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Soluble fms-Like Tyrosine Kinase 1 Is Increased in Preeclampsia But Not in Normotensive Pregnancies with Small-for-Gestational-Age Neonates: Relationship to Circulating Placental Growth Factor

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Context: An excess of the soluble receptor, fms-like tyrosine kinase 1 (sFlt-1) may contribute to maternal vascular dysfunction in women with preeclampsia by binding and thereby reducing concentrations of free vascular endothelial growth factor and placental growth factor (PlGF) in the circulation. The putative stimulus for increased sFlt-1 during preeclampsia, placental hypoxia due to poor perfusion, is common to both preeclampsia and idiopathic intrauterine growth restriction. However, the latter condition occurs without maternal vascular disease.

Objective: We asked whether, as with preeclampsia, sFlt-1 is increased and free PlGF is decreased in villous placenta and maternal serum of normotensive women with small-for-gestational-age (SGA) neonates.

Study Design: This was a case-control study using banked samples. Groups of women with SGA neonates (birth weight centile < 10th) and women with preeclampsia were matched to separate sets of normal pregnancy controls based on gestational age at blood sampling (serum) or gestational age at delivery (placenta).

Results: sFlt-1 levels were higher in preeclamptics than controls (serum, $P < 0.0001$; placental protein, $P = 0.03$; placental mRNA, $P = 0.007$) but not increased in SGA pregnancies. PlGF was lower in both preeclampsia (serum, $P < 0.0001$; placental protein, $P = 0.05$) and SGA (serum, $P = 0.0008$; placental protein, $P = 0.03$) compared with their controls. PlGF in preeclampsia and SGA groups did not differ.

Conclusions: These data are consistent with a role for sFlt-1 in the maternal manifestations of preeclampsia. In contrast to preeclampsia, sFlt-1 does not appear to contribute substantially to decreased circulating free PlGF in SGA pregnancies in the absence of a maternal syndrome. **(*J Clin Endocrinol Metab* 90: 4895–4903, 2005)**

PREECLAMPSIA IS THOUGHT to develop in two principal stages, the first being abnormal placentation and the second being the translation to the maternal systemic disorder including widespread endothelial dysfunction (1). The cause of this pregnancy syndrome remains unknown but is likely multifactorial, with involvement of both placental and maternal factors (2). Current evidence suggests that an excess of soluble fms-like tyrosine kinase 1 (sFlt-1), also known as soluble vascular endothelial growth factor receptor (sVEGFR)-1, contributes to the pathogenesis of preeclampsia (3–5). sFlt-1 is a secreted splice variant of Flt-1 that binds to VEGF and placental growth factor (PlGF) and thus prevents the interaction of these angiogenic growth factors with Flt-1 in target tissues (6–9). The placenta is thought to be the main source of the approximate 20-fold increase in circulating sFlt-1 levels by the third trimester of normal pregnancy, compared with nonpregnant values. Indeed, sFlt-1 mRNA is strongly expressed in the placenta, and maternal serum sFlt-1 concentrations decline rapidly after delivery (3, 10, 11).

Reduced placental perfusion with attendant placental hypoxia is considered to be an important early feature of pregnancies complicated by preeclampsia and/or pregnancies complicated by intrauterine fetal growth restriction (IUGR) (12–16). Hypoxia has been shown to increase sFlt-1 production by cytotrophoblasts and placental villous tissue in culture (17, 18), and other data suggest that hypoxia causes up-regulation of VEGF and down-regulation of PlGF in the placenta (18–20). Maternal serum concentrations of PlGF are reduced in IUGR pregnancies (21–23). Placental VEGF receptors, including sFlt-1, are reportedly increased (21), whereas circulating free (but not total) VEGF and free PlGF are decreased in preeclampsia (24, 25). If sFlt-1 were increased in the circulation of women with idiopathic IUGR pregnancies, the lack of significant maternal systemic disease in the face of such an increase might signify constitutional maternal resistance to the putative endothelial dysfunction-inducing effects of sFlt-1. However, the profile of sFlt-1 in

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Abbreviations: BMI, Body mass index; BP, blood pressure; IUGR, intrauterine fetal growth restriction; PlGF, placental growth factor; SDS, sodium dodecyl sulfate; sFlt-1, soluble fms-like tyrosine kinase 1; SGA, small-for-gestational-age; SGA-S, severe SGA; sVEGFR, soluble vascular endothelial growth factor receptor.

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relation to PlGF has not been adequately investigated in patients with growth-restricted fetuses and without preeclampsia or other maternal cardiovascular complications. Therefore, the purpose of this study was to determine the relationship between serum and placental sFlt-1 and PlGF in women with small-for-gestational-age (SGA) neonates in the absence of a maternal syndrome.

Subjects and Methods

Study population

We conducted a case-control study using banked serum and placental samples. We selected 26 women with preeclampsia and 24 with SGA infants. Each group was matched, based on gestational age at the time of sampling, to a set of normal pregnant women (n = 27 controls for preeclampsia; n = 31 controls for SGA). The Magee-Womens Hospital Institutional Review Board approved the study, and all women gave written informed consent.

Preeclampsia was defined using the criteria of gestational hypertension, proteinuria and hyperuricemia, and reversal of hypertension and proteinuria after delivery. Gestational hypertension was defined as systolic blood pressure (BP) more than 140 mm Hg or diastolic BP more than 90 mm Hg, arising after 20 wk gestation, in a previously normotensive woman. Proteinuria was defined as more than 300 mg protein in a 24-h urine collection or more than 2+ on a voided or more than 1+ on a catheterized random urine sample, or a random urine protein/creatinine ratio of more than 0.3. Hyperuricemia was defined as more than 1 SD above normal for the given gestational age [at term, >5.5 mg/dl (3.3 mM)]. SGA was defined as birth weight less than 10th centile in an otherwise normal pregnancy. Percentiles for growth parameters were obtained by comparison with local race- and gender-specific fetal growth data (adjusted for gestational age). Control groups were composed of women with uncomplicated, normotensive pregnancies and who delivered healthy, non-SGA babies. Patients with multiple gestations, chronic hypertension, diabetes, renal disease or other significant metabolic disorder, or a history of illicit drug use were excluded.

Table 1 shows the clinical characteristics of the study groups. The need for two control groups is evidenced by the earlier mean gestational weeks at delivery of the preeclampsia group. The patients from whom serum samples were available comprised a majority of the total (a few provided placentas only). Serum for sFlt-1 measurement was obtained from 22 with preeclampsia, 22 with SGA neonates, 24 normal pregnancy controls for preeclampsia, and 29 normal pregnancy controls for SGA. These cases and controls were group matched for gestational age at the time of blood sampling (Table 1). For most of these patients, sufficient serum sample was also available to measure PlGF (21 with preeclampsia, 21 with SGA, 21 controls for preeclampsia, 29 controls for SGA).

Cases and controls from whom placentas were obtained were group matched for gestational weeks at delivery (Table 2). These were comprised of 10 women with preeclampsia, 13 with SGA pregnancy, eight controls for preeclampsia, and 16 controls for SGA. Some of the placenta samples were from women who did not provide serum samples. However, the majority of study subjects who provided placentas had also provided serum samples (six of 10 preeclampsia, 11 of 13 SGA, five of eight controls for preeclampsia, 14 of 16 controls for SGA).

Most studies equate IUGR with SGA even though birth weight alone cannot distinguish constitutional smallness from failure to reach growth potential. To increase the likelihood of studying subjects with failed growth potential, we identified a potentially severe subset of SGA (SGA-S), namely those with an abnormal umbilical artery Doppler waveform and/or an asymmetric fetal growth profile (n = 15; Table 3).

The umbilical artery systolic/diastolic ratio was considered abnormal if it was elevated above the 95th percentile for gestational age or if diastolic flow was either absent or reversed (26). Percentiles for growth parameters were obtained by comparison with local race- and gender-specific fetal growth data. Asymmetric growth usually signifies a faltering of growth in the third trimester, resulting in a disproportionately low birth weight in comparison with length or occipital frontal head circumference. However, asymmetric growth has no universally accepted formula. Babies were considered to have asymmetric growth when the percentiles were disproportionate, generally following the pattern of weight being compromised before linear growth before brain growth (*i.e.* weight centile < length centile ≤ head circumference centile). For these data, we defined "significantly less than" to mean plotting in nonadjacent percentile categories (<3rd, 3–5, 5–10, 10–25, 25–50, 50–75, 75–90, 90–95, 95–97, and >97th), wherein weight centile must be at least two categories below length and/or head circumference centile to qualify as asymmetric. These fetal variables are listed for each SGA patient in Table 3. An example of asymmetric growth would be the infant of patient number 5 in Table 3. This infant has a birth weight centile of 9.0 (category 5–10), length centile 10–25, and head circumference centile 25–50 (two categories greater than birth weight).

Of the 24 SGA pregnancies listed in Table 3, three had abnormal umbilical Doppler (five had normal Dopplers, and 16 were without Doppler data), and 13 evidenced asymmetric growth (eight were symmetric, and three were missing fetal morphometric data), with one case positive for both abnormalities. The eight SGA infants with a symmetric

TABLE 1. Characteristics of the overall study population

	Preeclampsia (n = 26)	Preeclampsia control (n = 27)	SGA (n = 24)	SGA control (n = 31)
Age (yr)	26.5 ± 6.0	26.2 ± 7.1	22.7 ± 4.6	22.9 ± 5.0
Prepregnancy BMI (kg/m^2)	25.9 ± 4.5	24.8 ± 5.1	24.1 ± 5.7	25.5 ± 6.7
Gestational weeks at delivery	35.4 ± 3.5[b,d]	37.9 ± 2.1[e]	39.2 ± 1.6	39.9 ± 1.1
Gestational weeks at blood collection[a]	35.2 ± 3.8[d]	34.3 ± 5.0[e]	39.1 ± 1.6	40.0 ± 1.0
BP in early pregnancy (<20 wk)				
Systolic (mm Hg)	118 ± 7	114 ± 7	115 ± 5	114 ± 9
Diastolic (mm Hg)	72 ± 7[d]	69 ± 7	66 ± 4	68 ± 4
Pre-delivery BP				
Systolic (mm Hg)	159 ± 17[b,d]	125 ± 18	117 ± 10	120 ± 12
Diastolic (mm Hg)	94 ± 7[b,d]	73 ± 9	70 ± 7	73 ± 7
Birth weight centile	28.6 ± 27.3[b,d]	56.2 ± 31.7	3.4 ± 2.9[c]	66.0 ± 25.1
Birth weight (g)	2306 ± 804[b]	3233 ± 688	2455 ± 290[c]	3560 ± 474
Primigravida (%)	100	100	96	100
Smoking (%)	9	11	30	29
Presence of labor at blood sampling (%)	9	14	54	52
Maternal race (% white)	85	79	67	52

Continuous variables are shown as mean (SD). Dichotomous variables are given as percentage. Cigarette smoking status was self-reported. Race was self-reported; women were classified as white or black (no other races were represented). BP, Blood pressure.

[a] Subjects from whom serum measurements were available comprised a subset of the total (sFlt-1: preeclampsia, n = 22; preeclampsia control, n = 24; SGA, n = 22; SGA control, n = 29; PIGF: preeclampsia, n = 21; preeclampsia control, n = 21; SGA, n = 21; SGA control, n = 29).

Significant differences ($P < 0.0083$, Bonferroni/Dunn *post hoc*) are indicated as follows: [b] preeclampsia *vs.* their controls; [c] SGA *vs.* their controls; [d] preeclampsia *vs.* SGA; [e] preeclampsia control *vs.* SGA control. (Significant differences between other groups are not indicated.)

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TABLE 2. Characteristics of the study population from whom placental samples were obtained

	Preeclampsia (n = 10)	Preeclampsia control (n = 8)	SGA (n = 13)	SGA control (n = 16)
Age (yr)	28.4 ± 5.7	27.7 ± 4.9	21.8 ± 4.3	21.3 ± 4.2
Prepregnancy BMI (kg/m^2)	25.5 ± 3.6	23.0 ± 4.0	22.1 ± 4.8	23.2 ± 5.6
Gestational weeks at delivery (sampling)	36.4 ± 2.0[c]	37.6 ± 1.3[d]	39.5 ± 1.5	39.5 ± 0.9
BP in early pregnancy (<20 wk)				
Systolic (mm Hg)	118 ± 7	113 ± 7	115 ± 0	112 ± 8
Diastolic (mm Hg)	71 ± 6[c]	70 ± 7	64 ± 0	68 ± 5
Predelivery BP				
Systolic (mm Hg)	159 ± 23[a,c]	122 ± 18	115 ± 10	115 ± 11
Diastolic (mm Hg)	96 ± 9[a,c]	74 ± 4	68 ± 6	71 ± 7
Birth weight centile	30.3 ± 26.9[a]	68.7 ± 21.9	3.8 ± 3.3[b]	63.2 ± 20.7
Birth weight (g)	2502 ± 691[a]	3288 ± 452	2498 ± 248[b]	3414 ± 455
Primigravida (%)	100	100	92	100
Smoking (%)	29	43	58	50
Maternal race (% white)	90	88	69	44

Continuous variables are shown as mean (SD). Dichotomous variables are given as percentage. Smoking status was self-reported. Race was self-reported; women were classified as white or black (no other races were represented). BP, Blood pressure.

Significant differences ($P < 0.0083$, Bonferroni/Dunn *post hoc*) are indicated as follows: [a] preeclampsia *vs.* their controls; [b] SGA *vs.* their controls; [c] preeclampsia *vs.* SGA; [d] preeclampsia control *vs.* SGA control. (Significant differences between other groups are not indicated.)

growth profile (mean birth weight centile of 3.2; range, 0.03–7.6) may have been constitutionally small without growth restriction or, alternatively, may have been so severely affected that even head growth was compromised. Therefore, although we considered asymmetric growth to be evidence consistent with IUGR, we did not consider symmetric growth to preclude the possibility of IUGR.

Patient samples

Venous blood was withdrawn into sterile tubes in which the blood was allowed to coagulate for 45 min at room temperature, and the resulting serum was separated by centrifugation. For comparison of serum and plasma, in selected cases, blood was collected into sterile tubes containing 4 mmol/liter potassium-EDTA. The resulting serum and EDTA-plasma samples were stored at −70 C as 1.2-ml aliquots and thawed immediately before analysis. There were no differences by study group, regarding the time elapsed between venipuncture and freezer storage of serum (data not shown). Placental biopsies were obtained, immediately after cesarean delivery, from a site between the placental rim and cord insertion. The tissue was quickly washed three times in physiological saline, frozen in liquid nitrogen, and stored at −70 C until use. There were no between-group differences in the length of time of freezer storage of serum and placental tissues before use (data not shown).

ELISA of serum sFlt-1 and PlGF

The concentration of total sFlt-1 in serum was measured in duplicate using a specific ELISA with a sensitivity of 5 pg/ml, according to the

TABLE 3. Clinical characteristics of SGA pregnancies

Patient no.	Oligohydramnios	Placental weight centile <10%	Umbilical Doppler	Birth weight centile	Length centile	Head circumference centile	Growth profile	Research classification
1				0.9	3–5	3–5	Symmetric	SGA
2				0.4	10–25	10–25	Asymmetric	SGA-S
3	−		Normal	7.4	5–10	25–50	Asymmetric	SGA-S
4	−			8.2	50–75	50–75	Asymmetric	SGA-S
5				9.0	10–25	25–50	Asymmetric	SGA-S
6	+	+	Abnormal	3.4	<3	<3	Symmetric	SGA-S
7	−	+	Abnormal	1.0	3–5	10–25	Asymmetric	SGA-S
8	−	+		0.03	25–50	<3	Symmetric	SGA
9				0	<3	5–10	Asymmetric	SGA-S
10				3.6	<3	50–75	Asymmetric	SGA-S
11	+		Normal	3.9	<3	25	Asymmetric	SGA-S
12		−	Normal	3.5	>97	<3	Symmetric	SGA
13	+			7.6	10–25	10–25	Symmetric	SGA
14		+		3.9	3–5	<3	Symmetric	SGA
15	+		Normal	0.9	10–25	5–10	Asymmetric	SGA-S
16		+		4.5	<3	10–25	Symmetric	SGA
17	+		Normal	0	10–25	10–25	Asymmetric	SGA-S
18				1.5	<3	3–5	Symmetric	SGA
19	+			0	5–10	10–25	Asymmetric	SGA-S
20				4.8	5–10	25–50	Asymmetric	SGA-S
21		+		2.2	3–5	25–50	Asymmetric	SGA-S
22	+			4.0				SGA
23	−		Abnormal	3.0				SGA-S
24				7.8				SGA

Clinical data from each SGA pregnancy. Empty sections indicate unavailable data. +, Positive for oligohydramnios or placental weight centile < 10%; −, negative for oligohydramnios or placental weight centile < 10%. Birth weight percentile (centile) is listed as a continuous variable. Length and head circumference centiles are given as categories. Asymmetric growth was determined by comparison of birth weight, length, and head circumference centiles for each neonate as described in *Subjects and Methods*. Fifteen of the 24 SGA pregnancies are categorized as having a profile suggestive of "severe SGA" (SGA-S) on the basis of an abnormal umbilical artery systolic/diastolic ratio (n = 3) and/or an asymmetric growth profile (n = 13).

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manufacturer's instructions (R&D Systems, Inc., Minneapolis, MN). Two quality-control samples routinely used in our laboratory were included. The intra- and interassay coefficients of variation in our laboratory were 4 and 14%, respectively. Concentrations of the free (unbound) form of PlGF in serum were measured in duplicate by ELISA (R&D Systems, Inc.). The PlGF assay has a sensitivity of 7 pg/ml. The intra- and interassay coefficients of variation were 5.4 and 11.2%.

Validation of serum ELISAs

We validated the R&D Systems ELISAs for serum sFlt-1 and PlGF by performing sample dilution and spike-recovery tests on separate pools of preeclampsia, SGA, and normal pregnancy patient serum (n = 3 patient samples per pool). There was a high degree of linear correlation between the degree of sample dilution and measured sFlt-1 concentrations, using 1:3, 1:8, and 1:12 (vol/vol) dilutions of each preeclampsia, SGA, and normal pregnancy serum pool within the dynamic range of the assay ($r^2 > 0.99$ in each group). Likewise, there was a high degree of linear correlation between sample dilution and PlGF concentration, using 1:2, 1:4, and 1:6 (vol/vol) dilutions of each preeclampsia, SGA, and normal pregnancy serum pool within the dynamic range of the assay ($r^2 > 0.99$ in each case). Three separate spike-recovery tests were performed for each ELISA; the calculated recovery of excess sFlt-1 added to pooled SGA patient serum was 96 ± 5%, and recovery of excess PlGF added to pooled SGA patient serum was 93 ± 5%.

Concentrations of sFlt-1 and PlGF in EDTA-plasma were slightly lower than concentrations in serum; plasma from three preeclamptic and three SGA individual patients yielded mean sFlt-1 and PlGF values that were 86 ± 5 (SD) and 80 ± 11 percent, respectively, of the corresponding serum values. The correlations between plasma and serum concentrations of sFlt-1, and plasma and serum PlGF in these six pregnancy subjects were highly linear ($P < 0.001$; $r^2 = 0.99$ each) over their range of serum values (sFlt-1, 1053–6695 pg/ml; PlGF, 233-1023 pg/ml). This suggests that the magnitude of group differences in the overall study would have been essentially the same had the analyses been performed on plasma. Plasma and serum samples from each patient were processed in parallel, and there was no correlation between sFlt-1 or PlGF concentration and the time interval from venipuncture to freezer storage (data not shown).

RNA isolation

After removing the basal plate, placental tissues were pulverized using a tissue pulverizer (Spectrum Medical Industries, Inc., Laguna Niguel, CA) and then homogenized in 20 vol of RNAwiz (Ambion, Inc., Austin, TX) using a Tissuemizer (Tekmar, Cincinnati, OH) at speed 60 for two 30-sec bursts. After a 20-min incubation at 25 C, 0.2 vol of chloroform was added to the homogenate, mixed thoroughly, and allowed to incubate for an additional 10 min. The mixture was next centrifuged at 10,000 × *g* for 10 min. The clear aqueous phase was collected in a clean tube and diluted with an equal volume of ribonuclease free water. The RNA was precipitated by the addition of 1 vol of isopropanol. After 10 min of incubation at 25 C, the RNA was recovered by centrifugation at 10,000 × *g* for 15 min at 4 C. The pellet was washed with 70% ethanol, allowed to air dry, and dissolved in a minimal volume of ribonuclease free water. The amount of RNA was estimated by spectrophotometry (A260), and one absorbance unit was taken to be 40 μg/ml.

Northern blot analysis

Twenty micrograms of total RNA were separated on formaldehyde-containing 1.5% agarose gels and transferred to nylon membranes (MSI, Westborough, MA). The RNA was crosslinked to the membrane using a Fisher Biotech UV-crosslinker (Fisher Scientific, Pittsburgh, PA). A 650-bp region common for both Flt-1 and sFlt-1 genes using 5′-ATGGTCAGCTACTGGGACACCGGGGTC (forward primer at nucleotide 250) and 5′-ACTGTTGCTTCACAGGTCAGAAGC (reverse primer at nucleotide 881) was amplified from cDNA prepared from RNA obtained from normal pregnant placental villous explants by PCR. cDNA probes were made using 25–50 ng of the insert, 50 μCi of α-^{32}P-dCTP (3000 Ci/mmol; NEN Life Science Products-DuPont), and 2 U Klenow polymerase using the Multi-prime DNA-labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ). Unincorporated free ^{32}P was removed using a spin column (Bio-Spin P30; Bio-Rad Laboratories, Inc., Hercules, CA).

The membranes were washed once briefly in 2× standard saline citrate solution and were processed further for hybridization. Prehybridization was carried out for 12 h in 6 ml buffer containing 50% deionized formamide, 5× Denhardt's solution, 5× saline sodium phosphate with EDTA, 1% sodium dodecyl sulfate (SDS), 0.5 mg/ml denatured salmon sperm DNA, and 0.1 mg/ml tRNA at 42 C in a Hybaid oven with roller bottles (250 × 35 mm). After decanting the prehybridization solution, the labeled probe in 4 ml prehybridization buffer was added to the membrane (2×10^6 cpm/ml), and the hybridization was carried out for 12–24 h. The membranes were washed in 2× standard saline citrate/0.1% SDS twice for 5 min at 25 C, and then twice in the same solution at 37 C. The membranes were exposed to Kodak-Biomax-AR film (Eastman Kodak Co., Rochester, NY).

Densitometry was performed on the 18S RNA and sFlt-1, using the film negative of the ethidium bromide gel and the autoradiograph, respectively. The value of 18S RNA was used to normalize loading variations in the gel. The signal on the film was quantified using the UN-SCAN-IT densitometry program (Silk Scientific, Inc., Orem, UT).

ELISA of placental tissue lysates

Concentrations of free PlGF and total Flt-1 in placental villous homogenates were assessed in duplicate by ELISA (R&D Systems, Inc.) (27). Because the ELISA for sFlt-1 uses an antibody against the Flt-1 extracellular domain, both endogenous sFlt-1 and Flt-1 are measured when applying this assay to tissue homogenates. For assay of PlGF, 0.05 g tissue was homogenized in 1 ml PlGF ELISA calibrator diluent. After centrifugation at 5000 × *g* for 15 min, supernatant PlGF was measured by ELISA. For assay of placental sFlt-1 and Flt-1 protein, 0.05 g tissue was homogenized in 1 ml ELISA kit cell lysis buffer and incubated for 1 h at room temperature. Samples were centrifuged at 5000 × *g* for 15 min, and the supernatant was diluted 1:100 (vol:vol) in lysis buffer for assay.

Concentrations were normalized to tissue total protein (Bradford assay). We validated the R&D Systems ELISAs for placental sFlt-1 and PlGF by performing dilutional parallelism and spike-recovery tests on placental tissue homogenates from healthy women delivering at term (n = 3). Correlations between the degree of sample dilution and measured sFlt-1 and PlGF concentration were linear ($r^2 > 0.99$, each). Calculated recoveries of excess sFlt-1 and PlGF added to placental homogenates were 94% in both cases. Placental Flt-1 and sFlt-1 were further assessed by Western and Northern analyses as described below.

Western blotting

Total proteins were extracted from archived placental villous tissue and processed using methods as described, with slight modifications (28). In brief, 30 μg total protein was separated on SDS-containing 7.5% polyacrylamide gels (20 × 20 cm; Owl Separations Systems, Inc., Portsmouth, NH). The proteins were then transferred to polyvinylidene fluoride membranes (Immobilon; Millipore Corp., Bedford, MA) using a semidry transfer system (The Panther Semidry Electroblotter; Owl Separations Systems Inc.). Detection of proteins was carried out after blocking the membranes with a 5% solution of nonfat dry milk. Membranes were incubated with rabbit antisoluble VEGFR-1 antibody (against specific carboxy-terminal region to the sFlt-1 protein) (Zymed Laboratories Inc., San Francisco, CA) at 1:100 dilution, or rabbit anti-VEGFR-1 antibody (against carboxy terminus not present in sFlt-1) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:200 dilution, or mouse monoclonal β-actin antibody (Sigma, St. Louis, MO) at 1:1000 dilution. The membranes were then washed in Tris-buffered saline containing 0.05% Tween-20 three times for 10 min each and next incubated with alkaline phosphatase-conjugated secondary antibody (1:5000 dilution; Promega Corp., Madison, WI) for 30 min. The membranes were washed three more times in Tris-buffered saline containing 0.05% Tween-20 for 10 min each. They were further washed in buffer without Tween-20 for 10 min and allowed to equilibrate in alkaline phosphatase buffer (100 mM Tris, pH 9.5; 150 mM NaCl) for 5 min. Chemiluminescent detection was carried out using CDP-star substrate (Boehringer Mannheim, Indianapolis, IN) diluted 1:200 in alkaline phosphatase buffer for 5 min. Membranes were exposed to Kodak Bio-max–AR film. The bands were

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scanned using a Hewlett-Packard laser scanner (Scanjet 5370C; Hewlett-Packard Co., Palo Alta, CA). Densitometry was carried out using an automated digitizing software UN-SCAN-IT Gel Version 4.3 (Silk Scientific, Inc.).

Statistical analysis

Because the distributions for sFlt-1 and PlGF protein and sFlt-1 mRNA were skewed, statistical testing of these variables was conducted after logarithmic transformation. Median concentrations (interquartile range) are reported in text and figures. Data on clinical characteristics were normally distributed and therefore are tabulated as mean (SD) unless otherwise indicated. Group differences were assessed by ANOVA; Bonferroni/Dunn *post hoc* test was used to adjust for multiple comparisons where appropriate. Correlations between placental weight and serum variables were by Spearman rank.

Multivariable linear regression was used to assess the independent effect of pregnancy diagnosis (preeclampsia, SGA, or normal) on serum sFlt-1 and serum PlGF after adjusting for potential confounders [gestational age at blood sampling, presence of labor at blood sampling, smoking, and prepregnancy body mass index (BMI)]. Covariates that changed the coefficient for SGA or preeclampsia by at least 10% were included in the model. Dependent variables were log transformed to ensure normality. Results are presented as the percent change in the dependent variable, calculated as $[\exp(\beta) - 1] \times 100$.

Results

Serum sFlt-1 concentrations

As shown in Fig. 1A, we found no difference in serum sFlt-1 concentration between SGA cases [1987 pg/ml (interquartile range, 1439–3494)] and normal pregnancy controls for SGA [2472 pg/ml (1933–3204)] ($P = 0.56$). However, serum concentrations of sFlt-1 were nearly 3-fold greater in preeclampsia [5221 pg/ml (3972–7838)] compared with preeclampsia controls [1857 pg/ml (821–2696)] or SGA cases [1987 pg/ml (1439–3494)] ($P < 0.0001$, each). After adjusting for gestational age at blood sampling, preeclampsia was associated with a 2-fold increase in sFlt-1 compared with controls [coefficient (SE), 0.69 (0.15); $P < 0.001$] and a 2.3-fold increase compared with SGA cases [0.83 (0.16); $P < 0.001$]; sFlt-1 was not significantly different between SGA and SGA controls [−0.14 (0.12); $P = 0.22$].

Of the 22 women with SGA infants in which serum sFlt-1 was measured, 13 had abnormal umbilical artery Doppler waveforms and/or an asymmetric fetal growth profile. Serum sFlt-1 concentrations in this potentially severe SGA subgroup (SGA-S) also did not differ from SGA controls [2912 pg/ml (1425–3775) *vs.* 2472 pg/ml (1933–3204)] ($P = 0.99$) but were significantly lower than preeclamptics ($P < 0.001$). The subgroup of women with SGA infants without Doppler or growth asymmetry data to corroborate abnormality in growth (n = 9) had lower serum sFlt-1 concentrations than the SGA-S subgroup [1674 pg/ml (1395–1984) *vs.* 2912 pg/ml (1425–3775)] ($P < 0.04$). Similar to the SGA-S subgroup, the concentration of serum sFlt-1 in this potentially less severe SGA subgroup was not different from controls ($P = 0.13$) but was significantly less than preeclamptics ($P < 0.001$). Median sFlt-1 concentrations in the SGA subgroup delivering infants with birth weight centiles less than 1.0 (n = 8; median centile, 0.22) [1716 pg/ ml (1288–3032)]) were likewise not different from controls for SGA ($P = 0.31$) but were significantly lower than preeclamptics ($P < 0.001$).

To determine whether the serum sFlt-1 concentration in women with preeclampsia is related to birth weight centile, we compared women who had preeclampsia plus SGA infants with women who had preeclampsia without SGA infants. The median value of serum sFlt-1 in preeclampsia with SGA [7961 pg/ml (5491–9527)] (n = 5) was greater than that of preeclampsia without SGA [4759 pg/ml (3654–6933)] (n = 17) but not significantly so ($P = 0.08$).

Serum PlGF

As shown in Fig. 1B, serum concentrations of free PlGF were about 2.5-fold lower in women with preeclampsia [86 pg/ml (interquartile range, 49–132)] compared with preeclampsia controls [228 pg/ml (124–405)] ($P < 0.0001$). PlGF concentrations were significantly decreased in SGA cases [163 pg/ml (79–206)] compared with SGA controls [266 pg/ml (114–420)] ($P = 0.008$). PlGF concentrations in women with SGA infants did not differ significantly compared with women with preeclampsia ($P = 0.27$). After controlling for gestational age at sampling, presence of labor, and smoking, SGA and preeclampsia were both associated with a 64% decrease in free PlGF compared with controls [SGA, −0.44 (0.20), $P = 0.02$; preeclampsia, −0.44 (0.26), $P = 0.09$], although this was of borderline statistical significance for preeclampsia. In the multivariable model, there were no differences in free PlGF comparing SGA cases with preeclampsia [−0.002 (0.29); $P = 0.99$]. Although placental weights were of limited availability, significant correlations between PlGF concentration and placental weight were observed within SGA (n = 9; $r^2 = 0.56$; $P < 0.04$) and preeclampsia (n = 16; $r^2 = 0.52$; $P < 0.01$) groups but not controls.

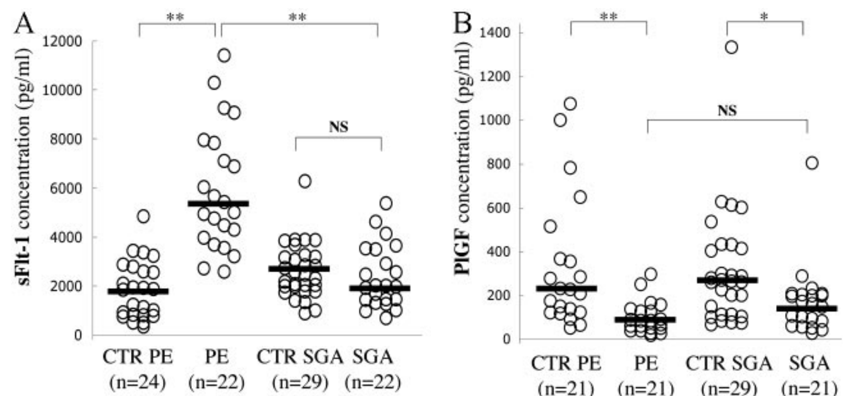

FIG. 1. Scatter plots demonstrating the distribution of serum total sFlt-1 (A) and serum free PlGF (B) concentrations among women with preeclampsia (PE), normal pregnancy controls group-matched for gestational age to women with preeclampsia (CTR PE), women with SGA infants, and normal pregnancy controls group-matched for gestational age to women with SGA pregnancies (CTR SGA). The *thick horizontal bars* denote the median value for each group. **, $P < 0.0001$, PE *vs.* CTR PE and PE *vs.* SGA; *, $P < 0.01$, SGA *vs.* CTR SGA; NS, not significant. For complete statistical comparisons, see *Results*.

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Similar to the SGA cases as a whole, serum PlGF was significantly lower (less than half) in the SGA-S subgroup [108 pg/ml (62–198)] (n = 13) than controls for SGA [266 pg/ml (114–420)] (*P* = 0.003). PlGF in the SGA cases with birth weight less than first centile [168 pg/ml (106–204)] (n = 8) was marginally less than controls for SGA (*P* = 0.06).

There was no difference in PlGF concentration between women with preeclampsia with SGA infants [86 pg/ml (72–182)]; n = 5] compared with women with preeclampsia without SGA infants [66 pg/ml (46–129); n = 16] (*P* = 0.82).

Placental sFlt-1 and Flt-1 mRNA

As illustrated by Fig. 2, placental sFlt-1 mRNA expression followed a pattern similar to that of serum sFlt-1 protein. sFlt-1 mRNA expression was increased 1.6 -fold in placentas of women with preeclampsia [median sFlt-1/18S band intensity, 3.92 (interquartile range, 3.03–5.19)] compared with preeclampsia controls [2.52 (2.37–3.05)] or SGA cases [2.66 (2.30–2.88)] (*P* < 0.001, each). However, placental sFlt-1 mRNAs in SGA cases and SGA controls did not differ [2.66 (2.30–2.88) *vs.* 2.95 (2.45–3.77); *P* = 0.31] (Fig. 2B). Flt-1 mRNA expression was also increased 1.4 -fold in placentas of women with preeclampsia [median Flt-1/18S band intensity, 1.47 interquartile range, (1.13–1.72)] compared with preeclampsia controls [1.03 (0.98–1.07)] or SGA cases [0.99 (0.81–1.07)] (*P* < 0.001, each). Flt-1 mRNA expression in SGA cases and SGA controls was not significantly different [0.99 (0.81–1.07) *vs.* 0.91(0.78–1.16); *P* = 0.31] (Fig. 2C). There was no difference in placental sFlt-1 mRNA in SGA-S patients (n = 9) compared with the remainder of SGA patients (n = 4) (*P* = 0.13).

ELISA of PlGF and Flt-1 in placental lysates

Median free PlGF concentration in lysates of placental villous tissue from women with SGA infants [965 pg/mg total protein (interquartile range, 728-1243); n = 13] was less than controls [1207 (930–1762); n = 16] (*P* = 0.03) but not different from preeclampsia [543 (360–906); n = 10] (*P* = 0.12). Women with preeclampsia had placental PlGF concentrations about half that of preeclampsia controls [1125 (1039–1188); n = 8] (*P* = 0.05). sFlt-1 protein (in lysates likely a combination of sFlt-1 and Flt-1 interacting with the antibody against the extracellular domain) was increased in placentas from preeclampsia [10,385 pg/mg total protein (6,850–11,350) n = 10] compared with controls [6,685 (4,775–8,695); n = 8] (*P* = 0.03) but not in SGA [6,420 (5,175–8,463); n = 13] compared with SGA controls [6,295 (4,835–7,960); n = 16] (*P* = 0.71).

SGA-S patients (n = 9) had marginally lower median values for placental PlGF protein than the remainder of SGA patients (n = 4) [813 (687–1035) *vs.* 1267 (1021–1382)] (*P* = 0.07). Placental sFlt-1 protein in SGA-S patients [6500 (5250–8463); n = 9] was not different from that in SGA patients [5860 (4095–8410); n = 4] (*P* = 0.59).

Western blot

As shown in Fig. 3A, placental sFlt-1 protein expression was increased 1.4-fold in placentas of women with preeclampsia [median sFlt-1/β-actin band intensity, 0.48 (interquartile range, 0.39–0.51); n = 10] compared with preeclampsia controls [0.34 (0.23–0.43); n = 8] (*P* = 0.04) or SGA cases [0.28 (0.23–0.41); n = 13] (*P* < 0.005). However, placental sFlt-1 proteins in SGA cases and SGA controls [0.33 (0.3–0.44); n = 16] did not differ (*P* = 0.19). As shown in Fig. 3B, placental Flt-1 protein expression was increased 1.9-fold in placentas of women with preeclampsia [0.94 (0.87–1.01); n = 6] compared with preeclampsia controls [0.42 (0.41–0.64); n = 6] (*P* < 0.0005) or SGA cases [0.41 (0.32–0.49); n = 6] (*P* < 0.0001). Placental Flt-1 expression in SGA cases and SGA controls [0.44 (0.37–0.51); n = 6] was not different (*P* = 0.8). These data are consistent with the data by ELISA of sFlt-1 and Flt-1 in placental lysates.

Discussion

We report that sFlt-1 is increased and free PlGF is decreased in maternal serum and placental villous tissue of

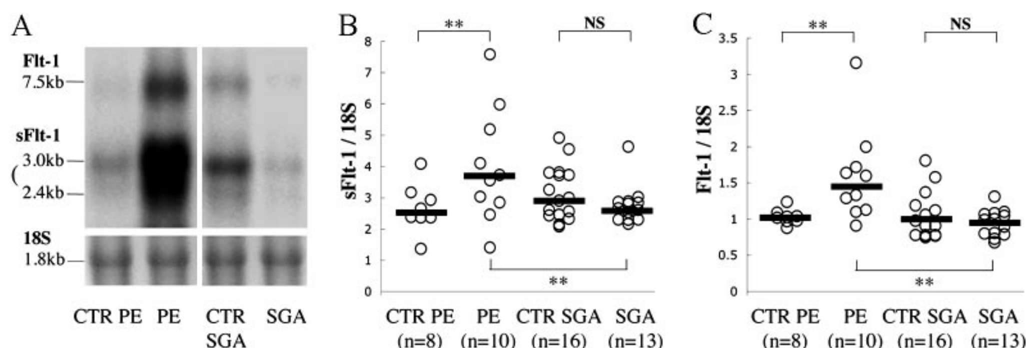

FIG. 2. Flt-1 and sFlt-1 mRNA expression in placental biopsy samples obtained from women with preeclampsia (PE; n = 8), normal pregnancy controls group-matched for gestational age to women with preeclampsia (CTR PE; n = 10), women with SGA infants (n = 13), and normal pregnancy controls group-matched for gestational age to women with SGA infants (CTR SGA; n = 16). Total RNA was isolated from frozen samples using RNAwiz (Ambion, Inc.). Twenty micrograms of RNA were electrophoresed and processed for Northern blot analysis as described in *Subjects and Methods*. A, Representative autoradiograph depicting Flt-1 mRNA (7.4 kb) and sFlt-1 mRNA (3.0 and 2.4 kb). B and C, Densitometry was performed on the Flt-1 mRNA and sFlt-1 separately and was normalized to the 18S densitometric value. Scatter plots demonstrate the distribution of quantified placental sFlt-1 (A) and Flt-1 (B) mRNA expression among these groups. The *thick horizontal bars* denote the median value for each group. **, *P* < 0.001, PE *vs.* CTR PE and PE *vs.* SGA; NS, not significant. For complete statistical comparisons, see *Results*.

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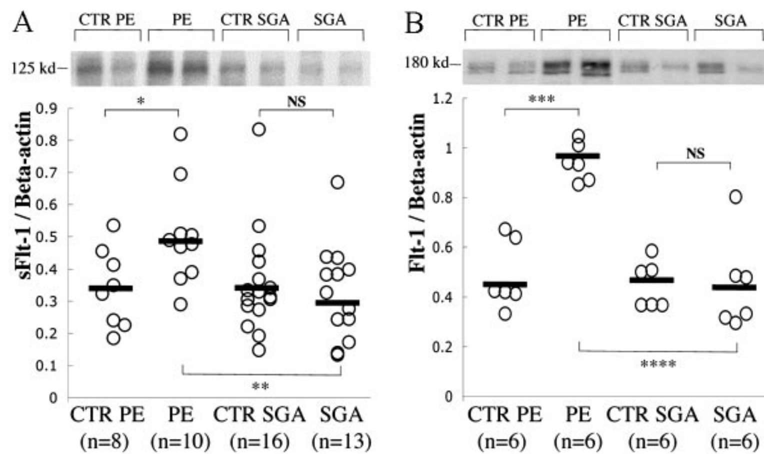

FIG. 3. Placental sFlt-1 (A) and Flt-1 (B) protein expression in placental biopsy samples obtained from women with preeclampsia [PE; n = 8 (sFlt-1) and n = 6 (Flt-1)], normal pregnancy controls group-matched for gestational age to women with preeclampsia [CTR PE; n = 10 (sFlt-1) and n = 6 (Flt-1)], women with SGA [n = 13 (sFlt-1) and n = 6 (Flt-1)], and normal pregnancy controls group-matched for gestational age to women with SGA infants [CTR SGA; n = 16 (sFlt-1) and n = 6 (Flt-1)]. Thirty micrograms of total protein were electrophoresed and processed for Western blot analysis as described in *Subjects and Methods*. Representative Western blot depicting placental sFlt-1 protein (A) (molecular mass, 125 kDa) and placental Flt-1 protein (B) (molecular mass, 180 kDa). Densitometry performed on the sFlt-1 and Flt-1 protein was normalized to the β-actin densitometric value. Scatter plots demonstrate the distribution of quantified placental sFlt-1 (A) and Flt-1 (B) protein expression among these groups. The *thick horizontal bars* denote the median value for each group. *, $P = 0.04$, PE *vs.* CTR PE; **, $P < 0.005$, PE *vs.* SGA; ***, $P < 0.0005$, PE *vs.* CTR PE; ****, $P < 0.0001$, PE *vs.* SGA; NS, not significant. For complete statistical comparisons, see *Results*

women with preeclampsia. These data confirm previous reports and are consistent with a postulated role for excess sFlt-1 as a circulating antagonist of VEGF and PlGF in preeclampsia (3, 11, 29, 30). A principal new finding of our study is that, in contrast to preeclampsia, placental and maternal serum sFlt-1 are not increased in normotensive pregnancies with SGA infants, including those with corroborating evidence of fetal growth restriction. Despite sFlt-1 not being increased, concentrations of free PlGF in women with SGA infants were significantly decreased to levels observed in women with preeclampsia. Therefore, we conclude that excess sFlt-1 does not explain the decreased free PlGF in the circulation of women with SGA pregnancies. Women with preeclampsia with or without SGA infants, but not women with isolated SGA, exhibited increased serum sFlt-1 concentrations even though free PlGF was reduced in all of these groups compared with controls. These data appear to be compatible with recent work suggesting fundamental placental differences in pregnancies with fetal growth restriction with and without preeclampsia (31, 32).

We did not attempt to measure concentrations of free VEGF because this growth factor has been previously reported to be virtually undetectable in the circulation of pregnant women, especially in women with preeclampsia (3, 33). Circulating free PlGF concentrations increase progressively with advancing normal gestation, peaking at around 30 wk gestation and then declining slightly near term (23, 29, 34–37). The degree of reduction in free PlGF in patients with SGA neonates to levels seen in women with preeclampsia, and the absolute concentrations that we report agree closely with data of Tsatsaris *et al.* (33), who used the same ELISA for PlGF measurement. Tests performed in our laboratory, including recovery of spiked PlGF and linearity upon dilution, support the validity of this assay. Other data likewise support a reduction of free PlGF in the maternal circulation of women with SGA fetuses. Chappell *et al.* (35) reported that plasma PlGF concentrations in patients with preeclampsia and in patients with SGA fetuses were decreased from the first point of observation (20–32 wk gestation). Taylor *et al.* (23) reported lower PlGF (albeit of borderline significance), by 27–30 wk gestation, in women with SGA fetuses. Bersinger and Odegard (36) reported decreased PlGF in pregnancies with SGA fetuses by 33 wk gestation, and at 17, 25, and 33 wk gestation in women who subsequently developed preeclampsia. Given that preeclampsia is distinguished from SGA/IUGR by maternal systemic vascular disease, the question remains as to what impact, if any, lowered circulating free PlGF might have on maternal vascular function.

We observed significant correlations between placental weight and serum concentrations of free PlGF (but not placental weight and serum sFlt-1) within both the SGA and preeclampsia groups. Thus, reduced placental size may contribute to reduced PlGF in both groups of women, with increased sFlt-1 further reducing circulating free PlGF in preeclampsia. However, placental PlGF concentration, expressed per milligram of placental total protein, was also reduced in these patients, so factors in addition to reduced placental size may result in reduced output of PlGF. Hypoxia down-regulates PlGF mRNA and protein in trophoblast-like (BeWo) cells (38). Hypoxia secondary to failure of extravillous trophoblast invasion might decrease placental output of PlGF and thus contribute to decreased circulating free PlGF (23). However, the concept of placental hypoxia has been disputed, especially in cases of severe IUGR with absent or reversed end-diastolic flow velocity in the umbilical artery. Indeed, it has been argued that, in these cases, the maternal blood in the intervillous space is hyperoxic (19, 39, 40) This might explain the report of increased PlGF expression in severe IUGR placentae (38). It is possible that divergent PlGF results reflect the heterogeneous etiology of IUGR.

The serum sFlt-1 concentrations (median picograms/milliliter) that we observe in women with preeclampsia (5,221;

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range, 2,586–11,423) and normal pregnancy (1,857; range, 353-4,835) agree closely with previous studies that also used the R&D Systems, Inc. ELISA (3, 11, 29). However, Tsatsaris *et al.* (33) reported much lower plasma values in women with preeclampsia at 30.5 gestational weeks (median pg/ml, 2690; range, 2310–2970), in controls at 38.3 gestational weeks (660; range, 410–930), and in controls matched for gestational age with the preeclamptic group (120; range, 0–290). Furthermore, they reported significantly increased plasma sFlt-1 (1810; range, 820-2760) and increased placental sFlt-1 mRNA in IUGR patients, at 32 gestational weeks, compared with controls. The reasons for this discrepancy with our data are not readily apparent. This difference cannot be attributed to the fact that we used patient serum rather than plasma, because plasma concentrations in our study were only slightly lower than, and correlated linearly with, serum concentrations. IUGR was defined in the aforementioned study as birth weight below the third centile; although this was a more stringent cutoff than we primarily used, the mean birth weight centile in our study was also quite low (mean birth weight centile, 3.1). On the other hand, Tsatsaris *et al.* (33) used an ELISA kit for sFlt-1 from a different manufacturer without accompanying validation data for use with pregnancy samples. None of the IUGR patients in the their study were hypertensive, but the reported mean predelivery systolic BP was 19 points higher in IUGR cases compared with the controls. This raises the question of whether some of their IUGR patients manifested significant BP increases during pregnancy.

The placenta is likely to be the principal source of the pregnancy-induced increase in circulating sFlt-1, given the strong placental expression of sFlt-1 mRNA, the high secretion of sFlt-1 protein by villous tissue in explant culture, and the rapid decline of maternal circulating sFlt-1 levels after delivery (3, 10, 11). Hypoxia has been shown to increase sFlt-1 production by placental villous tissue in explant culture and in cultured cytotrophoblasts (17, 18). One might speculate that excessive sFlt-1 in the maternal circulation can result from placentas subjected to reduced perfusion, *i.e.* insufficient remodeling of uterine spiral arteries by cytotrophoblasts (3, 11). Reduced uteroplacental perfusion is thought to frequently occur in preeclampsia and also in idiopathic IUGR (14). However, our divergent data regarding sFlt-1 in preeclamptic and SGA pregnancies raises the question of whether reduced uteroplacental perfusion alone is sufficient to increase sFlt-1in the maternal circulation. Differences between preeclamptic and normal pregnancy serum sFlt-1 (about 3-fold) appeared to be substantially greater than corresponding placental differences by Western blot for sFlt-1 (about 1.4-fold). Furthermore, sFlt-1 protein was not as dramatically increased as Flt-1 protein in preeclamptic placenta. These data are consistent with the notion that much of the placentally produced sFlt-1 protein is secreted into the maternal blood stream. It is noteworthy, however, that peripheral blood mononuclear cells may comprise a significant extraplacental source of excess sFlt-1 in preeclamptic pregnancies (28). Genetic differences influencing Flt-1 gene splicing might also contribute to group differences. Seemingly healthy women with a history of preeclampsia, 18 months postpartum, demonstrated significantly (37%) increased concentrations of serum sFlt-1, compared with women with prior normotensive pregnancies, even though postpartum sFlt-1 concentrations were 50-fold lower than intrapartum concentrations (41).

In conclusion, our data showing increased sFlt-1 in serum and placentae of women with preeclampsia, but not normotensive pregnancies with SGA fetuses, are consistent with a role for sFlt-1 in the maternal vascular dysfunction of preeclampsia and are consistent with excess production of sFlt-1 in preeclamptic placenta as a significant source of maternal circulating levels. In contrast to preeclampsia, sFlt-1 does not contribute substantially to decreased free PlGF in pregnancies with SGA fetuses in the absence of a maternal syndrome. Therefore, inasmuch as idiopathic IUGR and preeclampsia may share reduced placental perfusion, placental infarcts, or other aspects of placental pathology, these data raise more questions than answers regarding the factors responsible for up-regulation of sFlt-1 in women with preeclampsia.

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