Soluble Vascular Endothelial Growth Factor Receptor-1 (sFLT-1) Mediates Downregulation of FLT-1 and Prevents Activated Neutrophils From Women With Preeclampsia From Additional Migration by VEGF

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Abstract—Neutrophil activation and increased migration is associated with preeclampsia and is resolved after delivery. Preeclampsia is an inflammatory disorder where altered levels of vascular endothelial growth factor (VEGF) and the circulating soluble flt-like tyrosine kinase 1 (sFlt-1) have a pathogenic role. VEGF, by binding to FLT-1, induces leukocytic chemotaxis. We studied expression and function of FLT-1 in maternal neutrophils during preeclampsia and normal pregnancies. Analysis of maternal neutrophils showed the relationship between FLT-1 expression and week of gestation. Preeclamptic women express lower FLT-1 and sFLT-1 in neutrophils. In contrast, serum levels of s FLT-1 in patients with preeclampsia are increased and, therefore, inhibit upregulation of FLT-1 in neutrophils by neutralizing VEGF. VEGF-dependent FLT-1 expression is regulated by changing FLT-1-promoter activity. Promoter activity is decreased by sFLT-1. In vitro experiments demonstrated that migration of neutrophils is regulated by VEGF via FLT-1 and excess of sFLT-1. Thus, VEGF-dependent migration of neutrophils is decreased during preeclampsia as a consequence of excess circulating sFlt1. But, they still increase migration by fMLP and, therefore, migration of neutrophils from preeclamptic women is highly activated when compared with the normotensive group. In conclusion, besides being involved in inducing an antiangiogenic state in the serum, excess of sFLT-1 seems to prevent activated neutrophils from women with preeclampsia from additional migration by VEGF. We provide evidence that neutrophils may be involved in the pathophysiology of pregnancy-related hypertensive disorders. (Circ Res. 2005;97:1253-1261.)

Key Words: migration | neutrophils | preeclampsia | pregnancy | VEGF receptor 1

Preeclampsia (PE) is a hypertensive disorder of unknown etiology affecting 5% to 10% of all pregnancies. Pathophysiologic changes include elevated systemic vascular resistance, generalized vasoconstriction, activation of the coagulation cascade, maternal endothelial dysfunction, and a poorly perfused fetoplacental unit.1 Preeclampsia is characterized by altered cytokine production2 and marked neutrophil activation.3–6 They release proteases and reactive oxygen species, which can cause vascular endothelial injury, inappropriate endothelial activation or transformation.7–9 The latter processes are important for physiological adaptations during normal pregnancy creating a low-resistance arteriolar system, which allows adequate blood supply to the growing fetus.10,11

Recently, case-control studies have shown associations between polymorphisms of the vascular endothelial growth factor (VEGF) gene and preeclampsia.12 VEGF is a multifunctional cytokine that plays a pivotal role in angiogenesis in vivo.13 VEGF is expressed by different cells, eg, smooth muscle, endothelial cells, monocytes/macrophages, and polymorphonuclear neutrophils (PMNs). VEGF exerts its biological effects through Fms-like tyrosine kinase 1 (FLT-1)/VEGF receptor-1 and VEGF receptor-2 (FLK-1/KDR).13 KDR is considered as the principal receptor of the VEGF-dependent angiogenic signals resulting in normal vessel growth and maintenance.13 In contrast, FLT-1 has recently gained attention because of its overall implication in pathological angiogenesis and inflammation.13 Thus, VEGF by binding to FLT-1, induces monocyte chemotaxis and modulates transendothelial PMN migration and activation.14–16 Furthermore, VEGF-induced macrophage migration is strongly suppressed in FLT-1(TK−/−) mice.17

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Previous studies emphasized the physiological significance of VEGF during pregnancy. FLT-1 is found in the blood of pregnant but not in nonpregnant women and men. In the serum it is known as soluble FLT-1 (sFlt1),18 sFLT-1 functions as a “decoy” during development and prevents VEGF from binding to its signaling receptor KDR.19,20 This scavenger role might be involved in the pathogenesis of preeclampsia.13,21 Increased sFlt1 production by the preeclamptic placenta scavenges VEGF and placental growth factor (PIGF), thereby lowering circulating levels of unbound VEGF and PIGF. This altered balance causes generalized endothelial dysfunction resulting in multi-organ disease.13 It was demonstrated in pregnant rats, that sFLT-1 treatment induces hypertension, proteinuria, and glomerular endotheliosis.21

Because of the reports about the physiological impact of the VEGF-system during pregnancy and its role in activating monocytes, we hypothesize the existence of differences in the expression, regulation, and function of FLT-1 in maternal neutrophils between normotensive and preeclamptic pregnancies. We studied, therefore, the relation between expression of FLT-1 in PMNs and sFLT-1 of healthy and preeclamptic women. We asked how VEGF and sFLT-1 are able to regulate chemotaxis of maternal PMNs in healthy and preeclamptic women.

**Materials and Methods**

Detailed Materials and Methods are available in the online data supplement available at http://circres.ahajournals.org.

The investigation conforms with the principles outlined in the Declaration of Helsinki. Maternal blood was obtained during and immediately after delivery from healthy women (n = 179) and patients with severe PE (n = 42) at the Department of Obstetrics and was used for further analysis. Clinical data were collected at routine obstetric visits and are summarized in the Table. All pregnant women were normotensive and nonproteinuric before the week 24 of pregnancy. Preeclampsia was defined according to criteria of the American College of Obstetricians and Gynecologists. Patients with other medical complications were not included.

For RT-PCR analysis and cell culture experiments, PMNs were carefully isolated from whole blood samples immediately after obtaining them from the women.

**Flow Cytometry**

Blood samples were tested by direct immunofluorescence staining of whole blood using fluorochrome-conjugated antibodies. All analyses were performed on a FACSscan flow cytometer (Becton Dickinson). The results are expressed as fluorescence histograms plotted on a log scale.

Briefly, maternal PMNs were identified based on the typical morphology in the forward scatter/side scatter cytogram and with monoclonal antibodies against the neutrophil surface markers CD11b (aM-Integrin, MAC-1; Becton-Dickinson) and CD6 (Fc receptor IIb; Immunotech) according to the manufactures instructions. Antibodies against the lymphocyte surface marker CD4 (Becton-Dickinson) were used as negative control for PMNs. Human serum was used to minimize nonspecific antibody binding to Fc receptors. Nonspecific fluorescence was assessed by substituting a nonbinding isotype-matched control FITC conjugated to sheep F(ab)2 anti-mouse IgG (Sigma) for the primary mAb. A polyclonal (goat) primary antibody raised against the internal domain of FLT-1 (SC-316G; Santa Cruz) was conjugated with FITC and used for flow cytometry.

**Immunofluorescence**

Isolated maternal PMNs were fixed with 4% paraformaldehyde. The cells were subsequently incubated with the primary antibody raised against FLT-1 (SC-316G; Santa Cruz) and examined with a laser scanning confocal microscope (LSM-META, Carl Zeiss).

**RT-PCR**

Total cellular RNA was isolated from isolated maternal PMNs. Human microvascular endothelial cells were used as a positive control for FLT-1 and KDR expression.

For RT-PCR analysis, first-strand cDNA was synthesized as described previously.22 RT-PCR and quantitative RT-PCR (Tagman) was used to compare mRNA levels of FLT-1 and sFLT-1 in PMNs of normotensive and preeclamptic women. TaqMan analysis was performed according to the manufacturer’s instructions, with the use of an Applied Biosystems 7700 system (PerkinElmer).

**Enzyme-Linked Immunosorbent Assay**

Sandwich enzyme-linked immunosorbent assay (ELISA) for the detection of total sFLT-1 was performed according to manufacturer’s (RELIAtech) specifications. Aliquots of serum from normotensive and preeclamptic women were assayed in duplicate.

**Cell Culture and Assays**

**Comparison of VEGF-Mediated FLT-1 Induction on PMNs**

According to previous experiments with human vascular endothelial cells, where VEGF upregulates FLT-1 expression,24 we tested whether VEGF can affect FLT-1 expression in PMNs. Isolated maternal PMNs were cultured. Expression of FLT-1 on PMNs was assessed by incubating cultures with 0.6 nM recombinant VEGF165 (Biochrome AG) or preincubation with sFLT-1 (1,000 pg/mL) for 2 hours before incubating with VEGF or aqua bidest (negative control). After 24 hours FLT-1 expression was measured by quantitative flow cytometry.

**Measurement of Neutrophil Chemotaxis in Boyden Chamber**

To compare FLT-1-dependent migration of PMNs from preeclamptic and healthy women, chemotaxis was assessed using multidish 6-well cell culture plates with a 3-μm pore diameter polycarbonate membrane transwell apparatus (cell culture insert, NUNC) as directed by the manufacturer. Recombinant VEGF165 at different concentrations (0.3 and 0.6 nM; Biochrome AG) or aqua bidest (negative control) was applied to the lower wells of the chamber. To study the effects of sFLT-1 in maternal serum on PMN chemotaxis, neutrophils from normotensive women were preincubated with sFLT-1 (1000 pg/mL) for 2 hours before incubating with 0.6 nM VEGF, or pretreated with...
serum from normotensive and preeclamptic women for 2 hours. After incubation for 24 hours, the cells that had migrated through the filter were collected and counted. PMN response to other cytokines was investigated by using N-formylmethionyl-leucyl-phenylalanine (10 ng/mL; fMLP; Sigma), a positive control for a neutrophil chemoattractant.

Transcriptional Regulation of FLT-1

To examine the putative functional significance of enhanced FLT-1 expression after VEGF exposure on the transcriptional level, we studied its promoter activity in HL-60 cells. Based on the sequence of the human FLT-1 promoter [gi 1088437, D64016.1], we performed a genomic PCR. The resulting PCR products (1196 bp of the 5'-regulatory region directly upstream of the putative translation initiation start codon) were subcloned into the luciferase reporter vector pGL3basic (Promega) and the identity of the inserts was confirmed by sequencing.25

HL-60 cells (DSMZ) were cultured in RPMI1640 containing 10% FBS for 72 hours. Then, HL-60 cells were suspended in serum-free RPMI1640 medium, mixed with FLT-1 reporter-plasmid and electroporated in the Gene Pulser Xcell System (Bio-Rad). For standardizing the transfection efficiency, pSV-Beta-Galactosidase vector (Promega) was cotransfected. The cells were immediately cultured either with 0.6 nM recombinant VEGF165 or pretreated with sFLT-1 (1000 pg/mL) for 2 hours before incubating with VEGF or with aqua bidest (negative control) for 24 hours. We analyzed luciferase and beta-galactosidase activity as previously described.25

Screening of the Promoter of the FLT-1 Gene for Genetic Variations

Information about genetic variations were obtained either by database search in the NCBI’s dbSNP, HGMD (Human Gene Mutation Database), and HGBASE (Human Genic Bi-Allelic Sequences) or by Single-Strand Conformation Polymorphism (SSCP) analysis.

SSCP Analysis

Genomic DNA from all study subjects was extracted by standard techniques.25 From the published sequences of the FLT-1 promoter gene (D64016) overlapping fragments, ~320 bp in length were enzymatically amplified to cover the entire promoter region in 166 healthy (332 alleles) women and 33 patients (66 alleles) with preeclampsia.

All PCR amplifications were performed by standard techniques using specific primers (supplementary Table I), followed by single-strand conformation polymorphism (SSCP) analysis as previously described. DNA from patients presenting different migration patterns on the polyacrylamide gels were then sequenced twice (both DNA strands with sense and antisense primers) with the use of an automated sequencing device (ABI PRISM 377, Perkin-Elmer).

Statistical Analysis

Data analysis was performed using the Statistical Package for Social Sciences for Windows (SPSS; versions 11.0) and Sigma Plot for Windows Version 8.0 (Systat Software GmbH). Values are given as means±SEM if not otherwise indicated. Statistical analyses between the hypertensive and control group were performed using analysis of variance, the Mann-Whitney test or Student t test. In addition, statistical analysis of the migration assay was preformed using the Kruskal-Wallis test. P<0.05 was regarded as the level of significance.

Results

Identification of FLT-1 in Maternal Neutrophils

As shown in Figure 1A, forward scatter and side scatter were used to identify maternal PMNs. Figure 1B and 1C illustrate the gates used to characterize PMNs by CD16 and CD11b expression, whereas CD4 expression was not detected (Figure 1D). Our flow cytometry revealed specific FLT-1 expression in the maternal CD16+/CD11b+ PMNs (Figure 1E and 1F). Substituting control FITC conjugated to sheep F(ab)2 antihuman IgG instead of primary antibody revealed no immunofluorescence in the flow cytometry (Figure 1G).

Immunohistochemistry demonstrated specific staining for FLT-1 in the cytoplasm and cell membrane of PMNs (Figure 1H) by using laser scanning confocal microscopy.

Specificity of the immunoreactions used for both methods was assessed by using different amounts of primary antibody (data not shown) and peptide pre-absorbed antiserum against FLT-1-peptide and antiserum against CD11b pre-absorbed with FLT-1-peptide (supplemental Figure 1).

Characterization of FLT-1 Expression in Maternal Neutrophils of Hypertensive Pregnancies

Figure 2A and 2B present FLT-1, sFLT-1 and KDR expression on mRNA level in maternal PMNs of healthy women with the preeclamptic pregnancies. RT-PCR analysis revealed expression of FLT-1 and sFLT-1 only (Figure 2). Omission of the reverse transcriptase from the reaction mixture resulted in no amplification (data not presented). Sequence analysis of the obtained KDR, FLT-1, and sFLT-1 RT-PCR products confirmed total homology with the published human mRNA sequences.

Quantitative results of FLT-1 and sFLT-1 mRNA levels by realtime RT-PCR is given in Figure 2. Statistical analysis of the data showed a significantly decreased FLT-1 and sFLT-1 expression in PMNs of the preeclamptic group compared with normotensive controls, when related to β-actin mRNA expression.

Furthermore, FLT-1 protein expression in maternal PMNs from 179 healthy women were studied for between gestational week (GW) 6 and 42 and compared with the pre-eclamptic group between GW 23 and 41 using FACS analysis. Additionally, PMNs from 9 healthy women were studied 24 hours after giving birth. The plot in Figure 3A demonstrates the relationship between FLT-1 expression and week of gestation in healthy women. FLT-1 expression increased during the first two trimesters, peaked at 30 to 33 weeks, and decreased thereafter with the lowest expression after giving birth (Figure 3A).

In contrast, PMNs from patients with preeclampsia expressed significantly lower FLT-1 levels (Figure 3B) when compared with the healthy group (Figure 3B). In subgroup analysis in patients with and without preeclampsia of matched gestational age, FLT-1 expression in the PMNs was significantly lower in preeclamptic women (Figure 3C) as compared with healthy women.

Another subgroup analysis (gestational week 30 and 38) compared FLT-1 expression with sFLT-1 serum levels during normotensive and preeclamptic pregnancies (Figure 4A). Soluble FLT-1 serum concentrations are elevated in the PE group when compared with the normotensive women. In contrast, FLT-1 expression in PMNs is decreased in the PE group.

FLT-1 Expression in Maternal PMNs After VEGF Stimulation

It was demonstrated that VEGF exposure leads to higher levels of FLT-1 in cultured ECs,24 but nothing was known.
about a VEGF-dependent regulation of FLT-1 in leukocytic cells. Therefore, we determined whether VEGF is able to induce endogenous FLT-1 expression in PMNs of both study groups at the same GW. Within the control group FLT-1-expression increased in PMNs after treatment with VEGF for 24 hours. By contrast, PMNs from patients with preeclampsia were unable to upregulate FLT-1 expression (Figure 4B).

We investigated whether the increase in sFLT-1 levels in preeclampsia is responsible for the altered FLT-1 expression in PMNs (Figure 4A), because the activity of VEGF can be modulated by sFLT-1. Indeed, preincubation with exogenous sFLT-1 significantly attenuated FLT-1 expression in PMNs from normotensive women in response to VEGF (Figure 4C).

These results stimulated us to characterize in detail the VEGF-dependent FLT-1 upregulation in PMNs on the transcriptional level of the FLT-1 promoter. Reporter analysis was performed in HL-60, a leukocytic cell line with the ability to differentiate into neutrophils. We could demonstrate increased promoter activity after stimulation with VEGF (Figure 4D). Again, pre-incubation with exogenous sFLT-1 significantly attenuated FLT-1 promoter activity in HL-60 cells in response to VEGF (Figure 4D).

It has been reported that the gene for FLT-1 contains putative ETS-responsive elements in its promoter, rendering them potentially susceptible for transcriptional activation by VEGF via ETS-1.26,27 Therefore, we screened the two groups of patients for genetic differences within the promoter region of the FLT-1 gene, because a variation in one of the ETS-responsive elements of the FLT-1 promoter might change FLT-1 expression. Database search and SSCP analysis found no genetic variation within the ETS-responsive elements of the reported FLT-1 promoter region (data not shown).

**FLT-1-Dependent Migration of Maternal Neutrophils**

Next, we investigated VEGF-dependent PMNs migration. As shown in Figure 5A migration of PMNs from preeclamptic
women is significantly increased. VEGF is able to stimulate cell migration dose-dependently only in the healthy group (Figure 5A). The chemoattractant fMLP that was used as a positive control was still able to induce PMN migration in the preeclamptic group (Figure 5A).

Furthermore, we investigated whether increased sFLT-1 levels during preeclampsia are responsible for the compromised PMN migration after VEGF stimulation. Preincubation with exogenous sFLT-1 significantly attenuated migration in response to VEGF (Figure 5B) in PMNs from normotensive women. Likewise, the inhibitory effect of the serum from preeclamptic women on VEGF-dependent PMN migration could be demonstrated (Figure 5C). In contrast, PMNs preincubated with serum from normotensive women were still able to migrate after VEGF stimulation.

Again, a significant increase of cell migration was seen after preincubation with serum from preeclamptic patients (Figure 5C).

**Discussion**

Our results support previous findings demonstrating the impact of the VEGF system during preeclamptic pregnancy. Previous data emphasize that excessive placental sFLT-1 production, by neutralizing VEGF and PlGF, may play a causal role in the pathogenesis of the maternal syndrome in preeclampsia. sFLT-1 production induces an antiangiogenic
An adequate and organized interaction of VEGF with its receptors is essential for normal placental development and function as well as maintenance of the established maternal vasculature. Continuous low levels of VEGF are required for endothelial cells to survive prolonged periods and to function properly. Thus, when sFLT-1 plasma levels rise, they may reduce the circulating (or free) VEGF levels below a critical threshold required for vasculogenesis and the maintenance of the established vasculature during pregnancy. In pregnant women with preeclampsia, the placenta produces elevated levels of sFLT-1, which captures free VEGF and PlGF. First attempts to measure VEGF concentrations in the blood of preeclamptic women were confusing and contradictory. This contradiction exists because only some groups were able to measure the unbound VEGF and PlGF, while others detected total (unbound and bound) VEGF and PlGF. A recent study solved this problem. Preeclampsia is characterized by normal to high total VEGF levels but low physiologically more relevant unbound (free) VEGF and PlGF, owing to a vast excess of sFlt1. It was suggested that excess placental production of sFlt1 contributes to hypertension, proteinuria, and glomerular endotheliosis noted in patients with preeclampsia. Therefore, balance of VEGF, PlGF, and sFLT-1 is carefully regulated, because of the high level of angiogenesis that is necessary to maintain a successful pregnancy. Cross-sectional analysis of serum obtained within gestational-age intervals revealed that maternal sFLT-1, VEGF and PlGF concentrations increase during the first two trimesters. When sFLT-1 and VEGF concentrations peak pre-delivery and decrease after giving birth, PlGF levels peaked already at 29 to 32 weeks, and decreased thereafter. Interestingly, we found a similar expression pattern of FLT-1 in PMNs over the different weeks of gestation (Figure 3A), detected significantly lower levels of FLT-1 in PMNs during PE and confirmed previous findings of higher sFLT-1 blood levels (Figure 4A) during preeclampsia. These differences between sFLT-1 blood levels and FLT-1 expression in PMNs are not very surprising considering that the increase in the sFlt-1 level is paralleled by decreased free VEGF levels, especially, in the preeclamptic group when compared with normotensive women. It is suggested that the decrease in these factors may be attributable in part to binding by sFlt-1.

Facing the fact that maternal blood levels of VEGF as well as FLT-1 expression on cell surface of PMNs increased in the same manner during the first two trimesters (Figure 3A), we have to remember a recent study showing increased FLT-1 expression in endothelial cells after VEGF exposure. Therefore, we became interested to understand whether VEGF is also able to induce FLT-1 expression in maternal PMNs during normal and preeclamptic pregnancies. Increased FLT-1 expression in normal PMNs was found after incubation with VEGF conditioned medium (Figure 4B). In contrast, PMNs from preeclamptic patients were unable to upregulate FLT-1 expression (Figure 4B). Thus, we addressed whether the elevated sFLT-1 in patients with preeclampsia inhibits upregulation of FLT-1 in neutrophils by neutralizing VEGF.
Indeed, our experiments demonstrated that sFLT-1 inhibits VEGF-dependent upregulation of FLT-1 (Figure 4C) by changing its promoter activity (Figure 4D), because the gene for FLT-1 contains putative ETS-responsive elements in its promoter, rendering them potentially susceptible for transcriptional activation by VEGF via ETS-1. These results stimulated us to screen the two groups of patients for genetic differences within the promoter region of the FLT-1 gene. In fact, a variation in one of the ETS-responsive elements of the FLT-1 promoter could be responsible for changes of FLT-1 expression. Because the general catalog of genome variation with the complete contents of dbSNP, HGMD, and HGBase is available, we can easily screen those databases for published genetic variations. However, our bioinformatics analysis failed to find any kind of genetic variation within the whole FLT-1 promoter region (data not shown). Genotyping of the patients’ DNA by SSCP analysis and additional sequencing confirmed that there are no variations in the ETS-responsive elements (data not shown). Nevertheless, future studies performing large-scale associations with promoter analysis of genetic variations of the ETS-1 gene should be initiated for a better understanding of the impact of ETS-1 on the transcriptional regulation of FLT-1 during hypertensive pregnancies.

So far, the functional consequence of the attenuated FLT-1 expression in PMNs during preeclampsia remained unsolved.

Figure 4. Impact of sFLT-1 on FLT-1 expression in maternal PMNs after VEGF stimulation. A, Subgroup analysis (GW 30 to 38) compared FLT-1 expression with sFLT-1 serum levels during normotensive and preeclamptic pregnancies. (n=9). B, Isolated maternal neutrophils (at the same gestational week) were stimulated with 0.6 nM VEGF165 for 24 hours. FLT-1 expression was measured by FACS analysis before (FLT-1VEGF) and after stimulation (FLT-1VEGF). (n=9). C, Expression of FLT-1 on PMNs from normotensive pregnant women was assessed by incubating cultures with VEGF165 for 24 hours or by pretreating with sFLT-1 for 2 hours before incubating with VEGF. FLT-1 expression was measured by FACS analysis before (FLT-1VEGF) and after stimulation (FLT-1VEGF). (n=6). D, HL-60 cells transfected with FLT-1 promoter construct. The cells were cultured with VEGF165 or pretreated with sFLT-1 for 2 hours before incubating with VEGF or with aquabidest (used as negative control) for 24 hour. Relative light units (RLU) were determined as described in Materials and Methods. (n=3)
We knew that resting monocytes normally express low levels of FLT-1; binding to FLT-1, induces monocyte chemotaxis and modulates transendothelial migration. Our results confirmed that FLT-1 is involved in VEGF-induced chemotaxis of peripheral blood cells, because these cells express only FLT-1 but not KDR (Figure 2A and 2C) and migrate in response to exogenous VEGF (Figure 5A and 5B). Thus, when FLT-1 is downregulated in preeclamptic PMNs no VEGF-dependent chemotaxis could be observed (Figure 5A), although fMLP (a standard chemokine used as a positive control) was still able to induce normal PMNs migration. Furthermore, sFLT-1 (Figure 5B) or medium conditioned with preeclamptic serum rich in sFLT-1 (Figure 5C) decreased neutrophil migration by neutralizing VEGF. These findings are in accordance with recent reports showing decreased endothelial cell migration after incubation with sFLT-1 or conditioned medium from preeclamptic placenta.

How does the decreased FLT-1 expression in neutrophils of preeclamptic women fit into the understanding of the pathophysiology of the maternal syndrome of preeclampsia? In general, pregnancy is associated with a generalized inflammatory response. One reason is the maintenance of the peripheral tolerance to fetal alloantigen. Thus, during gestation physiological adaptation of innate and adaptive immune system is a necessary key element for maternal homeostasis. PMNs and monocytes as parts of the innate immune system appear to be the most profoundly affected cells as shown by distinct changes in their phenotype and function. In particular, proper receptor-driven cell functions such as apoptosis and VEGF/FLT1-dependent chemotaxis are required.

Compared with normal pregnancy, the systemic inflammatory response is much more pronounced during preeclampsia. The reason for this extensive systemic inflammation remains unknown. It is believed that a hypoxic and dysfunctional preeclamptic placenta releases increased amounts of proinflammatory components: plasma factors, like IL-6, TNF-α, and sFLT-1, cell debris, such as syncytiotrophoblast membrane microparticles, and even cytotrophoblasts into the maternal circulation. Increased plasma levels of sFLT-1, besides other released proinflammatory factors, then alter neutrophil function (Figures 4 and 5).

We therefore speculate that the consequence of this placental disposal plus the impaired VEGF response of neutrophils (Figures 4B and 5A) increases the maternal inflammatory burden. This leads to a higher PMNs activation and, in turn, a potentiated interaction with the vascular endothelium resulting in increased infiltration of the maternal systemic vasculature, and contributes to vascular endothelial dysfunction, which is central for the pathophysiology of preeclampsia. Indeed, our results based on absolute cell migration measurements revealed a significantly increased migration of PMNs from preeclamptic women when compared with controls (Figure 5A). Furthermore, medium conditioned with preeclamptic serum increased even migration of PMNs from normotensive pregnant women (Figure 5C). This could be attributable to increased plasma concentrations of cytokines, such as TNF-α.

Figure 5. Neutrophil chemotaxis in response to VEGF, sFLT-1 and fMLP. A, PMNs from control (n=7) and preeclamptic (n=6) women at same gestational age were exposed to medium with different doses of VEGF (0.3 and 0.6 nM) and aqua bidest (negative control). Neutrophil chemotactic response was expressed as percentage of migrated PMNs. Each result represents an average of two independent experiments per patient. B, VEGF-induced migration of PMNs from normotensive women (n=3) with and without preincubation of sFLT-1. (Relative cell migration; migration_{VEGF}/migration_{VEGF}). C, VEGF-induced migration of PMNs from healthy pregnant women (n=3) after preincubation with serum from normotensive and preeclamptic women.
In summary, increased sFLT-1 appears to alter the neutrophil function resulting in loss of VEGF-dependent migration during preeclampsia. Moreover, sFLT-1 appears to down-regulate FLT-1 expression in PMNs because of its VEGF neutralizing action.

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Soluble Vascular Endothelial Growth Factor Receptor-1 (sFLT-1) Mediates Downregulation of FLT-1 and Prevents Activated Neutrophils From Women With Preeclampsia From Additional Migration by VEGF

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Expanded Materials and Methods

For RT-PCR analysis and cell culture experiments PMNs were carefully isolated from whole blood samples immediately after obtaining them from the women. Neutrophils (>95% purity, >95% viability ± 2 as per trypan blue and propidium iodid exclusion) were purified over a gradient using Polymorphprep® (AXIS-SHIELD PoC AS, Norway) and characterized by flow cytometry for CD4, CD11b, CD16 and FLT-1 expression.

Flow cytometry

Blood samples were tested by direct immunofluorescence staining of whole blood using fluorochrome-conjugated antibodies. All analyses were performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, USA). The results were expressed as fluorescence histograms plotted on a log scale. Compensation and PMT voltages were standardized before each run using CalibriteTM beads (Becton Dickinson, Germany).

Maternal PMNs were identified based on the typical morphology in the FSC/SSC cytogram and with monoclonal antibodies against neutrophil surface markers conjugated with fluorochromes phycoerithrin for CD11B (αM-Integrin, MAC-1; Becton-Dickinson, Germany) and PC5 for CD16 (FC receptor IIIb; Immunotech, Germany) according to the manufactures instructions. Antibodies against the lymphocyte surface marker CD4, conjugated with FITC (Becton-Dickinson, Germany), were used as negative control for PMNs. Anti-mouse IgG1, conjugated with PE or FITC was used as isotype control. For the procedure, 10 µl of fluorochrome-conjugated antibody was added to 50 µl of whole blood according to the manufactures protocol.

Different monoclonal antibodies against the external domain of FLT-1 from commercial source (V4262, Sigma Aldrich, Germany) or donated by A. Menrad (Schering AG, Germany) were tested for characterization of FLT-1 expression in maternal PMNs. Binding was assessed either by the colour indirect method performing a secondary detection with FITC conjugated to sheep F(ab)2 anti-mouse IgG (Sigma, Germany) or direct FITC conjugated
primary antibodies against FLT-1. Human serum was used to minimize nonspecific antibody binding to Fc receptors. Nonspecific fluorescence was assessed by substituting a nonbinding isotype-matched control FITC conjugated to sheep F(ab)2 anti-mouse IgG (Sigma, Germany) for the primary mAb. Nevertheless, nonspecific binding to the Fc receptors on PMNs could not be prevented.

A polyclonal (goat) primary antibody raised against the internal domain of FLT-1 (SC-316G; Santa Cruz, Germany) was conjugated with FITC by using The Zenon Goat IgG Labeling Kit (Molecular Probes Europe BV, The Netherlands) and used for flow cytometry. Briefly, for the intracellular staining, the CD11b+ / CD16+ cells were permeabilized, washed with Cellwash (BD Biosciences Pharmingen, Germany), and stained sequentially with FITC-conjugated polyclonal antibody against FLT-1 containing Lysing solution G (BD Biosciences Pharmingen, Germany) at 4°C for 30 min.

Specificity of the obtained immunoreactions was assessed by using different amounts of first antibody against FLT-1, peptide pre-absorbed antiserum against FLT-1-peptide (SC-316P; Santa Cruz, Germany; 2:1 and 1:1 competition for 30 min at room temperature), peptide pre-absorption of antiserum against CD11b with FLT-1-peptide (negative control) or substituting control FITC conjugated to sheep F(ab)2 anti-mouse IgG (Sigma Aldrich, Germany) instead of primary antibody.

All samples were tested by quantitative flow cytometry for FLT-1- and CD16b-expression. The intensity levels for all fluorescence markers were standardized for each run using Calibrite beads (Becton-Dickinson Biosciences, Germany). The mean channel fluorescence (MCF) for each conjugated antibody binding to neutrophils was determined by comparing sample MCF of four levels of calibrated beads containing a known number of fluorochrome molecules per bead (Quantibrite Beads, Becton-Dickinson Biosciences, Germany). An MCF ratio of 101 or higher was voted as a "positive" reaction. This value invariably exceeded that obtained with normal control serum by at least 3.0 SD. Analysis was performed using the quantitative calibration option of “Win List” (Verity Software House, United States of America).
Immunofluorescence

Isolated maternal PMNs were fixed with fresh 4% paraformaldehyde, rinsed in phosphate-buffered saline and then incubated for 1 h in blocking buffer (PBS containing 1% BSA and 10% goat serum; DAKO, Germany). The cells were subsequently incubated with the polyclonal primary antibody raised against the internal domain of FLT-1, diluted in PBS containing 0.1 % Triton X-100 at a concentration of 1:100. Cells were washed with PBS, and incubated with a secondary FITC-conjugated anti-goat IgG Ab (Jackson ImmunoResearch Europe Ltd, UK) at a dilution of 1:200 in PBS containing 0.1 % Triton X-100. Subsequently, cells were washed, and mounted to be examined with a laser scanning confocal microscope (LSM-META, Carl Zeiss, Germany).

Negative control reactions for FLT-1-immunostaining were conducted on isolated PMNs with substitution of either PBS or normal goat serum or preabsorption of the primary antibody with an excess of FLT-1 peptide (SC-316P; Santa Cruz, Germany).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from isolated maternal CD11+/ CD16+/ CD4-/ FLT-1+ PMNs using TRIZOL reagent (Invitrogen, Germany). Human microvascular endothelial cells were used as a positive control for FLT-1 and KDR expression.

For RT-PCR analysis, first-strand cDNA was synthesized as described previously 1. Control PCR assays were performed to estimate cDNA amount, as well as RT efficiency and cDNA quality, using specific primers to human $\beta$-actin 2. Reactions were carried out as previously described 1.

RT-PCR and quantitative RT-PCR (Taqman) was used to compare mRNA levels of FLT-1 in PMNs of normotensive and preeclamptic women. Primer and probes (FLT-1 forward: 5´-TTCCCTGGATGAGCAGTGTGA-3´ and reverse: 3´-GTGGCCAATGTGGGGAAGATAAG-5´, probe:5-6-FAMTGATGCCAGCAAGTGGGAGTTTG-TAMRA-3; sFLT-1 forward: 5-GGCTGACTCTAGAATTCTGGAATCT-3 and reverse: 5-
GTGGTACAATCATTCTTGCTT-3, probe: 5-6-FAM-ACTTGGAAAAATGCGACGGAAGG-TAMRA-3; KDR forward: 5-GAA AGC ATC GAA GTC TCA TGC-3’ and reverse: 5-GTGGTACAATCATTCTTGCTT-3; β-actin forward: 5-ATCGTCCACCAGCAATGCTT-3; reverse: 5-CAACCGACTGCTGACCTTCA-3; probe: 5-6-FAM-CACCCTTTCTTGACAAAACCTAACTTGCGC-TAMRA-3) were designed with the use of the program Primer Express 1.0 (PE Applied Biosystems). RT-PCR was carried out as previously described. TaqMan analysis was carried out according to the manufacturer’s instructions, with the use of an Applied Biosystems 7700 system (PerkinElmer). The amplification was performed with the following time course: 94°C for 3 minutes and 40 cycles of 94°C for 30 s, 60°C for 1 minute and 72°C for 1 minute. Each sample was tested in triplicate. Quantitation of the amount of target in unknown samples is accomplished by using the Standard Curve Method. Plasmid DNA (pCR II vector; Invitrogen, Netherlands) containing either the sFLT-1, FLT-1- or human β-actin-RT-PCR product was used to prepare absolute standards. Expression levels of sFLT-1, FLT-1 were normalized to β-actin expression. For realtime PCR analysis of β-actin cDNA was diluted 1:10 due to the higher amount of β-actin expression. Specificity of the FLT-1, sFLT-1 and KDR RT-PCR products was confirmed by sequencing using an automated sequencing device (ABI PRISM 377, Applied Biosystems, Germany).

**Enzyme-Linked Immunosorbent Assay**

Sandwich ELISA for the detection of total (free and complexed) sVEGFR-1 was performed according to manufacturer’s (RELIATech) specifications. Aliquots of serum from normotensive and preeclamptic women were assayed in duplicate.

**Cell Culture and Assays**

Comparison of VEGF-mediated FLT-1 induction on PMNs

According to previous experiments with human vascular endothelial cells, where VEGF up-regulates FLT-1 expression, we tested whether or not VEGF can affect FLT-1 expression in
PMNs. Isolated maternal PMNs were cultured in RPMI 1640 (Seromed, Germany) containing 5 % FCS, 1% L-Glutamin (2mM) and 1% Penicillin/Streptomycin at 37 °C. Expression of FLT-1 on PMNs was assessed by incubating cultures with 0.6 nM recombinant VEGF165 (Biochrome AG, Germany) or by pretreating with sFLT-1 (1,000 pg/mL) for 2 hours before incubating with VEGF or aqua bidest (negative control). After 24 hours, FLT-1 expression was measured by quantitative flow cytometry.

Measurement of neutrophil chemotaxis in Boyden chamber
To compare FLT-1-dependent migration of PMNs from preeclamptic and healthy women, chemotaxis was assessed using multidish-6-well-cell culture plates with a 3-µm pore diameter polycarbonate membrane transwell apparatus (cell culture insert, NUNC, Germany) as directed by the manufacturer. 1.5 x 10^6 cells/well were placed over the filter in the upper wells of the chamber and chemotactic were agents added to the lower chamber. Recombinant VEGF165 at different concentrations (0.3 / 0.6 nM; Biochrome AG, Germany) or aqua bidest (negative control) was applied to the lower wells of the chamber. To study the effects of sFLT-1 and maternal serum on PMNs’ chemotaxis, neutrophils from normotensive women were pretreated with sFLT-1 (1,000 pg/mL) for 2 hours before incubating with VEGF (0.6 nM) or pretreated with serum from normotensive and preeclamptic women for 2 hours. After incubation for 24 h the cells that had migrated through the filter were collected by centrifugation and counted. The data were expressed as the average number of cells that had migrated into three wells. Viability at the end of the assay in both chambers remained >95 ± 2%. The cells that had migrated nonspecifically were excluded.

PMN responses to other cytokines was investigated by using N-formylmethionyl-leucyl-phenylalanine (10 ng/ml, fMLP; Sigma, Germany) instead of VEGF165 as a positive control for a neutrophil chemoattractant.

Transcriptional Regulation of FLT-1
To examine the putative functional significance of enhanced FLT-1 expression after VEGF exposure on the transcriptional level, we studied its promoter activity in HL-60 cells. Based on the sequence of the human FLT-1 promoter [gi 1088437,|D64016.1], we performed a genomic PCR (5'-GCGCACCGCTGTGGCAACTTTGGTACC-3' and 5'-GCGCAAGCTTTGTGAGAAGCAGACAGCTGAGCA-3' containing restriction sites for HindIII respectively MluI at their 5'-end) using genomic templates. The resulting PCR products (1196 bp of the 5'-regulatory region directly upstream of the putative translation initiation start codon) were subcloned into the luciferase reporter vector pGL3basic (Promega, Madison, USA) and the identity of the inserts was confirmed by sequencing.

HL-60 cells (DSMZ- Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) were cultured at 0.5 x 10^5 cells/ml RPMI 1640 (1% L-Glutamin, 1% Penicillin/Streptomycin) containing 10 % FBS for 72 hours. Next, HL-60 cells (2 x 10^7 cells) were suspended in serum-free RPMI 1640 medium, mixed with pGL3-FLT-1 reporter plasmid and electroporated in the Gene Pulser Xcell System (Bio-Rad, Germany). For standardizing the transfection efficiency, pSV-Beta-Galactosidase vector (Promega, Germany) was cotransfected. The cells were immediately transferred to a 12-well plate and cultured either with 0.6 nM recombinant VEGF165 or pretreated with sFLT-1 (1000 pg/mL) for 2 hours before incubating with VEGF or with aqua bidest (used as negative control) for 24h, respectively.

We analyzed luciferase and beta-galactosidase activity as previously described. The transfection data represent the mean relative luciferase activity (RLA) values of 3 independent experiments (each n=3). With respect to statistical analysis of transient transfection experiments, a two-tailed t-test was performed and statistical significance was assumed at P<0.05.

Screening of the promoter of the FLT-1 gene for genetic variations
Information about genetic variations were obtained either by database search in the NCBI's dbSNP, HGMD (Human Gene Mutation Database), and HGBASE (Human Genic Bi-Allelic Sequences) or by Single-Strand Conformation Polymorphism (SSCP) analysis.

SSCP-analysis

Genomic DNA from all study subjects was extracted by standard techniques. From the published sequences of the FLT-1 promoter gene (D64016) overlapping fragments, ~320 bp in length were enzymatically amplified to cover the entire promoter region in all 166 healthy (332 alleles) women and 33 patients (66 alleles) with preeclampsia. All PCR amplifications were performed by standard techniques using of specific primer (table S1), followed by single-strand conformation polymorphism (SSCP) analysis as previously described. DNA from patients presenting different migration patterns on the polyacrylamide gels were then sequenced twice (both DNA strands with sense and antisense primers) with the use of an automated sequencing device (ABI PRISM 377, Perkin-Elmer).

Statistical Analysis

Data analysis was performed using the Statistical Package for Social Sciences (SPSS, version 11.0 for Windows, United States of America) and Sigma Plot for Windows Version 8.0 (Systat Software GmbH, Germany). Values are given as means ± SEM if not otherwise indicated. Statistical analyses between the hypertensive and control group were performed using analysis of variance, the Mann-Whitney test or Student’s t test. In addition, statistical analysis of the migration assay was performed using the Kruskal-Wallis-test. P < 0.05 was regarded as the level of significance.
References


Additional Figures and supporting information:

Figure S1

Figure S1: FACS analysis of FLT-1 expression in maternal neutrophils using peptide pre-absorbed antibodies against FLT-1.

Specificity of the obtained immunoreactions within the flow cytometry was assessed by using different amounts of peptide pre-absorbed antiserum against FLT-1 (2:1 and 1:1 competition for 30 min at room temperature; A, C and E). In contrast, pre-absorption of the CD11b antibody with the FLT-1 peptide (2:1 and 1:1 competition for 30 min at room temperature; B, D and F) did not change its immunoreaction. We conclude, that immunoreactions against FLT-1 are highly specific. Data are representative of three independent experiments.
### Additional Tables:

**Table S1: FLT-1 Primer sequences for SSCP-analysis**

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