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Overexpression of the Soluble Vascular Endothelial Growth Factor Receptor in Preeclamptic Patients: Pathophysiological Consequences

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Several growth factors such as vascular endothelial growth factor (VEGF)-A and placental growth factor (PlGF) are involved in the placental vascular development. We investigated whether dysregulation in the VEGF family may explain the defective uteroplacental vascularization characterizing preeclampsia. We compared pregnancies complicated by early onset severe preeclampsia or intrauterine growth retardation to normal pregnancies. Maternal plasma, placentas, and placental bed biopsies were collected. The mRNA levels of VEGF-A, PlGF, and their receptors were quantified in placentas and placental beds. Levels of VEGF-A, PlGF, and soluble VEGF receptor (VEGFR) were assessed in maternal plasma. In compromised pregnancies, elevated levels of VEGF-A and VEGFR-1 mRNAs may reflect the hypoxic status of the placenta. On contrast, the membrane-bound VEGFR-1 was decreased in the placental bed of preeclamptic patients. Preeclampsia was associated with low levels of circulating PlGF and increased levels of total VEGF-A and soluble VEGFR-1. Free VEGF-A was undetectable in maternal blood. Immunohistochemical studies revealed that VEGF-A and PlGF were localized in trophoblastic cells. Altogether, our results suggest two different pathophysiological mechanisms associated with preeclampsia. The first one is related to an overproduction of competitive soluble VEGFR-1 that may lead to suppression of VEGF-A and PlGF effects. The second one is the down-regulation of its membrane bound form (VEGFR-1) in the placental bed, which may result in the defective uteroplacental development. (*J Clin Endocrinol Metab* 88: 5555–5563, 2003)

PREECLAMPSIA IS AN important cause of maternal morbidity and mortality and is responsible for more than 40% of iatrogenic deliveries (1). The underlying pathogenetic mechanisms of this maternal syndrome are much debated. Current hypotheses include inflammatory disease, vascular-mediated factors, placental ischemia, genetic predisposition, and immune maladaptation (2–5). Preeclampsia is also associated with defective uteroplacental vascularization (2). Angiogenesis and vascular transformation of the uteroplacental unit, which are crucial to normal fetal development, are impaired (6). However, molecular pathways responsible for normal angiogenesis and vascular remodeling in the fetomaternal unit are still poorly understood. Knockout studies performed in mice demonstrate the crucial role of vascular endothelial growth factor (VEGF) in embryonic development (7–9). Inactivation of a single VEGF gene resulted in embryonic lethality in heterozygous embryos at d 11–12, and significant defects in placental vasculature were observed. VEGF seems thus to be involved in placental vascular development (10).

VEGF-A is one of the most important growth factors for endothelium. It induces angiogenesis and endothelial cell proliferation and plays an important role in regulating vasculogenesis (11, 12). Other proteins are related in structure and receptor specificity to VEGF-A. The VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF) (13). There are several splice variants of VEGF-A. The major ones include 121, 165, 189, and 206 amino acids, each comprising a specific exon addition. VEGF 165 is the most predominant protein. The 165, 189, and 206 amino acids splice variants have heparin-binding domains, which are involved in presentation to VEGF receptors (VEGFRs). PlGF is a member of the VEGF family closely related to VEGF-A (10).

VEGF-A transcription is potentiated in response to hypoxia and by activated oncogenes (14). Hypoxia-inducible factor (HIF)-1 is a transcriptional complex that plays a central role in oxygen homeostasis. HIF binds to hypoxia response elements of the VEGF-A gene as a heterodimer of proteins designated HIF-1α and HIF-1β subunits (15). HIF-1α subunits are specific to the response to hypoxia, whereas HIF-1β subunits are constitutive nuclear proteins (16). In normoxia, HIF-1α subunits are rapidly destroyed by the ubiquitous proteasome pathway such that the transcription complex cannot form (17, 18). Recent studies have shown that degradation of HIF-1α is secondary to its recognition by the von Hippel Lindau tumor suppressor protein. Interactions between HIF-1α and von Hippel Lindau tumor suppressor

Abbreviations: CTR, Internal control; HIF, hypoxia-inducible factor; IUGR, intrauterine growth retardation; NRP, neuropilin; PlGF, placental growth factor; sVEGFR-1, soluble form of VEGFR-1; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

protein are regulated through enzymatic hydroxylation (17). In hypoxia this process is suppressed and HIF heterodimers activate the transcription of the VEGF-A gene (16). On the contrary, PlGF expression is not up-regulated by hypoxia (19).

There are two main receptors for VEGF-A and PlGF, VEGFR-1 (also known as Flt-1) and VEGFR-2, (also known as KDR or flk-1) (10, 20). They have the common properties of multiple IgG-like extracellular domains and tyrosine kinase activity. Both VEGFR-1 and VEGFR-2 are essential for development of the embryonic vasculature in mice (21). They have different transduction properties. VEGFR-2 undergoes strong ligand-dependent tyrosine phosphorylation and mediates mitogenesis and chemotaxis in response to VEGF, whereas VEGFR-1 reveals weak responses. Because VEGFR-1 has strong affinity to VEGF and its kinase activity is weak, the regulatory role of VEGFR-1 appears to be due to VEGF-trapping activity (13, 22). The tyrosine kinase domain of VEGFR-1 is implicated in inhibition of VEGF-dependent endothelial cell migration (23). VEGFR-1 is expressed by trophoblast cells and thought to play a physiological function during pregnancy (24, 25). A soluble form of VEGFR-1 (sVEGFR-1) can be detected in peripheral blood. Soluble VEGFR-1 has a strong antagonistic activity and neutralizes the effects mediated by VEGF and PlGF effects (26–28). It is known that villous and extravillous trophoblastic cells produce sVEGFR-1 (29).

VEGF-A and PlGF are implicated in several pathological conditions associated with enhanced angiogenesis such diabetic retinopathy, carcinogenesis, rheumatoid arthritis, and wound repair (30). In preeclampsia and some cases of fetal growth restriction, deficient uteroplacental angiogenesis is usually reported, leading to the classic notion of "placental hypoxia" in these two disorders (31, 32). Recently the idea of placental hypoxia has been challenged, especially in cases of severe early-onset growth-restricted pregnancies with absent or reverse end-diastolic flow velocity in the umbilical artery. It is supposed that in such cases, oxygen transport to the fetal blood is compromised because of reduced villous placental angiogenesis resulting in hyperoxic status of maternal blood in the intervillous space (33, 34). Several studies (35–41) aimed to analyze the expression of VEGF-A in such pathological conditions during pregnancy but results are conflicting, some authors reporting reduced levels of VEGF-A in maternal circulation and others showing increased levels of VEGF-A in maternal circulation, compared with normal pregnancies. The aim of this study was therefore to attempt to explain the discrepancies between the results of the available studies. We analyzed modifications in pregnancies complicated by preeclampsia or intrauterine growth retardation

TABLE 1. Clinical characteristics of the study subjects

	SPE	IUGR	Control
n	19	10	31
Age [mean (yr)]	30	34	30
Gravida/paragravida (mean)	2/1	2/0	2/1
BP (mm Hg) (systolic/diastolic)	180/110[a]	128/79	109/77
Gestational age (wk)	30.5[a]	32.2[a]	38.3
Birth weight (g)	1,250[a]	1,300[a]	3,064
Placental weight (g)	327[a]	316[a]	582
Abnormal OD	7 (36%)	7 (70%)	0
OD = 0 and RDF	4 (21%)	4 (20%)	0
Proteinuria > 3 g/24 h	10 (52%)	0	0
Platelets	154,000[a]	216,000	230,000

BP, Blood pressure; OD, ombilical doppler; SPE, severe preeclampsia; RDF, reverse diastolic flow.

[a] $P < 0.05$ compared to control group.

TABLE 2. Sequence of primers and TaqMan probes used for RT-PCR studies

Gene (accession no.)	Position	Sequence	Size PCR product	Cycles (no. of PCR amplifications)
			479 bp	
VEGF-FP	1208F	5′-CCTGGTGGACATCTTCCAGGAGTA-3′	407 bp	33
VEGF-RP (AH001553)	1687R	5′-CTCACCGCCTCGGCTTGTCACA-3′	347 bp	
28S rRNA-FP	12403F	5′-GTTCACCCACTAATAGGGAACGTGA-3′	275 bp	19
28S rRNA-RP (U13369)	12614R	5′-GATTCTGACTTAGAGGCGTTCAGT-3′	212 bp	
VEGFR1-FP	2438F	5′-TCCCTTATGATGCCAGCAAGT-3′	79 bp	40
VEGFR1-RP	2516R	5′-CCAAAAGCCCCTCTTCCAA-3′		
VEGFR1 Probe (AF063657)	2469	5′-CCGGGAGAGACTTAAACTGGGCAAATCA-3′		
sVEGFR1-FP	2209F	5′-ACAATCAGAGGTGAGCACTGCAA-3′	180 bp	40
sVEGFR1-RP	2388R	5′-TCCGAGCCTGAAAGTTAGCAA-3′		
sVEGFR1 Probe (U01134)	2257	5′-TCCAAATTTAAAAGCACAAGGAATGATTGTACCAC-3′		
VEGFR2-FP	791F	5′-CTTCGAAGCATCAGCATAAGAAACT-3′	156 bp	40
VEGFR2-RP	946R	5′-TGGTCATCAGCCCACTGGAT-3′		
VEGFR2 Probe (AF063658)	820	5′-AACCGAGACCTAAAAACCCAGTCTGGGAGT-3′		
PlGF-FP	668F	5′-CCTACGTGGAGCTGACGTTCT-3′	77 bp	40
PlGF-RP	744R	5′-TCCTTTCCGGCTTCATCTTCT-3′		
PlGF Probe (X54936)	702	5′-CTGCGAATGCCGGCCTCTGC-3′		
NRP1-FP	1831F	5′-CACAGTGGAACAGGTGATGACTTC-3′	112 bp	40
NRP1-RP	1942R	5′-AACCATATGTTGGAAACTCTGATTGT-3′		
NRP1 Probe (XM_034725)	1883	5′-CCACAGAAAAGCCCACGGTCATAGACA-3′		

(IUGR) of VEGF-A family members in the placenta, uterus, and maternal blood.

Materials and Methods

Sample collection

Samples were collected from 60 pregnant women recruited between January 1999 and December 2001 from the department of Obstetrics and Gynecology at the hospital La Citadelle, Liège, Belgium. The protocol for this study was approved by the local ethics committee, and informed consent was obtained from the patients.

Nineteen subjects had severe preeclampsia, defined using the criteria of hypertension, edema, and proteinuria defined as below. Hypertension was defined as systolic and diastolic blood pressures above 160 mm Hg and 100 mm Hg, respectively, in at least two consecutive measurements. Proteinuria was defined as more than 1000 mg per 24 h collection or at least 3+ on urine sample when urine could not be collected for 24 h. Ten subjects had IUGR defined as birth weight below the third centile according to gestational age. None of these patients had hypertension or proteinuria. Thirty-one patients had noncomplicated pregnancy and constituted the control group. Nineteen patients from the control group were recruited just before planned cesarean delivery and 12 were enrolled in the study at their seventh-month visit to obtain a control group match for gestational age for plasma measurements. None of the patients had chronic hypertension or renal or endocrine disease.

Placenta samples, placental bed samples, and maternal plasma were collected during cesarean section. None of the patients were in labor. After extraction of the newborn, placental insertion was verified and the placenta was rapidly delivered. Basal plate was removed from the placenta. Placenta villi were collected in the center part of the placenta avoiding infarcted areas. Placental bed biopsies were performed in the uterine wall as close as possible to the center part of the implantation site. All blood samples were centrifuged at 3000 rpm during 20 min at 4 C. Aliquots of plasma were stored at −80 C until analysis. Tissue samples were frozen in liquid nitrogen within 30 min after birth and stored at −20 C until analysis. Clinical details of the patients are presented in Table 1.

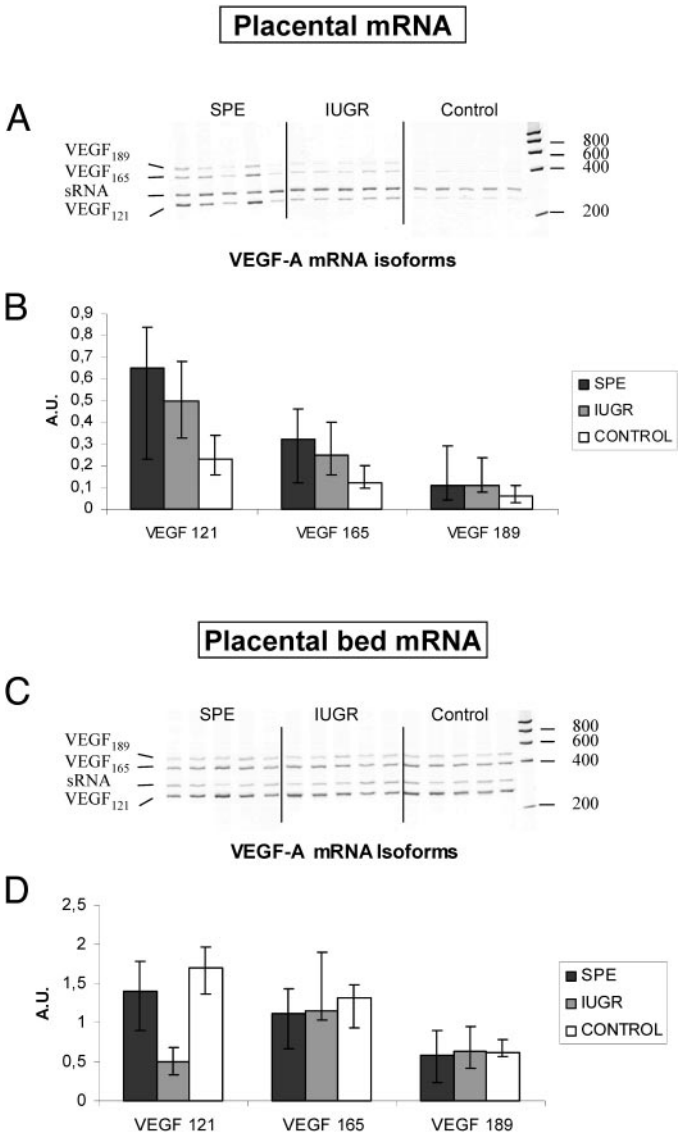

FIG. 1. VEGF-A mRNA isoforms in placentas and placental beds. Representative aspect of multiples RT-PCR for $VEGF_{189}$, $VEGF_{165}$, $VEGF_{145}$, $VEGF_{121}$ isoforms in placentas (A) and placental beds (C). Quantification of VEGF mRNA levels performed by end point quantitative RT-PCR is shown for placentas (B) and placental beds (D). Levels of mRNAs are expressed as arbitrary units (A.U.). SPE, Severe preeclampsia.

RT-PCR

RNA extraction. The frozen tissues were pulverized using a Dismembrator (B. Braun Biotech International, Gmbh Melsungen, Germany) and suspended in lysis buffer (5 M guanidine thiocyanate, 25 mM sodium citrate, 17 mM *N*-lauroylsarcosine and 0.1 M 2-mercaptoethanol, pH 7.0). Total RNA was purified by cesium chloride ultracentrifugation according to the procedure of Chirgwin *et al.* (42).

Primers

Primers pairs used in this study are described in Table 2. Primers for the VEGF-A gene were chosen to distinguish among $VEGF_{189}$, $VEGF_{165}$, $VEGF_{145}$, and $VEGF_{121}$ mRNA isoforms. Intron-spanning primers and probes for the TaqMan system (primers for VEGFR-1 (Flt-1), sVEGFR-1, VEGFR-2 (KDR/Flk-1), PlGF, and neuropilin (NRP)1 were designed to meet specific criteria by using Primer Express software (Perkin-Elmer, Foster City, CA). All primers were synthesized by Eurogentec (Liège, Belgium). The 5′- and 3′-end nucleotides of the probe were labeled with a reporter (FAM = 6-carboxy-fluorescein) and a quencher dye (TAMRA = 6-carboxy-tetramethylrhodamine). We conducted BLASTn (National Center for Biotechnology Information, Bethesda, MD) searches against dbEST and the nonredundant set of GenBank, EMBL, and DDBJ database sequences to confirm the total gene specificity of the

TABLE 3. mRNA levels (arbitrary units) of VEGF, PlGF, and their receptors in the placenta

Probe	Group	Median	25th centile	75th centile	P^a
VEGF	SPE	1.21	0.42	1.57	0.01
	IUGR	0.83	0.59	1.34	0.006
	Control	0.4	0.29	0.62	
PlGF	SPE	0.38	0.33	0.65	0.34
	IUGR	0.47	0.34	0.77	0.15
	Control	0.47	0.34	0.78	
VEGFR-1	SPE	1.16	0.49	1.87	0.0002
	IUGR	0.49	0.31	1.35	0.04
	Control	0.31	0.26	0.48	
VEGFR-2	SPE	0.31	0.18	0.44	0.47
	IUGR	0.17	0.12	0.27	0.03
	Control	0.27	0.22	0.4	
SVEGFR-1	SPE	0.41	0.18	0.94	0.0002
	IUGR	0.23	0.12	0.52	0.0019
	Control	0.08	0.06	0.12	
NRP	SPE	0.47	0.27	0.33	0.73
	IUGR	0.5	0.26	0.76	0.72
	Control	0.51	0.33	0.67	

SPE, Severe preeclampsia.
[a] *P* value for comparison *vs.* the control group.

nucleotide sequences chosen for the primers. The 18S rRNA was measured using the Pre-Developped TaqMan assay reagents endogenous control kit (PE Applied Biosystems, Inc., Foster City, CA).

End point quantitative RT-PCR for VEGF-A mRNA isoforms

$VEGF_{189}$, $VEGF_{165}$, $VEGF_{145}$, and $VEGF_{121}$ mRNA isoforms and 28S rRNA were measured in 10-ng aliquots of total RNA using the GeneAmp Thermostable rTth reverse transcriptase RNA PCR kit (Applied Biosystems) and two pairs of primers (Table 2). The efficiency of the RT-PCR was monitored with an internal control (CTR) as previously described (43). RT-PCR products were resolved on 10% acrylamide gels and analyzed using a Fluor-S MultiImager (Bio-Rad Laboratories, Hercules, CA) after staining with Gelstar dye (FMC BioProducts, Rockland, ME). The expected size for $VEGF_{189}$, $VEGF_{165}$, and $VEGF_{121}$ is, respectively, 479, 407, 347, and 275 bp and 212 bp for 28S rRNA.

Ratios VEGF:CTR and 28S:CTR were determined. VEGF mRNA levels were expressed as the ratio of VEGF transcripts:28S transcripts.

Real-time quantitative RT-PCR VEGFR-1, sVEGFR-1, VEGFR-2, PlGF, and NRP1 mRNA

Real-time quantitative RT-PCR analyses for VEGFR-1 (Flt-1), sVEGFR-1 (sFlt-1), VEGFR-2 (KDR/Flk-1), PlGF, NRP1 mRNAs, and 18S rRNA were performed using the ABI PRISM 7700 sequence detection system instrument and software (PE Applied Biosystems). The sequences of the PCR primer pairs and fluorogenic probes that were used for each gene are shown in Table 2. The relative quantitation of our data has been performed using the standard curve method according to the manufacturer recommendation (PE Applied Biosystems in User Bulletin #2). The relative expression level of the gene of interest was computed with respect to 18S rRNA to normalize for variation in the quality of RNA and the amount of input cDNA. For each experimental sample, the amount of target was determined from a standard curve. The latter was constructed with 5-fold serial dilutions of pooled placenta cDNA (50,000 ng to 80 ng) and was run in duplicate during every experiment. The cycle threshold obtained for each amount of pooled placenta cDNA allowed to draw a linear standard curve of amplification for each target gene. The cycle threshold of any given sample was reported to this standard curve both for the 18S RNA and each mRNA. The amount of target gene was divided by the 18S rRNA amount to obtain a normalized target value (as described by the manufacturer PE Applied Biosystems in User Bulletin #2). PCR was performed with the TaqMan Universal PCR Master Mix (PE Applied Biosystems) using 5 μl diluted cDNA (equivalent to 10 ng total RNA), 200 nM of the probe, and 400-nM primers in a 25-μl final reaction mixture. After a 2-min incubation at 50 C to allow for uracil-*N*-glycosylase cleavage, AmpliTaq Gold was activated by an incubation for 10 min at 95 C. Each of the 40 PCR cycles consisted of 15 sec of denaturation at 95 C and hybridization of probe and primers for 1 min at 60 C.

To confirm amplification specificity, the PCR products were also examined by subsequent 2% agarose gel electrophoresis. Experiments were repeated at least three times in duplicate.

Immunoassays

Levels of free VEGF-A and free PlGF in maternal plasma were measured by the sandwich enzyme immunoassay technique (R&D Systems Europe Ltd., Abington, UK). Total VEGF-A (free and bound VEGF-A)

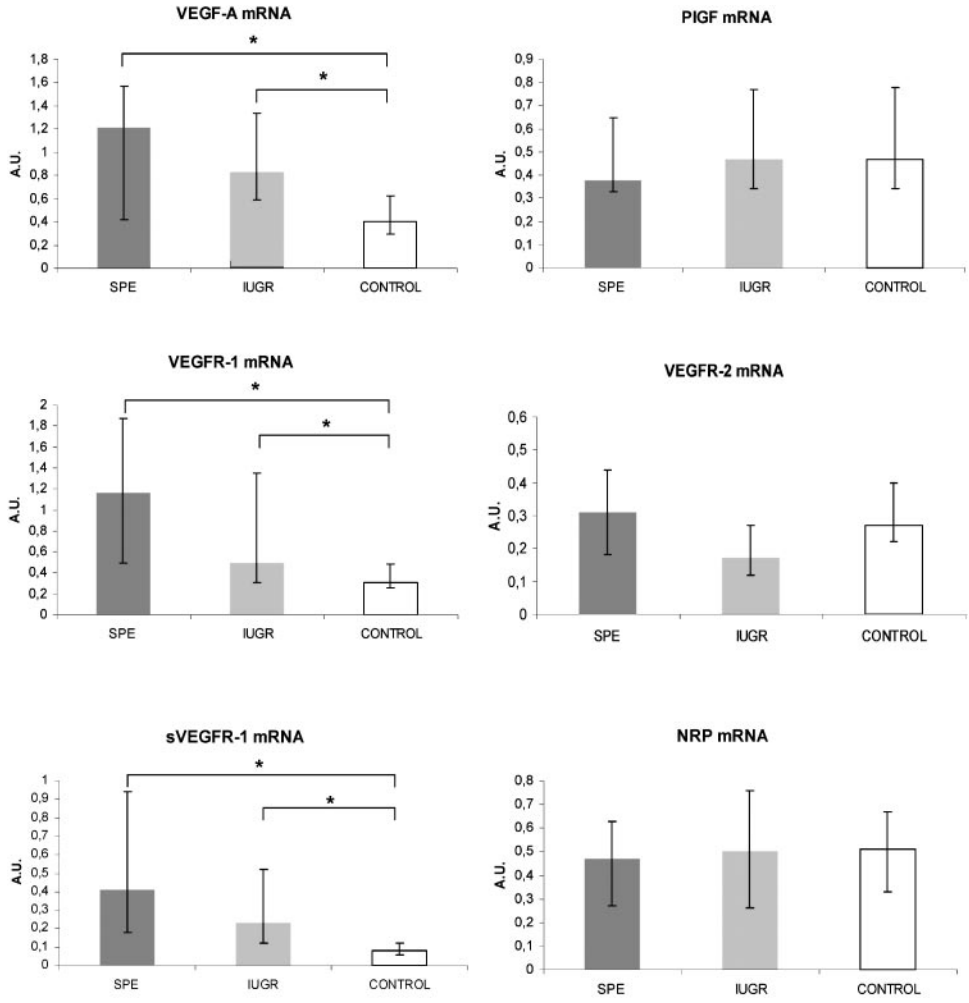

FIG. 2. mRNA levels of VEGF, PlGF, and their receptors in placentas. All RNA quantifications were performed by real time RT-PCR except for VEGF mRNA isoforms. Levels of mRNA are expressed as arbitrary units (A.U.). SPE, Severe preeclampsia. * , $P < 0.05$.

in maternal plasma were measured by competitive enzyme immunoassay (Cytimmune, College Park, MD). Levels of the soluble form of VEGF-R1 in maternal plasma were measured by sandwich enzyme immunoassay technique (Bender MedSytems, Vienna, Austria). All samples were measured in duplicate. Sensitivity for free VEGF, free PlGF, total VEGF, and sVEGF-R1 are, respectively, 9 pg/ml, 7 pg/ml, 0.2 ng/ml, and 0.1 ng/ml.

TABLE 4. mRNA levels (arbitrary units) of VEGF, PlGF, and their receptors in the placental bed

Probe	Group	Median	25th centile	75th centile	P^a
VEGF	SPE	3.03	1.75	4.15	0.12
	IUGR	3.47	2.89	5.1	0.5
	Control	3.62	2.79	4.12	
PlGF	SPE	0.015	0.01	0.022	0.29
	IUGR	0.007	0.003	0.01	0.04
	Control	0.012	0.006	0.041	
VEGFR-1	SPE	0.06	0.04	0.1	0.0003
	IUGR	0.08	0.04	0.14	0.01
	Control	0.14	0.09	0.19	
VEGFR-2	SPE	0.27	0.12	0.59	0.22
	IUGR	0.26	0.22	0.48	0.4
	Control	0.34	0.22	0.43	
SVEGFR-1	SPE	0.01	0.007	0.038	0.88
	IUGR	0.009	0.0035	0.034	0.5
	Control	0.01	0.007	0.021	
NRP	SPE	0.34	0.21	0.69	0.93
	IUGR	0.28	0.23	0.68	0.85
	Control	0.35	0.25	0.5	

SPE, Severe preeclampsia.
[a] *P* value for comparison *vs.* the control group.

Immunohistochemistry (VEGF-A and PlGF)

Placentas were fixed in 4% formalin for 4–12 h and then embedded in paraffin. Paraffin sections (4 μm) were mounted on aminopropyltriethoxysilane (Tespa)-coated glass slides.

Sections were dewaxed in xylene and rehydrated. Nonspecific antibody-binding was blocked by incubation for 20 min in a blocking reagent containing 3% H_2O_2 and for 60 min in 10% serum albumin solution. Then 4 μg/ml VEGF-A antibody (rabbit antihuman VEGF (A-20), Santa Cruz Biotechnology, Heidelberg, Germany), 50 μg/ml PlGF antibody (mouse monoclonal antihuman PlGF, R&D Systems, MAB264) and 4 μg/ml cytokeratin antibody (mouse antihuman CK-07, DakoCytomation, Glostrup, Denmark) were incubated with the sections for 1 h at room temperature. Sections were washed in Tris-buffered saline-0.1% Tween 20 and incubated with a secondary antibody peroxidase conjugated (goat antimouse Envision, Dako K 4000, DakoCytomation) and goat antirabbit Envision (Dako K 4003) was applied for 30 min. Staining was detected with the diamino-benzidine chromogen after 5 min. Nuclei were counterstained by incubation for 2 min with hematoxylin. Sections were mounted, examined, and photographed under a Leica-DMLS microscope (Leica Microsystems, Wetzlar, Germany) couple to a digital camera (Nikon Coolpix 990, Nikon Instruments Europe, Badhoevedorp, The Netherlands). Controls were performed by omitting the primary antibody or by incubating the sections with nonspecific IgG at the same concentration as the primary antibody.

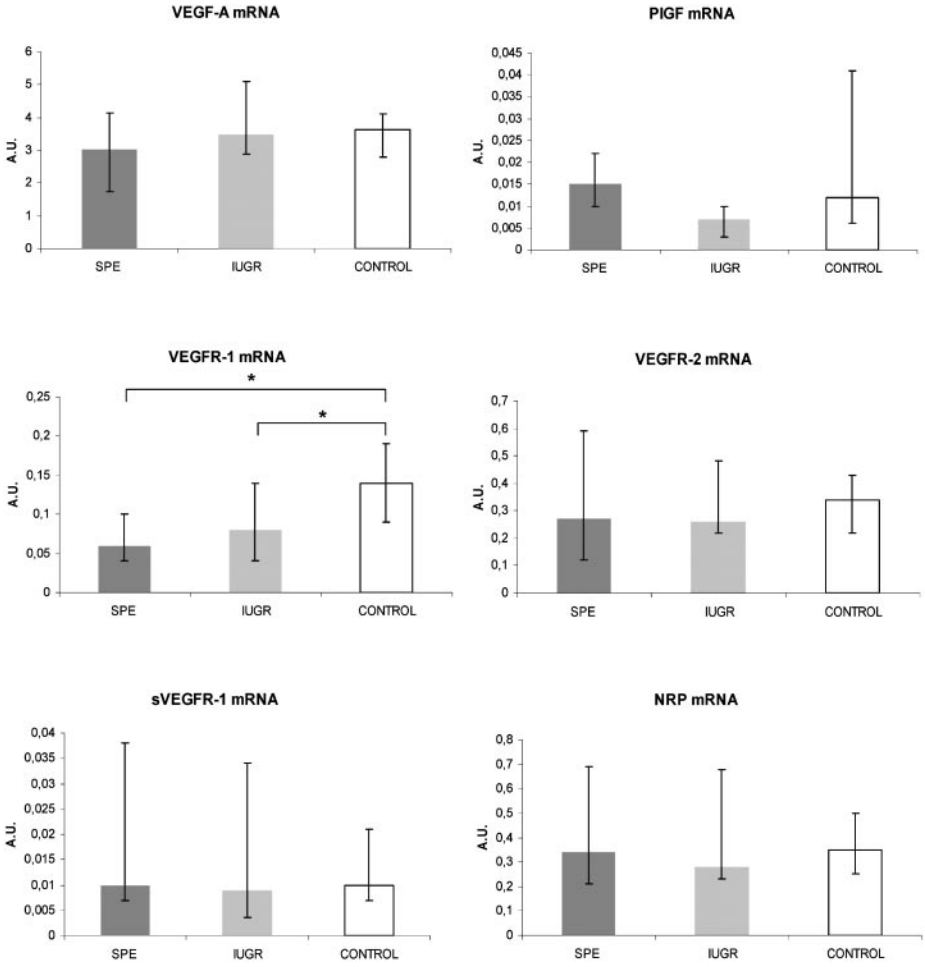

FIG. 3. mRNA levels of VEGF, PlGF, and their receptors in placental beds. All RNA quantifications were performed by real-time RT-PCR except for VEGF mRNA isoforms. Levels of mRNA are expressed as arbitrary units (A.U.). SPE, Severe preeclampsia. *, $P < 0.05$.

Statistics

Comparisons between the different groups were made with the Kruskall-Wallis test, and significant differences were further analyzed by pairwise comparison with the Mann-Whitney *U* test. Results are shown as median ± quartiles (25^{th}–75^{th} centile). $P < 0.05$ were considered statistically significant.

Results

mRNA quantification in placentas

The three main isoforms (121, 165, 189) of VEGF-A were expressed in the placenta, isoform 121 being predominant (Fig. 1A; values for the median and quartiles are detailed in Table 3). mRNA levels of VEGF-A isoforms were significantly higher in pregnancies complicated by preeclampsia and IUGR, compared with controls (Fig. 1B). Similarly, total VEGF-A mRNA levels (sum of the three isoforms) were higher in placentas from preeclamptic patients and patients with IUGR, compared with controls (Fig. 2). There was no difference in PlGF mRNAs among the three groups. VEGFR-1 mRNA and sVEGFR-1 mRNA levels were significantly increased in placentas from preeclamptic patients, compared with normal pregnancies (Fig. 2). On the contrary, no significant difference was found among the three groups for VEGFR-2 mRNAs and NRP mRNAs.

mRNA quantification in placental beds

VEGF 121, VEGF 165, and VEGF 189 mRNAs were expressed in the placental bed (Fig. 1C; values for the median and quartiles are detailed in Table 4). The isoform 121 was again the predominant one (Fig. 1D). Levels of VEGF, PlGF, sVEGFR-1, VEGFR-2, and NRP mRNAs were similar in samples from preeclamptic patients and normal pregnancies (Fig. 3). When considering VEGF isoforms, no significant difference was detected between both groups (Fig. 1). Compared with controls, VEGFR-1 mRNA expression was significantly decreased in preeclamptic patients and patients with IUGR. Although the levels of PlGF mRNA were decreased in the IUGR group in comparison with controls, the difference did not reach significance (Fig. 3).

VEGF-A measurements in maternal plasma

Free VEGF-A was below the detection limit (9 pg/ml) in 80% of the samples (Fig. 4). Compared with uncomplicated pregnancies, total VEGF-A (free + bound VEGF-A) was significantly higher in the plasma from preeclamptic patients [25.24 (21.22–42.92) *vs.* 6.83 (5.32–8.94) ng/ml, $P < 0.0001$] and patients with IUGR [22.67 (15.93–30.03) *vs.* 6.83 (5.32–8.94) ng/ml, $P < 0.0001$] (Fig. 4). There was no difference in VEGF-A levels between controls at term during cesarean section and controls matched for gestational age [6.67 (4.32–8.91) *vs.* 6.83 (5.32–8.94) ng/ml, $P = 0.5$].

Compared with uncomplicated pregnancies, free PlGF was significantly lower in the plasma from preeclamptic patients [67.41 (57.1–118.5) *vs.* 585.9 (356–1011) pg/ml, $P < 0.0001$] and patients with IUGR [101.5 (60.65–145.3) *vs.* 585.9 (356–1011) pg/ml, $P < 0.0001$] (Fig. 4). Similar PlGF levels were observed in controls at term during cesarean section and in controls matched for gestational age [344.3 (210.8–735.2) *vs.* 585.9 (356–1011) pg/ml, $P = 0.2$].

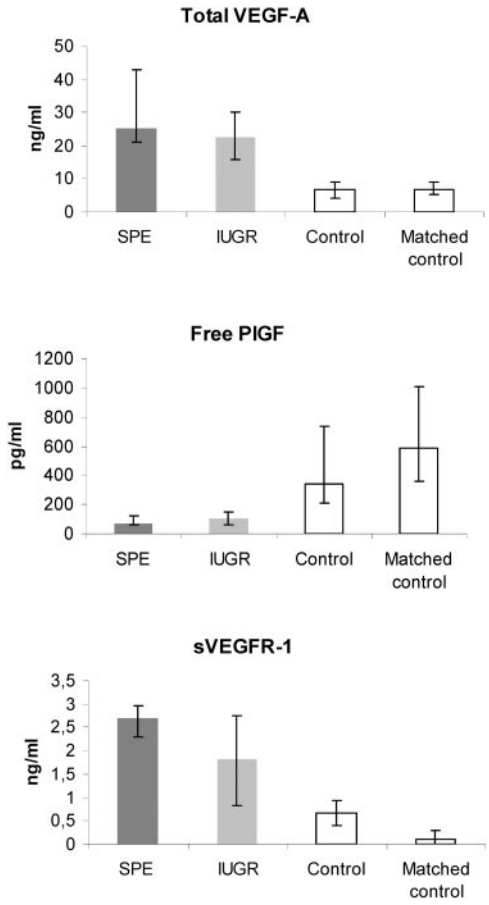

FIG. 4. Levels of total VEGF, free PlGF, and sVEGFR-1 in maternal plasma. Quantification of total VEGF, free PlGF, and sVEGFR-1 in maternal plasma from patients with IUGR, severe preeclampsia (SPE), and uncomplicated pregnancies matched for gestational age (matched control group). Patients with uncomplicated pregnancy at term are represented as control group. *, $P < 0.05$.

Soluble VEGFR-A levels were significantly higher in the plasma from preeclamptic patients [2.69 (2.31–2.97) *vs.* 0.12 (0–0.29) ng/ml, $P < 0.0001$] and patients with IUGR [1.81 (0.82–2.76) *vs.* 0.12 (0–0.29) ng/ml, $P < 0.0001$], compared with uncomplicated pregnancies (Fig. 4). Moreover, sVEGFR-1 levels were lower in the control group matched for gestational age than in the control group at term [0.12 (0–0.29) *vs.* 0.66 (0.41–0.93) ng/ml, $P = 0.002$].

Immunolocalization of VEGF and PlGF in the placenta

Cells producing VEGF-A and PlGF in the placenta were identified by immunohistochemistry. VEGF-A (Fig. 5, A and B) and PlGF (Fig. 5, C and D) are produced by villous cytotrophoblast, syncytiotrophoblast, and extravillous trophoblast. Cytokeratin was used to localize cytotrophoblasts (Fig. 5F). Negative controls are illustrated in Fig. 5F.

Discussion

In the present study, we have shown an important dysregulation of the expression of VEGF, PlGF, and their receptors by the placenta in pregnancies complicated by severe early-onset preeclampsia or IUGR. VEGF-A, PlGF, and

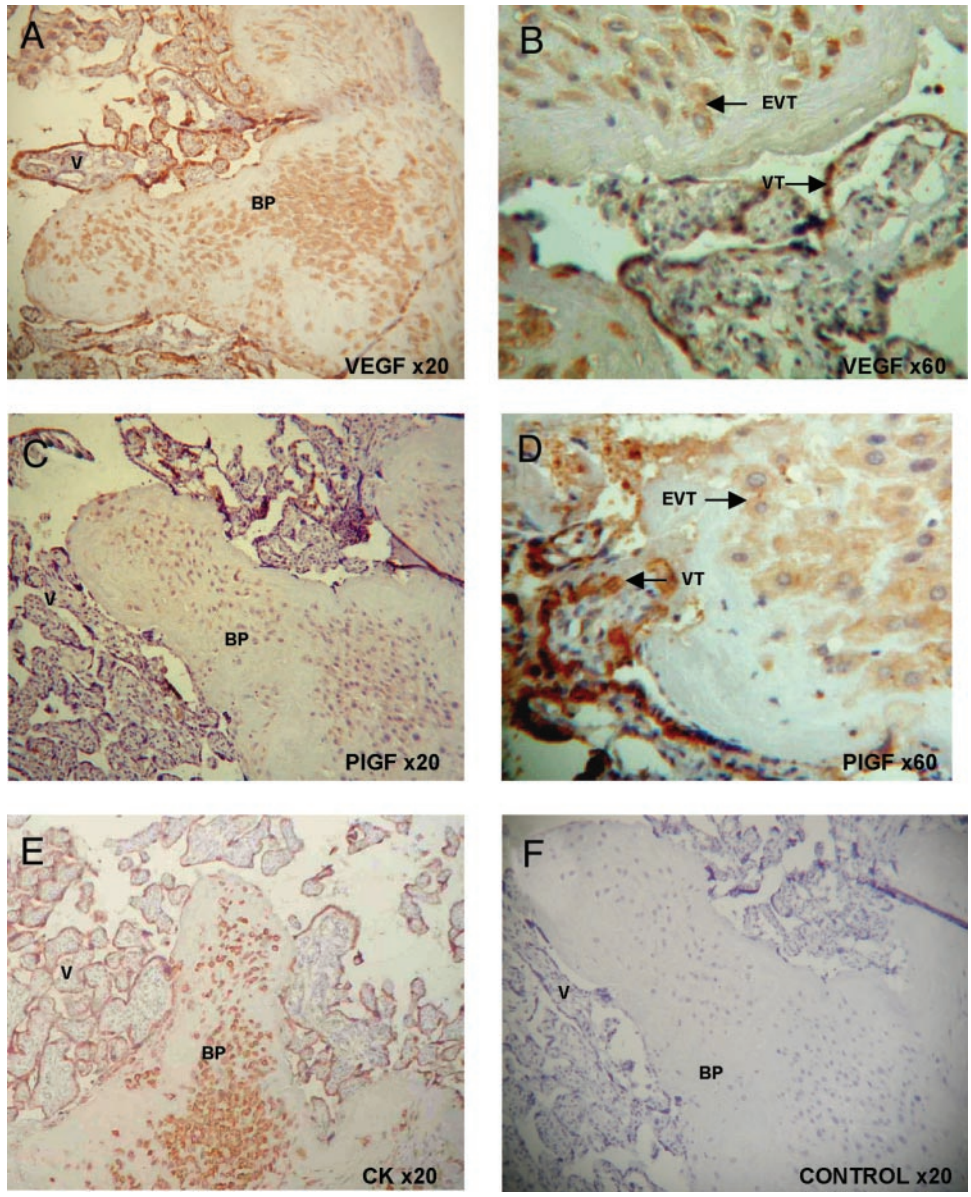

FIG. 5. Immunolocalization of VEGF and PlGF in the placenta. Immunolocalization of VEGF in the villi (V) and basal plate (BP) (A, magnification ×20; B, magnification ×60). Immunolocalization of PlGF in the V and BP (C, magnification ×20; D, magnification ×60). Immunolocalization of cytokeratin (CK) in the V and BP (E, magnification ×20). Negative control (F, magnification ×20). EVT, Extravillous trophoblast; VT, villous trophoblast.

VEGFR-1 are produced by villous and extravillous trophoblastic cells. VEGF-A, VEGFR-1, and sVEGFR-1 were significantly up-regulated in placentas from preeclamptic women and patients with IUGR, whereas no modification was found for PlGF, VEGFR-2, or NRP. High levels of VEGF, VEGFR-1, and sVEGFR-1 in preeclampsia are probably related to the hypoxic status of the placenta (44). We found the same increase of expression of VEGF, VEGFR-1, and sVEGFR-1 in placentas from pregnancies complicated by early-onset IUGR, suggesting that in these cases the placenta may also be hypoxic. It has been postulated that in severe IUGR with absent or reverse end-diastolic flow velocity in the uterine artery, the transfer of oxygen across the placenta from the maternal to the fetal circulation may be reduced, leading to a hyperoxic placenta and hypoxic fetus (33, 34). Our results do not support this hypothesis, but only 20% of our patients had absent or reverse end-diastolic flow velocity in the uterine artery.

VEGF, PlGF, and sVEGFR-1 produced by the placenta are released in maternal circulation. We therefore investigated whether the levels of these angiogenic factors were increased or decreased in maternal plasma because this information is conflicting in the literature (36–41). We chose to assess the expression of these factors in maternal plasma rather than maternal serum as suggested in the literature (45). Serum levels of VEGF are higher than those found in the plasma. The difference between plasma and serum concentrations have been ascribed to the release of VEGF from platelets and other blood cells during clotting (45). We used different assays to differentiate free circulating VEGF (capture immunoassay) to the VEGF bound to circulating proteins such as sVEGFR-1 or α-macroglobulin (competitive immunoassay). We found that free VEGF was undetectable in most samples. These results are consistent with data from Vuorela *et al.* (36). On the contrary, total circulating VEGF was detectable in all samples. Levels of total VEGF were significantly higher in

preeclampsia and pregnancies with IUGR, compared with normal pregnancies at term or matched for gestational age. These results are similar to those previously reported by Kupferminc *et al.* (39) and Sharkey *et al.* (37). Free PlGF was found to be significantly lower, whereas sVEGFR-1 was significantly higher in preeclampsia and pregnancies with IUGR, compared with normal pregnancies at term or matched for gestational age. These results are in concordance with recent studies showing elevated levels of sVEGFR-1 in preeclampsia (46, 47).

Three studies (35, 36, 38) reported measurements of free VEGF with the same technique as ours, but all samples were maternal serum. In these studies, free VEGF was detectable and decreased in preeclampsia, compared with normal pregnancies. Other studies reported measurements of total VEGF in maternal serum (41) or maternal plasma (37, 39) and showed a significant increased in preeclampsia, compared with normal pregnancies. Here we show a parallel increase of total VEGF and sVEGFR-1 in preeclampsia, compared with normal pregnancy. It seems that during pregnancy most of the circulating VEGF is bound to sVEGFR-1 produced in high amounts by the placenta. During preeclampsia placental production of VEGFR-1 and sVEGFR-1 is increased probably because of the hypoxic status of the placenta. This increase of sVEGR-1 in preeclampsia explains discrepancies between studies in the literature. Compared with normal pregnancies, levels of free VEGF (circulating at concentrations around 2–10 pg/ml) are lower, whereas levels of total VEGF (circulating at concentrations around 20 ng/ml) are higher in maternal circulation from preeclamptic patients, compared with controls.

Levels of circulating PlGF (circulating at concentrations around 400 pg/ml) are lower in preeclampsia than in normal pregnancies. This difference may be attributed to the fact that PlGF is also bound to sVEGFR-1 in maternal circulation. In this study, we show that the depressed concentrations of free VEGF and free PlGF in preeclampsia are due to an excessive production of sVEGFR-1, as suggested by Lyall (38) and Maynard *et al.* (46). In their recent study, Maynard *et al.* (46) have shown in a rodent model that VEGF, PlGF, and sVEGFR-1 are likely to play a role in the pathogenesis of preeclampsia. Excess placental production of sVEGFR-1 contributes to hypertension, proteinuria, and glomerular endotheliosis. Recently Koga *et al.* (47) reported that concentrations of sVEGFR-1 in serum from women with preeclampsia were more than 6-fold higher than those from control. Eremina *et al.* (48) provided additional evidence for a critical role of VEGF in renal disease during preeclampsia. In this study, mice lacking one VEGF allele in renal podocytes developed a renal disease, characterized by proteinuria and endotheliosis, the renal lesion seen in preeclampsia.

Results from the placental bed biopsies show no differences between the study groups for the expression of VEGF, PlGF, VEGFR-2, and NRP, whereas VEGFR-1 expression was significantly reduced in patients with preeclampsia (VEGFR-1 expression was reduced in the IUGR group but not significantly). Although it is not possible to determine whether this modification is cause or consequence of the pathologic status of the pregnancy, low levels of VEGFR-1 in the placental bed may explain the defective uterine vascularization frequently associated with early-onset preeclampsia.

Altogether our study indicates the involvement of sVEGFR-1 as an antagonist to VEGF and PlGF in preeclampsia. The down-regulation of the membrane-bound form of VEGFR-1 in the placental bed may also result in the decreased maternal vascular adaptation to pregnancy.

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