Angiotensin Receptor Agonistic Autoantibody–Mediated Tumor Necrosis Factor-α Induction Contributes to Increased Soluble Endoglin Production in Preeclampsia

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Background—Preeclampsia is a prevalent life-threatening hypertensive disorder of pregnancy. The circulating antiangiogenic factor, soluble endoglin (sEng), is elevated in the blood circulation of women with preeclampsia and contributes to disease pathology; however, the underlying mechanisms responsible for its induction in preeclampsia are unknown.

Methods and Results—Here, we discovered that a circulating autoantibody, the angiotensin receptor agonistic autoantibody (AT1-AA), stimulates sEng production via AT1 angiotensin receptor activation in pregnant mice but not in nonpregnant mice. We subsequently demonstrated that the placenta is a major source contributing to sEng induction in vivo and that AT1-AA–injected pregnant mice display impaired placental angiogenesis. Using drug screening, we identified tumor necrosis factor-α as a circulating factor increased in the serum of autoantibody-injected pregnant mice contributing to AT1-AA–mediated sEng induction in human umbilical vascular endothelial cells. Subsequently, among all the drugs screened, we found that hemin, an inducer of heme oxygenase, functions as a break to control AT1-AA–mediated sEng induction by suppressing tumor necrosis factor-α signaling in human umbilical vascular endothelial cells. Finally, we demonstrated that the AT1-AA–mediated decreased angiogenesis seen in human placenta villous explants was attenuated by tumor necrosis factor-α–neutralizing antibodies, soluble tumor necrosis factor-α receptors, and hemin by abolishing both sEng and soluble fms-like tyrosine kinase-1 induction.

Conclusions—Our findings demonstrate that AT1-AA–mediated tumor necrosis factor-α induction, by overcoming its negative regulator, heme oxygenase-1, is a key underlying mechanism responsible for impaired placental angiogenesis by inducing both sEng and soluble fms-like tyrosine kinase-1 secretion from human villous explants. Our results provide important new targets for diagnosis and therapeutic intervention in the management of preeclampsia. (Circulation. 2010;121:436-444.)

Key Words: angiogenesis ▪ antibodies ▪ endothelium ▪ hypertension ▪ inflammation ▪ pregnancy ▪ signal transduction

Preeclampsia is a prevalent life-threatening hypertensive disorder of pregnancy with high maternal and fetal rates of morbidity and mortality.1,2 A growing body of evidence indicates that a circulating maternal autoantibody, the angiotensin II type I receptor agonistic autoantibody (AT1-AA), is a prominent component in the pathogenesis of preeclampsia. Numerous early studies demonstrated that AT1-AAs activate AT1 receptors on a variety of cell types and provoke biological responses relevant to the pathophysiology of preeclampsia.3–8 Recently, we have extended these in vitro studies by showing that key features of preeclampsia are generated in pregnant mice injected with either total immunoglobulin G (IgG) or affinity-purified AT1-AAs from preeclamptic women.9 These studies provided the first direct evidence for the pathogenic nature of AT1-AAs in preeclampsia.

Clinical Perspective on p 444

Recently, Levine et al10 showed that a soluble form of endoglin (sEng) is present at significantly elevated levels in the circulation of women with preeclampsia compared with women with normotensive pregnancy and that the level of sEng correlated with disease severity. Endoglin is a cell-surface coreceptor for transforming growth factor-β1 and -β3 that is expressed mainly on endothelial cells and syncytiotrophoblasts.11–13 The introduction of recombinant adenovirus vectors encoding sEng into pregnant rats resulted in mild hypertension and proteinuria. Notably, the introduction of viral vectors encoding sEng and soluble fms-like tyrosine kinase-1 (sFlt-1, a soluble form of vascular endothelial growth factor receptor-1) together into pregnant rats resulted
in nephrotic-range proteinuria, severe hypertension, and the HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets), a severe form of preeclampsia. These studies demonstrate that sEng contributes to preeclampsia. However, factors and signaling pathways responsible for elevated sEng in women with preeclampsia were not determined.

Here, we show that AT1-AA induces the production of sEng in pregnant mice but not in nonpregnant mice by activation of AT1 receptors and that the placenta is a major source of its induction in vivo. We further provide compelling mouse and human evidence that AT1-AA leads to impaired placental angiogenesis via AT1 receptor activation. More important, we reveal that AT1-AA–mediated tumor necrosis factor–α (TNF–α) induction, by overcoming its negative regulator, heme oxygenase-1 (HO-1), is a key underlying mechanism responsible for impaired placental angiogenesis by inducing both sEng and sFlt-1 secretion from human villous placental explants. Overall, our findings are the first to link maternal autoantibodies with induction by AT1-AA, we injected pregnant or nonpregnant mice with a single dose (gestation day 13) or a double dose (gestation days 13 and 14) of IgG from women with preeclampsia (PE) or normotensive control (NT) mice for 5 days with a single injection or double injection. A, Plasma was collected at different time points as indicated, and the concentration of sEng was determined by ELISA. Data are expressed as mean±SEM. *P<0.05 vs pregnant mice injected with normotensive IgG (gestation day 18). B, Coinjection of losartan or the 7-aa epitope peptide inhibited the increase in sEng production by IgG from women with preeclampsia. **P<0.01 vs pregnant mice injected with IgG from women with preeclampsia (gestation day 13); ***P<0.05 vs pregnant mice injected with AT1-AA IgG (gestation day 18). C, No effect on sEng production by IgG from women with preeclampsia in nonpregnant mice. Data are expressed as mean±SEM. n=8 for each group.

Results

IgG From Women With Preeclampsia Stimulates sEng Production in Pregnant Mice but Not Nonpregnant Mice via AT1 Receptor Activation

To determine the potential role of pregnancy in the sEng induction by AT1-AA, we injected pregnant or nonpregnant mice with a single dose (gestation day 13) or a double dose (gestation days 13 and 14) of IgG from women with preeclampsia or women with a normotensive pregnancy. The single injection of IgG from women with preeclampsia did not induce a significant increase in sEng production by gestation day 18 in the pregnant mice compared with mice injected with IgG from women with a normotensive pregnancy (Figure 1A). However, a 4-fold increase in sEng was observed by gestation day 18 in pregnant mice that received a double injection of IgG from preeclamptic women compared with mice that received 2 injections of IgG from normotensive pregnant women (Figure 1A), which is similar to the increase seen in preeclamptic women. Because 2

in pregnant mice but not in nonpregnant mice by activation of AT1 receptors and that the placenta is a major source of its induction in vivo. We further provide compelling mouse and human evidence that AT1-AA leads to impaired placental angiogenesis via AT1 receptor activation. More important, we reveal that AT1-AA–mediated tumor necrosis factor–α (TNF–α) induction, by overcoming its negative regulator, heme oxygenase-1 (HO-1), is a key underlying mechanism responsible for impaired placental angiogenesis by inducing both sEng and sFlt-1 secretion from human villous placental explants. Overall, our findings are the first to link maternal autoantibodies with increased TNF–α, a prominent inflammatory cytokine, to contribute to impaired placental angiogenesis by inducing 2 key antiangiogenic factors, sFlt-1 and sEng. This study provides important new targets for diagnosis and therapeutic intervention in the management of preeclampsia.

Methods

For an expanded Methods sections, please refer to the online-only Data Supplement.

Patients

Patients admitted to Memorial Hermann Hospital were identified by the obstetric faculty of the University of Texas Medical School at Houston. Detailed patient information has been given previously.6,9 The research protocol was approved by the Institutional Committee for the Protection of Human Subjects.

Introduction of Antibody Into Mice

All animal studies were reviewed and approved by the Animal Welfare Committee, University of Texas Houston Health Science Center. Forty-eight C57BL/6J pregnant and 32 nonpregnant mice (18 to 22 g; Harlan, Indianapolis, Ind) were used in our study. Experiments were performed in the following groups of mice: pregnant mice (n=32) or nonpregnant mice (n=32) with single (n=16) or double (n=16) injection of IgG purified from normotensive control subjects (n=8) or preeclamptic patients (n=8). In addition, some of the pregnant mice were cojected with losartan (0.24 mg, a generous gift from Merck & Co, Inc, Rahway, NJ; n=8) or a 7–amino acid (7-aa) peptide (AFHYESEQ; n=8) corresponding to an epitope on the second extracellular loop of the AT1 receptor (1.5 mg). All of the mice were anesthetized with sodium pentobarbital (50 mg/kg IP), and concentrated IgG purified from 200 μL normotensive control subjects (n=12) or patients’ serum (n=14) (either combined or single individual) was introduced into pregnant mice (single injection on gestation day 13; for double injection, second injection on gestation day 14) or in nonpregnant mice (2 consecutive days by orbital sinus injection). IgG was prepared as previously described.6,9 We collected plasma and serum on multiple gestation days for determination of sEng and TNF–α concentration.

Figure 1. IgG from women with preeclampsia induces sEng secretion in pregnant but not nonpregnant mice via AT1 receptor activation. IgG from women with preeclampsia (PE) or normotensive pregnant women (NT) was introduced by retro-orbital injection into pregnant or nonpregnant mice for 5 days with a single injection or double injection. A, Plasma was collected at different time points as indicated, and the concentration of sEng was determined by ELISA. Data are expressed as mean±SEM. *P<0.05 vs pregnant mice injected with normotensive IgG (gestation day 18). B, Coinjection of losartan or the 7-aa epitope peptide inhibited the increase in sEng production by IgG from women with preeclampsia. **P<0.01 vs pregnant mice injected with IgG from women with preeclampsia (gestation day 13); ***P<0.05 vs pregnant mice injected with AT1-AA IgG (gestation day 18). C, No effect on sEng production by IgG from women with preeclampsia in nonpregnant mice. Data are expressed as mean±SEM. n=8 for each group.
autoantibody injections were required to achieve a significant increase in sEng levels in pregnant mice, we chose the double-injection protocol for the remainder of the experiments presented here. Antibody-mediated induction of sEng production was blocked by coinjection with losartan, an AT1 receptor antagonist, or with a 7-aa epitope peptide that blocks autoantibody-mediated AT1 receptor activation (Figure 1B). These results indicate that IgG from preeclamptic women induced sEng production in pregnant mice via AT1 receptor activation. In contrast to what we observed in pregnant mice, sEng in nonpregnant mice was not induced by IgG from women with preeclampsia or normotensive pregnant women after single or double injections (Figure 1C). Thus, these findings demonstrate that IgG from women with preeclampsia is capable of inducing sEng production via AT1 receptor activation in pregnant mice but not in nonpregnant mice.

Placenta Is a Major Organ Contributing to sEng Production in Autoantibody-Injected Pregnant Mice

Next, to determine whether the placenta is a major source of sEng production and secretion, we measured Eng mRNA and protein levels in the mouse placenta and kidneys from pregnant mice injected with IgG as described above. We found that total Eng mRNA levels were increased in placenta tissue of mice injected with IgG from women with preeclampsia compared with placenta tissue of mice injected with IgG from women with normotensive pregnancies (Figure 2A and 2B), which suggests that AT1-AA–mediated sEng induction is at the mRNA level, a finding consistent with earlier human studies.14 Similarly, we found that the abundance of intact Eng protein and the small amount of sEng remaining in the placentas were also induced in pregnant mice injected with IgG from preeclamptic women but not IgG from normotensive pregnant women (Figure 2C through 2E). We consistently found that Eng protein levels were much lower in kidney samples and that there was no difference in mice injected with IgG from normotensive pregnant women or those with preeclampsia (Figure 2C through 2E). Thus, these results provide direct evidence that placenta is a major organ contributing to sEng synthesis and secretion in autoantibody-injected pregnant mice.

Angiogenesis Is Impaired in Placentas of Autoantibody-Injected Pregnant Mice

To address whether AT1-AA–induced placenta-derived angiogenic factors led to impaired placental angiogenesis, we analyzed the vasculature of isolated mouse placentas by immunostaining using antibody recognizing CD31, an endothelial cell–specific marker. The results show that CD31 staining was less prominent in the labyrinth zone of the placentas of mice injected with IgG from women with preeclampsia than in those injected with IgG from normotensive pregnant women (Figure 3A). Coinjection of antibody with losartan or the 7-aa epitope peptide reduced this effect (Figure 3A). Quantitative image analysis of CD31 immunostaining demonstrated significantly
less immunoreactivity in placenta sections from mice injected with IgG from preeclamptic women than in those from mice injected with IgG from normotensive pregnant women. Co-injection with losartan or the 7-aa epitope peptide significantly reduced the antiangiogenic effects of autoantibody injection (Figure 3B). Taken together, these results indicate that IgG from women with preeclampsia is capable of inducing placenta-derived antiangiogenic factor production via AT1 receptor activation and thereby contributes to decreased angiogenesis in the placentas of injected mice.

**Serum From Mice Injected With IgG From Preeclamptic Women Stimulates sEng Secretion From Endothelial Cells**

It is difficult to decipher the signaling pathways involved in AT1-AA–mediated sEng induction in intact animals; therefore, we chose endothelial cells, a recognized source of sEng production, as a cellular model system. Unexpectedly, we found that neither angiotensin II nor IgG from preeclamptic and normotensive pregnant women stimulated sEng production by the cultured endothelial cells (Figure 4A). These results suggest that increased sEng in autoantibody-injected pregnant mice did not result from direct AT1 receptor activation. However, the production of sEng secretion was significantly increased by endothelial cells treated with serum of pregnant mice injected with IgG from preeclamptic women compared with that of cells cultured with serum of pregnant mice injected with normotensive IgG (Figure 4B). The increase in sEng production did not occur when serum from mice that were co-injected with losartan or the 7-aa epitope peptide was used (Figure 4B). These results imply that serum from pregnant mice injected with IgG from preeclamptic women contains a factor that stimulates human sEng secretion from human endothelial cells. Furthermore, the causative factor present in the serum of antibody-injected mice is the result of AT1 receptor activation.

**TNF-α Is the Serum Factor in the Autoantibody-Injected Pregnant Mice Responsible for sEng Secretion From Endothelial Cells**

To determine the circulating factors involved in AT1-AA–mediated sEng induction in endothelial cells, we performed a drug screen to identify drugs that blocked the induction of sEng by serum from autoantibody-injected mice. Among all the drugs we have tested, we found that Enbrel, (Amgen, Thousand Oaks, CA 91320 and Wyeth Pharmaceuticals Inc., Collegeville, PA 19426) a soluble form of the TNF receptor, blocks serum-mediated sEng production by human endothelial cells (Figure 4B), indicating that TNF-α is the serum factor responsible for inducing sEng production by endothelial cells. Next, we demonstrated that the ability of serum from autoantibody-injected mice to stimulate sEng production by cultured endothelial cells was also inhibited by anti-TNF-α, a neutralizing antibody. Finally, we found that TNF-α directly stimulated sEng production by endothelial cells and that the induction was blocked by the presence of anti-TNF-α or Enbrel, a soluble form of the TNF receptor that also blocks TNF-α signaling. Taken together, these findings provide strong evidence that TNF-α was the serum factor in the autoantibody-injected pregnant mice responsible for AT1-AA–mediated sEng induction.

**TNF-α Expression Is Increased in the Blood Circulation and Placentas of Autoantibody-Injected Pregnant Mice**

The level of TNF-α is elevated in the blood circulation of women with preeclampsia,15,16 and it is possible that TNF-α may be induced by AT1-AA. To address this possibility, we measured TNF-α in autoantibody-injected pregnant mice. As shown in Figure 4D, the level of TNF-α was elevated in pregnant mice injected with IgG from preeclamptic women compared with that in mice injected with IgG from normotensive pregnant women. The autoantibody-mediated induction of TNF-α was prevented by coinjection with losartan or the 7-aa epitope peptide (Figure 4D), indicating that IgG from preeclamptic women induces TNF-α production in pregnant mice via AT1 receptor activation.
To determine whether TNF-α/H9251 expression is upregulated in the placentas of antibody-injected mice, the placentas were collected on gestation day 18, 5 days after the initial IgG injection; then, total RNA was isolated, and real-time PCR was used to analyze the TNF-α/H9251 transcript levels. The results (Figure 4E) showed that the level of TNF-α/H9251 transcripts increased nearly 5-fold in the placentas of mice injected with IgG from preeclamptic women compared with that in the placentas of mice injected with IgG from normotensive pregnant women. The autoantibody-mediated increase in the abundance of placental TNF-α RNA was attenuated by coinjection with losartan or the 7-aa epitope peptide. These data indicate that IgG from women with preeclampsia stimulates an increase in the abundance of TNF-α transcripts in the placentas via AT1 receptor activation.

**AT1-AA–Induced TNF-α Overcomes Its Downstream Negative Regulator, HO-1, to Induce sEng Secretion by Endothelial Cells**

Among all the drugs we screened, we unexpectedly found that hemin, a well-known inducer of HO-1, blocked the ability of TNF-α to stimulate sEng production in human endothelial cells (Figure 4C). These results indicate that HO-1 functions as a break to control TNF-α–induced sEng production.
secretion. More important, hemin also blocked the ability of serum obtained from pregnant mice injected with preeclamptic IgG to stimulate sEng production by human endothelial cells (Figure 4B). Thus, these findings provide the first evidence that HO-1 functions downstream of AT1-AA–mediated TNF-α signaling to regulate sEng production.

**AT1-AA–Induced TNF-α Production Contributes to sEng Induction From Human Placental Villous Explants**

To extend mouse findings to humans and to assess the direct pathological role of autoantibody-mediated TNF-α signaling on sEng secretion in humans, we took advantage of human villous explants as an investigative tool. Immunohistostaining showed that Eng is evident in the syncytiotrophoblasts (fetus-derived cells) and endothelial cells (maternal origin) (Figure 5A), consistent with results published earlier. Quantitative image analysis revealed that the intensity of immunostaining of Eng was higher in the samples treated with IgG from women with preeclampsia and that the increase in intensity was prevented by the presence of losartan or the 7-aa epitope peptide, indicating that the increase was mediated through AT1 receptor activation (Figure 5A and 5B). The increase in Eng immunostaining was also prevented by the presence of anti-TNF-α, Enbrel, or hemin, indicating the requirement for TNF-α to overcome the negative regulation by HO-1 (Figure 5A and 5B).

In this study, we have provided both in vivo mouse and in vitro human evidence that AT1-AA contributes to impaired placental angiogenesis via AT1 receptor activation. Mechanistically, we have identified for the first time that the induction of sEng and sFlt-1 by AT1-AA is mediated through TNF-α signaling, which is negatively regulated by HO-1. Overall, both the mouse and human studies reported here provide strong evidence that AT1-AA–mediated TNF-α induction is an underlying mechanism for increased secretion of antiangiogenic factors and suggest that these signaling pathways contribute to impaired placental angiogenesis in preeclampsia and provide novel therapeutic targets for the disease (Figure 6).

Although the molecular basis for sEng induction in preeclampsia is poorly understood, hypoxia-inducible factor-1, transforming growth factor-β, and TNF-α are reportedly involved in regulating the endoglin gene expression and the release of sEng from the placenta. We have shown here that IgG harbored by women with preeclampsia induces TNF-α production via AT1 receptor activation, which in turn stimulates sEng production and its release into the maternal circulation from placenta but not kidney. These results indicate that AT1-AA is the causative factor responsible for sEng induction via TNF-α signaling and that the placenta is a major organ contributing to increased sEng secretion. Moreover, we provide compelling evidence that hemin, a well-known inducer of HO-1, attenuated AT1-AA–induced impaired human placental angiogenesis by blocking increased secretion of both sFlt-1 and sEng from human placenta villous explants. Thus, our studies provide the first evidence that HO-1 is a key intracellular molecule to control AT1-AA–induced dysregulated placental angiogenesis by inhibiting TNF-α–mediated sFlt-1 and sEng induction.

HO-1 is an inducible, endoplasmic reticulum–bound enzyme that catalyzes the nicotinamide adenine dinucleotide phosphate–cytochrome P450 reductase-dependent oxidation of heme to biliverdin in a 3-step process that liberates CO and Fe²⁺. Thus, our present findings are strongly supported by
Figure 5. AT1-AA–mediated TNF-α induction contributes to impaired placental angiogenesis by inducing both sEng and sFlt-1 secretion from human villous placental explants. Normal human placental villous explants were collected and treated with IgG from women with preeclampsia (PE) or normotensive pregnant women (NT) in the presence or absence of various reagents for 72 hours. A, At the end of treatment, human placental villous explants were collected, fixed, and stained with hematoxylin and eosin (H&E), anti-human Eng, and anti-human CD31 antibody. Scale bar=100 μm (H&E and CD31) or 200 μm (Eng). Syncytiotrophoblast (syn) is indicated by the arrowhead. B and C, Expression of endoglin (B) and CD31 (C) was quantified with Image-Pro Plus image analysis software. D and E, Cell culture supernatants were collected for sEng (D) and sFlt-1 (E) measurements by ELISA. Data are expressed as mean±SEM of >4 experiments performed in duplicate (IgG from 12 to 14 patients was obtained for each category). *P<0.001 vs villous explants treated with normotensive IgG; **P<0.05 vs villi treated with preeclamptic IgG.
Although there is general agreement that plasma levels of TNF-α are higher in women with preeclampsia, conflicting reports have appeared about the placental contribution to increased levels of TNF-α in women with preeclampsia. Two early reports provided evidence for increased TNF-α in placentas from women with preeclampsia. Two subsequent reports did not find any significant difference in TNF-α levels between placentas from women with preeclampsia and placenta from normotensive pregnant women. Because TNF-α is synthesized and released from the placenta, the amount of TNF-α remaining in placental tissue may not accurately reflect the amount of TNF-α produced and released by the placenta. The abundance of TNF-α mRNA will more likely reflect the potential for TNF-α protein production. Data presented here show that autoantibody-mediated induction of sEng occurs only in pregnant animals and that TNF-α is a critical signaling intermediate. We saw a significant increase in TNF-α mRNA in the placentas of pregnant mice injected with IgG from women with preeclampsia. The increase was prevented by coinjection with losartan or the 7-aa epitope peptide, indicating that the increase was due to AT₁-AA-mediated AT₁ receptor activation. Thus, our AT₁-AA-induced model of preeclampsia in mice and the data from human placenta villous explants suggest that the placenta is a major contributor to the resulting increase in circulating TNF-α.

**Conclusions**

The work reported here is the first to link AT₁-AA with the inflammatory system to regulate placenta-derived factors in both human and murine pregnancy and suggests that TNF-α is likely an important mediator of autoantibody-induced pathophysiology associated with preeclampsia resulting from increased production of sEng and sFlt-1. Therefore, the use of TNF-α-neutralizing antibodies, soluble forms of TNF-α receptors, or inducers of HO-1 to blunt the effects of elevated TNF-α may be useful in the treatment of preeclampsia.

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**Disclosures**

None.

**References**


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SUPPLEMENTAL MATERIAL

Expanded Materials and Methods

ELISA for sEng and TNF-α

sEng, sFlt1 and TNF-α levels in cell culture medium, mouse serum (TNF-α) and mouse plasma (sEng) were determined using ELISA kits (R&D Systems, Minneapolis, MN).

RNA isolation, semi-quantitative PCR and real-time PCR

RNA isolation, semi-quantitative PCR and real-time PCR were performed as described.1 TNF-α primer sequences and PCR conditions were as described; sense primer, 5’-TTTGCATAGCTTCAATAAGTTG, and antisense primer, 5’-CATGACAGTCTAAAGTGGTG GGAAC. Eng primer sequences: sense primer, 5’-CCCTGCTGTTTGTGTCATCTA and antisense 5’-GGGTGTCCTTGGAAGATGA. β-actin was used as an internal control and primer sequences were as described.1 TNF-α mRNA expression was represented by the ratio of TNF-α mRNA/β-actin mRNA.

Western Blot Analysis

Western blots were performed as described.1 Blots were incubated with polyclonal rabbit anti-mouse endoglin antibody (1:1000 dilution) or rabbit anti-β-actin antibody (1:1000 dilution) and
secondary anti-rabbit antibody (1:5000 dilution) for 1 hour each. The signals were visualized using ECL chemiluminescent western blotting detection.

**Eng, CD-31 staining and quantification**

Human placenta villous explants were fixed in 10% formalin overnight and then were paraffin embedded and cut at 4 μM thickness. Slides were stained using the standard method. Briefly, slides were stained with either mouse anti-human Eng (1:50 dilution, BD Pharmingen, Franklin Lakes, NJ) or mouse anti-human CD31 (DAKO) at 4°C for overnight. Signal was detected with anti-mouse IgG horseradish peroxidase kit (BD Pharmingen, Franklin Lakes, NJ). Counterstain is hematoxylin. For mouse tissue, rat anti-mouse CD31 antibody was incubated with the zinc-fixed mouse placental section overnight at room temperature with a 1:500 dilution and an anti-rat IgG horseradish peroxidase kit was used for detection. Counterstain is methyl green. N=10-14 patient’s IgG for each category. Quantification of the immunohistochemical staining was performed using the Image-Pro Plus software (Media Cybernetics, Bethesda, MD). The density of the brown staining (positive for CD-31) was measured. The average densities of ten areas per placenta were averaged and the SEM is indicated. Four placenta were chosen from each mice for staining and eight mice (n=8) were used for each group.

**Treatment of endothelial cells**

Human umbilical vein endothelial cells (HUVEC) cells (Cascade Biologics, Portland, Org) were cultured with EMC2 complete medium (Cambrex, Walkersville, MD) overnight. The next day,
cells were changed to serum free medium and treated with 100 nM Ang II, TNF-α (10 ng/ml), purified IgG (1:10 dilution) from normotensive or preeclamptic pregnant women and with serum (1:3 dilution) from mice injected with preeclamptic or normotensive IgG. Anti-mouse TNF-α antibody, Etanercept (Enbrel, 10 µg/ml, Amgen, Thousand Oaks, CA) and Hemin (5 µM, Sigma, St. Louis, MO) was added to cells at 2.5 µg/ml concentration for 30 minutes before the addition of serum from pregnant mouse injected preeclamptic IgG treatment. After 24 hours treatment, the cell culture media (conditioned cell culture media) were harvested and concentration of sEng was determined by ELISA as described above.

**Human placental villous explants treated with IgG purified from human serum**

Human placental tissue was obtained from women with normal term pregnancies delivered by elective cesarean section for breech presentation or a recurring indication in otherwise uncomplicated pregnancies, as described previously. Informed consent was obtained from the patients, and the study had the approval of the University of Texas-Houston Medical School Ethical Committee. Placenta villi were isolated, cultured and treated as described. Anti-human TNF-α antibody (2.5 µg/ml), Etanercept (Enbrel, 10 µg/ml) and Hemin (5 µM) was added to tissue culture for 30 minutes before treatment. After 72 hours treatment, the concentrations of sEng and sFlt-1 in cell culture media (conditioned cell cultured media) were determined by ELISA.

**Statistical analysis**
All data were expressed as the mean ± SEM. Statistical significance of the difference between the mean values of multiple groups was tested by one-way ANOVA, followed by Tukey-Kramer post-tests. Comparisons of multiple groups of plasma sEng levels of pregnant mice at different time points were assessed by two-way repeated-measures ANOVA, followed by Bonferroni post-tests. In addition, multiple factors and multiple groups comparisons of sEng/or Eng protein expression levels by western blot analysis and plasma sEng levels in non-pregnant mice were performed with two-way ANOVA and followed by Bonferroni post-tests. Data were analyzed for statistical significance using GraphPad Prism 4 software (GraphPad Software, San Diego, CA). A value of $P < 0.05$ was considered significant.

Supplemental References