANCY AND EPCS

The association of increased EPCs with cardiovascular health prompts the hypothesis that EPCs allow the endothelium to remain healthy. Pregnancy presents a physiologic challenge to this system that depends on the successful adaptation and plasticity of the endothelium. It is biologically plausible that upregulation

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of EPCs contributes to the adaptive cardiovascular changes of normal pregnancy.

### 4.1. Normal adaptation to pregnancy

Maternal cardiovascular adaptation occurs during normal human pregnancy to accommodate the needs of the maternal-fetal pair. Systemic vascular resistance decreases by approximately one-third, cardiac output and plasma volume increase nearly two-fold, and vascular reactivity and responses to vasopressor agonists diminish (59). Both neoangiogenesis, especially in the uteroplacental vascular bed, and vasodilation of existing vessels contribute to these adaptive changes. Factors elaborated from the endothelium contribute to the vasodilation, including a lesser contribution from prostaglandins and a more significant effect of NO, as we will discuss further below (60). Current knowledge regarding the function of EPCs in non-pregnant subjects, as well as the role of the endothelium in cardiovascular adaptation to pregnancy, raise the question of whether EPCs may be involved in this adaptation. Evidence that estrogen stimulates EPC mobilization and function further substantiates this theory (27). While speculation is appealing, the existing literature regarding EPCs in pregnancy is extremely sparse. The few published studies will be reviewed here.

### 4.2. Evidence of EPC changes in pregnancy

In 2002, Gussin *et al* (61) showed that peripheral blood mononuclear cells from both pregnant and nonpregnant women yielded early outgrowth (1 week culture) EPCs; however, late outgrowth (8-10 week culture) proliferative EPCs were observed only in samples from pregnant women. In a recent study designed to examine the relationship between estrogen during pregnancy and EPC number (62), the mean number of EPC colony forming units (CFU) in peripheral blood was found to be increased in pregnant patients as gestational age increased from 12 weeks to 41 weeks gestational age. The investigators also found that the increase in EPC CFU correlated with increasing estradiol levels ( $r=0.72$ ), consistent with the hypothesis that the vascular effects of estrogen during pregnancy include an increase in circulating EPCs. The cross-sectional, descriptive design of this study limited its comparisons. As the rise in estrogen parallels the increase in placental mass, mechanistic studies will be needed to delineate the role of estrogen versus other placental factors in EPC function.

Our group has recently demonstrated an increase in EPCs (defined using flow cytometric techniques as CD34+/KDR+ cells) in normal pregnancy compared to a control group of women who have never been pregnant (abstract 63). These EPCs were increased no later than 18-23 weeks' gestation, to a degree similar to that reported after ovarian hyperstimulation in the context of planned *in vitro* fertilization (32). Studies are underway that follow patients longitudinally through pregnancy.

In pregnancy, EPCs in the maternal circulation may be either maternal or fetal in origin. Studies thus far suggest primarily or perhaps exclusively maternal derivation. Gussin *et al* have investigated this issue using

both EPC-selective culture techniques and magnetic cell separation techniques (using CD133+ and CD105+, an endothelial marker also known as endoglin). EPCs from pregnant women with male fetuses were evaluated for the presence of a Y chromosome by fluorescence *in situ* hybridization and polymerase chain reaction techniques and, so far, their results have not detected any cells of fetal origin (61, 64, 65). Interestingly, EPCs (defined as CD34+ cells) have also been isolated from fetal umbilical cord blood (66).

The knowledge regarding the role of EPCs in normal pregnancy adaptation remains far from complete. Only a few studies have been published investigating EPCs in pregnancy. These have included select populations of EPCs or were limited in breadth of technique. Further, we are not aware of any laboratory animal studies dealing with pregnancy-induced changes in EPCs. Despite these limitations, studies completed thus far are consistent with the hypothesis that EPCs act to promote the vascular changes that occur during normal pregnancy.

## 5. PREECLAMPSIA AND EPCS

Because preeclampsia is a pregnancy-specific disorder characterized by endothelial dysfunction, it has been reasoned that mechanisms causing either endothelial injury or impeding endothelial repair may contribute to its pathogenesis. It has been further hypothesized that an abnormal number or dysfunction of EPCs limits the success of maternal adaptation to pregnancy and thereby plays a role in the development of the disease.

### 5.1. Endothelial dysfunction of preeclampsia

Clinically, preeclampsia is defined by hypertension and proteinuria, and can show variable degrees of organ system involvement, including disturbances of hematologic, hepatic, renal, neurologic, and uteroplacental systems, all of which may be influenced by endothelial dysfunction. Multiple lines of investigation substantiate the centrality of the endothelium in the pathophysiology of preeclampsia. Histologic changes, circulating markers of endothelial activation, and *in vitro* functional studies all support this concept (67). However, new information is continually emerging regarding the cardiovascular pathophysiology of preeclampsia. For example, one study found that functional and structural skin capillary density increases by mid-gestation in normotensive pregnant women but that women with preeclampsia exhibit pronounced peripheral capillary rarefaction compared to both gestational age-matched pregnant and non-pregnant controls (68).

The histologic renal lesion pathognomonic of preeclampsia, glomeruloendotheliosis, is characterized by swelling and hypertrophy of the glomerular endothelium that can substantially or completely occlude the capillary lumen. The endothelial fenestrae are often obliterated by this process (69).

In terms of circulating markers of endothelial activation, preeclampsia is associated with decreased prostacyclin and increased endothelin-1, cellular

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fibronectin, and thrombomodulin. Hypercoagulability is characteristically associated with preeclampsia as well; an altered balance of factors elaborated by the endothelium contributes to this predisposition, including increased expression of the procoagulants tissue factor, von Willebrand's factor, and platelet activating factor. Decreases in anticoagulant factors derived from the endothelium are also associated, including antithrombin III, protein C, and protein S. Expression of endothelial cellular adhesion molecules provides further evidence of endothelial activation, including ICAM-1, VCAM-1, and cFN (70).

The first functional evidence of endothelial dysfunction came from assessments of pressor responsiveness in pregnant women with preeclampsia; it was found that the physiologic suppression of pressor responsiveness characteristic of normal pregnancy is lost in the disease (71). Furthermore, other studies have shown that exposure of human umbilical vein endothelial cells to serum from preeclamptic patients results in endothelial activation (72). Investigation of arterial vasodilation in women with preeclampsia has shown an impaired vasodilatory response (73, 74). Likewise, arteries from women with normal pregnancy, upon exposure to plasma from women with preeclampsia also show impaired vasodilation (75). Mechanistically, it has been suggested that pathologic metabolism of NO may contribute to the endothelial dysfunction of preeclampsia, as we will discuss further below.

The significance of endothelial dysfunction in the development of preeclampsia highlights a potential link between preeclampsia and EPCs. Abnormal EPC function or number prior to or during pregnancy could lead to compromised endothelial repair, persistent or accelerated endothelial injury, and the subsequent pathologic changes seen in preeclamptic patients. While the evidence regarding EPCs in preeclampsia remains preliminary in nature, it does support such a link; the existing studies will be reviewed here.

### 5.2. Evidence of EPC changes in preeclampsia

Sugawara *et al* conducted a case-control study to investigate EPC number and function in preeclampsia. Eight patients with preeclampsia were compared to seven controls with normal pregnancies. EPCs were quantified by CFU techniques. The preeclamptic group had significantly fewer CFUs compared to the normal pregnant group. In addition, beta-galactosidase activity (a marker of cellular senescence) was higher in the preeclamptic group. The authors concluded that preeclamptic pregnancies were associated with not only a decreased number of EPCs, but also increased cellular aging. This indicates an association of a change in function of EPCs with preeclampsia (76).

In a study by Kim *et al*, twelve patients with preeclampsia were compared to twelve patients with normal pregnancies, using CFU methodology. The mean number of EPC colonies was decreased in the patients with preeclampsia, consistent with the findings of Sugawara. In addition, corresponding VEGF and VEGF receptor levels

were measured. Although total VEGF levels were not different between the two groups, VEGFR-1 levels were higher in the preeclamptic groups, while VEGFR-2 (KDR) levels were lower (abstract 77).

Our group found compatible results when comparing the number of EPCs from peripheral blood of gestational age-matched women with normal and preeclamptic pregnancies by flow cytometry. We found that both CD34+/KDR+ and CD133+/KDR+ cells were significantly decreased in preeclamptic patients compared to those with normal pregnancies. Interestingly, the numbers of circulating EPCs in the preeclamptic group were not significantly different from those who had never been pregnant (abstract 63).

In addition to the noted decrease in maternal circulating EPCs in preeclampsia, one group has also found a decrement in cord blood EPCs in preeclampsia compared with controls. This was associated with a decreased in free VEGF and a trend toward increased concentrations of the soluble form of the VEGF receptor, sFlt-1 (abstract 78).

In contrast to the studies described above, Matsubara *et al* (79) report that 1) the number of CD34+/CD133+/VEGFR-2+ EPCs in maternal peripheral blood is increased in the luteal phase compared with the follicular phase of the menstrual cycle, 2) the number of these EPCs during the first trimester of pregnancy is similar to the luteal phase, and 3) EPCs decrease significantly during the course of pregnancy, reaching a nadir during the third trimester. Furthermore, the number of circulating EPCs in women with preeclampsia was not different from third trimester normal pregnant. These apparent differences in results may relate to the sub-types of EPCs analyzed. For example, under most conditions, peripheral blood CD34+/VEGFR-2+ EPCs are several-fold more frequent than CD133+/VEGFR-2+ EPCs, and the latter, in turn, are more frequent than CD34+/CD133+/VEGFR-2+ EPCs. In a recent study of these progenitor cell subtypes by flow cytometry, only CD34+/VEGFR-2+ cells were significantly reduced in patients with high carotid intima-media thickness (marker of subclinical atherosclerosis) compared to healthy subjects (8).

The results from the above studies provide initial support for an association between impaired number and function of certain EPC subtypes with preeclampsia. Further research is needed to clarify 1) if there are distinct populations of EPCs that may have varying roles in pregnancy-related endothelial repair, 2) which populations of EPCs are involved in the disease process, 3) if the change in number of EPCs is accompanied by a change in function of these cells, and 4) if there is a potential opportunity to intercept or modify these alterations.

## 6. MECHANISTIC SPECULATION

The mechanisms by which EPCs may functionally contribute to the physiologic adaptation to pregnancy remain indistinct. Further, the pathways by which EPC impairment contributes to cardiovascular disease and preeclampsia also requires further

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investigation. As previously discussed, the angiogenic factors VEGF and PlGF directly stimulate NO production in the bone marrow and subsequent mobilization of EPCs. Further, rodent models suggest that VEGF-mediated mobilization of EPCs depends upon NOS (28, 29). One might therefore speculate that in normal pregnancy, angiogenic factors stimulate NO, therefore enabling EPC function and maintaining endothelial health. Deficiencies along this pathway may result in impaired mobilization of EPCs and thus inadequate endothelial repair, ultimately predisposing to disease states such as preeclampsia and cardiovascular disease. We propose a model whereby NO and angiogenic factors such as VEGF and PlGF may play central roles in the pathogenesis of preeclampsia.

### 6.1. Nitric oxide in normal pregnancy and preeclampsia

Evidence from animal models suggests a clear role for NO in adaptation to pregnancy, including increased NO synthesis, increased serum and urine concentrations of cGMP (a second messenger of NO), and increased urine concentrations of nitrate (NO<sub>x</sub>, a metabolite of NO). The NO synthase inhibitor, L-NAME, blocks the conversion of L-arginine to NO. Administration of L-NAME decreases urinary NO<sub>x</sub>, and specifically inhibits the physiologic attenuation of pressor responsiveness, suggesting that this adaptation can be attributed to NO. Human studies investigating NO levels in pregnancy have been more complicated and controversial. While there does seem to be an increase in plasma and urinary cGMP, studies addressing NO or its metabolites directly have revealed mixed findings, perhaps in part because dietary influences that can significantly alter the concentrations of NO<sub>x</sub> are much more difficult to control in humans (80). Conrad *et al* (81) reported that plasma and urinary NO<sub>x</sub> do not rise in parallel with cGMP during pregnancy in women on a controlled (reduced) nitrite diet. Furthermore, in contrast to gravid rats, NO-hemoglobin was not detected in the red blood cells of these women. Several explanations for the apparent divergence of cGMP and NO<sub>x</sub> were noted in addition to the possibility that agents other than NO may be primarily responsible for the increase in cGMP and the maternal vasodilation in women. For example, circulating NO<sub>x</sub> may not adequately reflect NO biosynthesis in the vasculature (local NO involved in regulation of vascular tone).

Although the cause of endothelial dysfunction in preeclampsia is clearly multifactorial, increasing evidence implicates reduction in the bioavailability of NO (oxidative destruction of NO, inappropriate sequestration of NO, and/or reduced NO synthesis) locally, i.e., failure of NO to reach its intended targets, as a root cause of maternal endothelial dysfunction. Chronic administration of L-NAME to pregnant rats results in the clinical manifestations of hypertension, proteinuria and other findings similar to preeclampsia. This is reversible with administration of L-arginine infusion (80, 82). In human preeclampsia, available evidence suggests an overall decrease in circulating NO<sub>x</sub>, though this remains controversial. While some studies have shown no clear difference in circulating levels, this is confounded by concurrent impaired renal excretion due to preeclamptic

changes as well as by the difficulties regulating dietary NO<sub>x</sub> in humans (80).

Assessment of true NO functionality in preeclampsia is further confounded by local oxidative reactions. In the local intravascular environment, NO can react with superoxide, forming the potent oxidant peroxynitrite. Peroxynitrite subsequently attacks proteins to form the stable reaction product nitrotyrosine, which then accumulates locally. This marker of oxidative damage is increased in placental villous vascular endothelium of women with preeclampsia or diabetes, and in maternal subcutaneous microvessels from women with preeclampsia (83-85). The diversion of NO from its physiologic pathway would suggest that the normal effects of NO may be diminished in these conditions. In support of this, there is evidence that in diabetes, NO production per se may not be reduced, but may not be bioavailable because of its destruction by superoxide produced in the vascular wall (86).

Further indirect support for the concept that NO may be implicated in the pathogenesis of preeclampsia via an EPC-related mechanism comes from the diabetic population. EPCs from individuals with type I or type II diabetes or coronary disease appear to "remember" their dysfunctional phenotype when in culture, as they exhibit impaired migratory capacity, proliferation, colony formation, and incorporation into tube-like structures compared to controls (87-90). Furthermore, conditioned media from diabetic patient EPCs were significantly reduced in their capacity to support endothelial (HUVEC) tube formation compared to control EPCs (89). Intriguingly, the suppressed migratory response in response to VEGF or SDF-1 was reversed by incubating the EPCs from diabetic patients with an NO donor (DETA/NO), but not with an NO donor in combination with an NO scavenger (carboxy-PTIO) (88).

### 6.2. Angiogenic factors in preeclampsia

As mentioned, angiogenic factors appear to play an important role in EPC mobilization. The importance of angiogenic factors in the pathophysiology of preeclampsia in general has been the subject of a great deal of recent investigation, and the evidence in this regard will be reviewed elsewhere in detail in this journal. In brief, the soluble form of the VEGF receptor, sFlt-1, antagonizes the functional activity of both of the angiogenic factors VEGF and PlGF. In mice, exogenous administration of sFlt-1 induces a preeclampsia-like syndrome (91). In human preeclampsia, and preceding clinically evident disease, the proangiogenic factors VEGF and PlGF are decreased compared with normal pregnancy, and sFlt-1 is increased (92). This excess sFlt-1 appears to derive primarily from the placenta, but peripheral blood mononuclear cells or other blood cells from preeclamptic women may also produce increased amounts of this soluble factor (93). Another antiangiogenic factor, soluble endoglin, may play a role in preeclampsia pathogenesis as well (94). Transforming growth factor (TGF)-β1 regulates the expression of matrix metalloproteinases 2 and 9 (enzymes involved in angiogenesis), and soluble endoglin appears to

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inhibit TGF-beta1 signaling in endothelial cells and blocks TGF-beta1-mediated activation of nitric oxide synthase (94, 95). It seems biologically plausible, therefore, that an excess of these two soluble receptors would interfere with EPC function.

### 7. CONCLUSIONS AND PERSPECTIVES

It has become clear in the past decade that bone-marrow derived EPCs play a critical role in maintenance of endothelial health. We propose that they also play a role in the normal maternal adaptation to pregnancy and that deficiencies in EPC function may lead to the pregnancy-specific disorder, preeclampsia. The mechanisms by which deficits in EPC function occur remain speculative, but there is indirect evidence consistent with the notion that an imbalance in angiogenic factors in preeclampsia may limit mobilization of EPCs via a pathway involving NO.

The clinical association between preeclampsia and later-life cardiovascular disease, as well as the apparent shared pathophysiologic processes point to a potential therapeutic opportunity. Interventions early in these processes would have greater potential for success. Might preeclampsia represent a window into processes that would otherwise only become apparent later in life? If so, might the identification of these patients at this stage in their lives enable interventions that modulate endothelial progenitor cell function such as exercise, statin therapy, and weight management that could significantly alter the trajectory of their endothelial disease?

The investigation of these mechanisms has the potential capacity to enable understanding of a disease that significantly affects maternal and child health. It may further allow for early intervention and prevention of cardiovascular disease in women.

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