Heparin Elevates Circulating Soluble fms-Like Tyrosine Kinase-1 Immunoreactivity in Pregnant Women Receiving Anticoagulation Therapy

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Background—Alterations in circulating levels of pro- and antiangiogenic factors have been associated with adverse pregnancy outcomes. Heparin is routinely administered to pregnant women, but without clear knowledge of its impact on these factors.

Methods and Results—We conducted a longitudinal study of 42 pregnant women. Twenty-one women received prophylactic heparin anticoagulation, and 21 healthy pregnant women served as controls. Compared with gestational age-matched controls, heparin treatment was associated with increased circulating levels of soluble fms-like tyrosine kinase-1 (sFlt-1) in the third trimester (P<0.05), in the absence of preeclampsia, placental abruption, or fetal growth restriction. Heparin had no effect on circulating levels of vascular endothelial growth factor, placenta growth factor, or soluble endoglin as assessed by ELISA. In vitro, low-molecular weight and unfractionated heparins stimulated sFlt-1 release from placental villous explants, in a dose- and time-dependent manner. This effect was not due to placental apoptosis, necrosis, alteration in protein secretion, or increased transcription. Western blot analysis demonstrated that heparin induced shedding of the N-terminus of Flt-1 both in vivo and in vitro as indicated by a predominant band of 100–112 kDa. By using an in vitro angiogenesis assay, we demonstrated that serum of heparin-treated cases inhibited both basal and vascular endothelial growth factor-induced capillary-like tube formation.

Conclusions—Heparin likely increases the maternal sFlt-1 through shedding of the extracellular domain of Flt-1 receptor. Our results imply that upregulation of circulating sFlt-1 immunoreactivity in pregnancy is not always associated with adverse outcomes, and that heparin’s protective effects, if any, cannot be explained by promotion of angiogenesis. (Circulation. 2011;124:2543-2553.)

Key Words: anticoagulation ■ angiogenesis ■ sFlt-1 ■ heparin ■ pregnancy

A successful pregnancy outcome requires the proper functioning of the molecular mechanisms responsible for regulating vascular endothelial homeostasis at both the systemic and local decidual/placental levels. Derangements of these mechanisms can lead to major pregnancy-related complications. Recent studies have focused on how alterations in placental trophoblast production of angiogenic proteins may help to explain the pathogenesis of preeclampsia, placental abruption, and stillbirth. A unifying theory is that excessive release of antiangiogenic factors, such as soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng) by hypoxic trophoblasts, antagonize proangiogenic factors such as vascular endothelial growth factor (VEGF) and placenta growth factor (PIGF). This phenomenon causes aberrant placental angiogenesis and vasculogenesis. The end result is an increasingly dysfunctional placenta with local and systemic vascular endothelial damage, activation of inflammatory pathways, and enhanced platelet turnover, which result in the aforementioned clinical manifestations.

Clinical Perspective on p 2553

Genetic (inherited thrombophilias) and acquired (antiphospholipid syndrome) thrombophilias carry the potential for causing vascular thromboembolism. Yet, the link between inherited thrombophilia and adverse pregnancy outcomes including miscarriage, abruption, severe preeclampsia, intrauterine growth restriction, or stillbirth remains controversial,
with prospective studies showing little or no association.\textsuperscript{11,12} Moreover, most studies have found no better outcome among patients with recurrent pregnancy loss treated with heparin compared with those left untreated.\textsuperscript{13–15} Heparin therapy has also not consistently been shown to improve pregnancy outcomes in patients with antiphospholipid syndrome with either high or low risk of preeclampsia.

Heparin reduces trophoblast invasion and modulates angiogenesis in vitro.\textsuperscript{16,17} A potential point of control is placental heparanase, an endogenous enzyme that cleaves heparin and heparan sulfate chains of proteoglycans and facilitates release of sFlt-1 and proangiogenic growth factors from the extracellular matrix or, as shown in vitro, from their tissue reservoirs.\textsuperscript{18,19}

In the current study, we investigated whether heparin altered the circulating profile of specific angiogenic factors (VEGF, PI GF, sFlt-1, and sEng) in women who required thromboprophylaxis during gestation. We present evidence that in vivo, during the third trimester, heparin was associated with increased maternal circulatory levels of sFlt-1 but not of other angiogenic factors. This effect was not due to placental apoptosis, necrosis, alterations in protein secretion, or increased sFlt-1 transcription, but seemed to be related to predominance of a 112-kDa sFlt-1 protein variant, which may be the result of shedding of the extracellular domain of Flt-1 receptor. Lastly, the angiogenic ability of serum from heparin-treated women was tested in vitro with the use of an endothelial tube formation assay. We demonstrated that the serum of heparin-treated cases with absent anticoagulant activity inhibited both basal and VEGF-induced capillary-like tube formation.

Methods

Selection of Cases and Controls

In a longitudinal study design, we analyzed serum samples retrieved across gestation from 42 pregnant women. Subjects were recruited in the high- and low-risk clinics at Yale-New Haven Hospital from December 2006 until May 2008. The Human Investigation Committee of Yale University approved the research protocol.

The study group consisted of 21 women in whom anticoagulation therapy was clinically indicated for: (1) personal history of maternal or prior adverse pregnancy outcome in the setting of a hereditary thrombophilia (Factor-V Leiden, n=8; prothrombin G20210A mutation, n=2; protein-C deficiency, n=1; angiotensin converting enzyme gene mutation, n=1); (2) antiphospholipid syndrome (n=4); (3) past history of maternal thromboembolic disease (deep venous thrombosis, n=3; sagittal sinus thrombosis, n=2; stroke, n=1).

Subjects were enrolled consecutively based on the availability of several investigators (V.A.R., M.J.P., C.S.B.). Of the targeted women, 90\% consented to participate. The diagnosis and indication for anticoagulant therapy was based on recognized clinical and laboratory criteria.\textsuperscript{20,21} Gestational age was established based on menstrual date and/or ultrasonographic examination prior to 20 weeks' gestation. Inclusion criteria for the heparin study group included: gestational age <14 weeks, absence of anticoagulant therapy at enrollment, and a plan for clinically indicated anticoagulant treatment during the current pregnancy. Exclusion criteria included history of transfusion, steroid and/or anticoagulant treatment prior to enrollment, known bleeding diathesis, bleeding within the past 3 months, associated medical conditions (ie, pregestational diabetes, chronic hypertension, thyroid disease), and renal or liver dysfunction.

Eligible women had their first blood sample drawn before anticoagulation, which consisted of enoxaparin (low molecular-weight heparin [LMWH]) in all but 1 case, in which treatment with unfractionated heparin (UFH) was initiated due to patient preference. Of the study group, 90\% (19 of 21) women had their second blood draw in the first trimester (gestational age \( \leq 13^{6/7} \) weeks), after initiation of therapy. Maternal blood samples were further retrieved during the second (gestational age \( 14^{1/7–27^{6/7} } \) weeks) and third (gestational age: \( 28^{1/7–39^{6/7} } \) weeks) trimester for all heparin-treated women.

Individualized risk assessment dictated that 80\% (17 of 21) of the patients required prophylactic doses of anticoagulation (Table 1). Per institutional protocol, patients who reached 35–36 weeks of gestation (16 of 21) were switched from LMWH to UFH. Patients with antiphospholipid syndrome (n=4) also received aspirin (81 mg/d). Following enrollment, all heparin-treated women were followed by

<table>
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<th>Heparin Group (n=21)</th>
<th>Control Group (n=21)</th>
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prospectively until delivery. Their clinical management was independent of our study protocol.

Twenty-one healthy pregnant women enrolled contemporaneously in the first trimester served as controls. Similar to the heparin-treated group, blood samples were retrieved serially from each patient during the first, second, and third trimesters. Inclusion criteria for the control women included uncomplicated pregnancies and delivery of a healthy newborn at term. Exclusion criteria were similar to those described above.

Blood Collection and Assessment of the Anti-Factor Xa Activity
Maternal blood samples were retrieved by venipuncture. Plasma was collected on sodium citrate. Serum and plasma samples were spun at 3000 g at 4°C for 20 minutes, the supernatant aliquoted and immediately stored at -80°C. Plasma specimens were assayed for heparin activity utilizing a commercially available chromogenic assay intended for the quantitative determination of LMWH and UFH levels by measurement of Factor-Xa inhibition (ANTICHROME; American Diagnostica, Stamford, CT) (Data Supplement).

sFlt-1, sEng VEGF, PlGF, and Heparanase Immunoassays
Biochemical analyses of human serum, plasma, and placental explant conditioned media were conducted by investigators blinded to pregnancy outcome and the in vivo or in vitro treatments. ELISA assays for human unbound serum sFlt-1, sEng, VEGF, PlGF (R&D Systems, Minneapolis, MN), and plasma heparanase (Cedarlane Labs, Burlington, NC) were performed according to manufacturer instructions. Details regarding technical aspects of the immunoassay procedures are provided in the Methods section of the online-only Data Supplement.

Placental Villous Explant Cultures
Placentas (n=7) were obtained from healthy term women without any significant medical history undergoing scheduled elective cesarean delivery in the absence of labor (gestational age, median [IQR] 39 [39–40] weeks). Delivery indications included elective repeat cesarean section and fetal breech presentation. All patients had infants appropriately grown for gestational age and normal fetal heart rate patterns prior to surgery.

Placental cotyledons were removed randomly from the central portion of the placental disk. Villi were incubated for 1.5, 3, 6, 18, and 24 hours with either UFH (0.5, 5, 50 IU/mL) or LMWH (0.5, 5, 50 IU/mL). The 5-IU/mL dose was chosen to mimic anticoagulant levels reached in vivo. The other doses were incorporated to allow performance of a dose-response curve analysis. In additional experiments, monensin (20 μmol/L, protein secretion blocker at the level of Golgi membrane) or amiloride (2 μmol/L, cellular Na/H+ pump blocker) was added to the tissues incubated with either UFH or LMWH (5 IU/mL). Control wells received an equivalent volume of water (diluent). The analytes’ concentrations in the explant medium were normalized to total protein in tissue extract. Tissue viability was assessed through the release of lactate dehydrogenase (LDH) into the incubation medium (LDH Liqui-UV® Assay; Stanbio, Boerne, TX). All drugs and chemicals were from Sigma-Aldrich unless otherwise specified.

Evaluation of Heparin’s Apoptotic Activity
Villous trophoblast explants were exposed to UFH and LMWH (0.5, 5, and 50 IU/mL) for 1.5 and 3 hours. Following tissue homogenization and DNA extraction, the apoptotic activity of heparin was evaluated as described in the Methods section of the Data Supplement.

Western Blotting
Molecular variants of sFlt-1 are thought to result through differential alternative splicing23 and shedding of the ectodomain of the Flt-1 receptor.23 To determine whether differences in sFlt-1 variants are associated with heparin treatment and could account for the observed changes in sFlt-1 immunoreactivity as determined by ELISA, we employed Western blotting of human serum (heparin-treated, n=8; control, n=8; samples matched for gestational age, 2 μL/lane) and placental explant conditioned medium (0.5 and 5 μg medium protein/lane). Two sFlt-1 primary antibodies were used: (1) the Sigma-Aldrich mouse monoclonal anti-Flt-1 (1:500 dilution, Clone Flt-11, Sigma-Aldrich is similar to the ELISA antibody and recognizes an N-terminus epitope present in both Flt-1 [Uniprot#: P17948-1] and alternatively spliced sFlt-1 [Uniprot#: P17948-2]; (2) the Zymed rabbit polyclonal antiFlt-1 (1:500 dilution, Zymed, InVitrogen, Carlsbad, CA) recognizes a C-terminus epitope unique to alternatively spliced sFlt-1. In Western blots, the Zymed antibody identifies a band at 130 kDa as well as few bands of lower molecular weight (http://tools.invitrogen.com). Details regarding technical aspects of the Western blotting procedure are provided in the Methods section of the Data Supplement.

Quantitative RealTime RT-PCR Procedures and Primer Sequences
Villous trophoblast tissues exposed to 0.5, 5, and 50 IU/mL UFH and LMWH were snap frozen in liquid nitrogen after 3 or 18 hours of incubation. RNA samples were treated with heparinase (Sigma-Aldrich H2519: Heparinase I from Flavobacterium heparinum) as previously recommended to remove heparins’ interference with PCR reactions.24 The following primers were used: sFlt-1 forward primer 5’-TTTTTGTTGCAGTGCTCACCT-3’; reverse primer 5’-TTTTGTCTGAGTCTACAAG-3’ (366 bp). This primer pair amplifies soluble, alternatively spliced variants of sFlt-1 (sFlt-1: NM 00159920.1 and U01134) but not the transmembrane form of Flt-1 (Flt-1: NM 002019) (for details see the Methods section of the online-only Data Supplement).

In Vitro Angiogenesis
The tube formation assay, a surrogate marker of angiogenesis, was performed as previously described.23 Technical details are presented in the Methods section of the online-only Data Supplement. To exclude any direct interference of heparin with angiogenesis, we selected serum samples only from third-trimester heparin-treated women who had elevated sFlt-1 levels (mean [SD]: 19 141 [4203] pg/mL, versus controls: 4353 [1127] pg/mL; P<0.001, n=5/group) but no detectable anti-Factor Xa activity. This objective was reached at the point at which the patients were switched from LMWH to UFH per clinical protocol.

Statistical Analysis
Distribution of data was tested for using the Shapiro-Wilk normality test. A 2-step clustering method complemented by receiver operating curve analysis allowed us to identify an unbiased gestational age separation point that partitioned the levels of sFlt-1 into 2 clusters (low versus high).24 A sample size calculation was performed based on the differences in circulating angiogenic factors in women with preeclampsia.21 It was estimated that 21 patients per group would be necessary to detect differences equal to the standard deviation observed for blood sFlt-1 concentrations with 80% power and a confidence coefficient of 95%. P<0.05 was considered to indicate statistically significant difference. Further details are provided in the Methods section of the online-only Data Supplement.

Results
Patients and Outcome Characteristics
The clinical and outcome characteristics of the study participants are presented in Table 1. Heparin-treated women delivered at term (albeit at earlier gestational age) and their neonates had lower birth weights compared with controls. Four women receiving anticoagulation therapy delivered preterm in the setting of either spontaneous preterm birth (n=3) or because of a nonreassuring fetal heart rate status (n=1). No patients developed preeclampsia or placental abruption or delivered a growth-restricted fetus.

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Villous trophoblast explants were exposed to UFH and LMWH (0.5, 5, and 50 IU/mL) for 1.5 and 3 hours. Following tissue homogenization and DNA extraction, the apoptotic activity of heparin was evaluated as described in the Methods section of the Data Supplement.

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Anticoagulation Therapy

Figure 1A demonstrates that women receiving anticoagulation therapy had significantly higher plasma anti-Factor Xa activity across gestation (2-way ANOVA: heparin treatment, \( P<0.001 \); trimester, \( P=0.018 \)). The highest level of anticoagulation was reached during the first trimester, with no differences between second and third trimesters.

Levels of Angiogenic Factors and Heparanase by ELISA

The systemic circulating levels of sFlt-1, sEng, VEGF, PI GF, and heparanase for women receiving anticoagulant therapy and their controls are presented in Table 2. During normal gestation, the levels of sFlt-1 increased significantly with advancing gestational age, reaching the highest level during the third trimester (second versus third trimester, \( P=0.003 \)). However, in women receiving anticoagulation therapy, heparin was associated with maternal sFlt-1 elevated above its expected level for gestational age (\( P=0.038 \)). In physiological pregnancy, circulating sEng but not VEGF or heparanase increased with gestational age, with the highest levels reached during the third trimester (sEng second versus third trimester, \( P<0.001 \)). Levels of PI GF increased during the first part of gestation and remained unchanged thereafter (PI GF first versus second trimester, \( P=0.008 \); and second versus third trimester, \( P=0.245 \)). Heparin treatment was not associated with alterations in the circulating levels of sEng, VEGF, PI GF, sFlt-1/PI GF ratio, or heparanase (\( P>0.05 \) for all).

Nonlinear modeling determined that the best-fitting curve for the relationship between gestational age and sFlt-1 was an exponential equation for both groups (Figure 1B, control: \( r=0.835, P<0.001 \); heparin: \( r=0.796, P<0.001 \)). The rate of increase in maternal serum concentration of sFlt-1 was significantly higher in the heparin-treated compared with the control group (\( F \text{ statistic} = 29.4, P<0.001 \)). Next, by using the 2-step clustering method, we found that in normal pregnancy, 37 weeks represented the third trimester inflection point, at which sFlt-1 changed from a relatively low to a high concentration (Figure 1C). This trend was significantly amplified in heparin-treated women. In multiple stepwise regression analysis, circulating sFlt-1 levels were dependent on the combination of sEng (\( r=0.654, 95\% \) confidence interval, 0.437–0.799, \( P<0.001 \)), gestational age (\( r=0.564, 95\% \) confidence interval, 0.314–0.740, \( P<0.001 \)), and heparanase (\( r=0.424, 95\% \) confidence interval, 0.138–0.644, \( P<0.001 \)) as independent variables. Excluded from the model were anti-Factor Xa activity and PI GF.

Heparin and Release of sFlt-1 In Vitro

Next, we aimed to determine if heparin has similar effects in vitro. In placental explants, UFH upregulated the release of sFlt-1 in a dose- and time-dependent manner (Figure 2A, 2-way ANOVA: dose: \( P=0.001 \), time: \( P<0.001 \), interaction \( P<0.001 \), with maximal effect at 18 hours. There was no significant difference between sFlt-1 concentration at 18 versus 24 hours (\( P>0.05 \)). The stimulatory effect of 5- and 50-IU/mL UFH doses, but not a 0.5-IU/mL dose, was significant even at 1.5 hours (Figure 2B, 1-way ANOVA, \( P=0.016 \)). Similar results were obtained with LMWH (Figure 2C, dose: \( P=0.004 \), time: \( P<0.001 \), interaction \( P<0.001 \)). Like UFH, the LMWH doses of 5 and 50 IU/mL were also able to elevate sFlt-1 immunoreactivity at 1.5 hours (Figure 2D, \( P=0.005 \)).

We then compared the ability of the 2 heparin formulations to upregulate sFlt-1 concentration at the time of maximal stimulation (18 hours). LMWH appeared more effective than UFH for all doses (Figure 3A, heparin type: \( P=0.031 \), dose: \( P<0.001 \), interaction \( P=0.008 \)). Neither UFH nor LMWH altered the explant medium levels of PI GF, VEGF, or sEng (\( P>0.05 \) for all).

Because endogenously expressed placental heparanase has the potential to inactivate heparin, we measured the residual heparin concentration, via antifactor Xa activity, in the explants at 18 hours (peak stimulation point) (Figure 3B). No detectable heparin activity was found in explants treated with 0.5 and 5 IU/mL UFH, suggesting full metabolism of UFH by 18 hours. Explants exposed to 50 IU/mL UFH had \( \approx 36 \) IU/mL (72%) remaining activity. Explants treated with 0.5, 5, and 50 IU/mL LMWH had a remaining activity of 0.08.
Results may explain the heightened effectiveness of LMWH in raising sFlt-1 levels when compared with UFH. In addition, it supports the biological relevance of a 5-IU/mL LMWH dose, because the residual heparin level at the end of the 18-hour incubation (0.5-IU/mL dose) fell within the range of heparin plasma concentration for LMWH-treated women, as shown in Figure 1A.

**Effect of Heparin on the Placental mRNA Expression of sFlt-1**

Villous trophoblast sFlt-1 expression was investigated in vitro following incubation of the tissues with heparin (all 3 doses) for 3 and 18 hours. Neither UFH nor LMWH changed sFlt-1 mRNA expression levels (heparin type: $P=0.928$; dose: $P=0.595$; interaction: $P=0.452$), suggesting that a transcriptional mechanism is not responsible for the elevation of sFlt-1 protein immunoreactivity.

**Effect of Monensin and Amiloride on Basal and Heparin-Induced Release of sFlt-1 From Placental Explants**

Placental explant basal and heparin-induced sFlt-1 production remained unaffected by either monensin (UFH: $P=0.991$; LMWH: $P=0.855$) or amiloride (UFH: $P=0.954$; LMWH: $P=0.284$). Therefore, disruption of the Golgi-mediated protein secretion and alteration of the intracellular pH through blockade of Na/H+ cellular pump were excluded as possible mechanisms.

**Effect of Heparin on Cell Death**

Treatment of the tissue with heparin did not alter the DNA fragmentation pattern compared with untreated controls (Figure 4A). Moreover, there was no difference in the cytoplasmic levels of nucleosomes measured in either tissue lysate or incubation medium (not shown). Neither heparin treatment nor the dose modified LDH activity in the explant media.
Figure 2. A, Time course of sFlt-1 immunoreactivity as measured by ELISA in the conditioned medium of placental explants exposed to unfractionated heparin (UFH 0.5, 5, and 50 IU/mL). B, sFlt-1 immunoreactivity measured at 1.5 hours of incubation in the absence of, or in the presence of, increasing concentrations of UFH. C, Time course of sFlt-1 immunoreactivity in the conditioned medium of placental explants exposed to low molecular-weight heparin (LMWH 0.5, 5, and 50 IU/mL). D, sFlt-1 immunoreactivity measured at 1.5 hours of incubation in the absence of, or in the presence of, increasing concentrations of LMWH. Error bars depict SEM (n=3 placental explants). *Means are significantly different (P<0.05) from control (CRL) wells. Results analyzed by 2-way repeated-measures ANOVA (A and C) or 1-way ANOVA (B and D).

Effect of Heparin on Flt-1 and sFlt-1 Immunoreactivity by Western Blotting

Figure 5A demonstrates the Western blot antigenic recognition pattern of the Sigma-Aldrich antibody (specific for an

(heparin type: P=0.277, dose: 0.597, interaction: P=0.856, Figure 4B). This suggests that heparin did not preferentially disrupt the cellular membrane, eliminating passive leakage as the mechanism responsible for release of sFlt-1.
N-terminus epitope on the extracellular domain of Flt-1) on serum samples of heparin-treated women (gestational age ≥37 weeks) and gestational age-matched controls. At least 3 immunoreactive bands (145, 112, and 100 kDa) were noted in serum of heparin-treated women. Additional bands were detected at 85 and 35 kDa, but these did not appear to change with heparin treatment. The 100- and 112-kDa bands were most conspicuous, and their precise separation on gels was not possible. The 100- to 112-kDa band complex was significantly increased in heparin-treated women compared with controls (P<0.001, Figure 5B). The Zymed antibody (specific for the C-terminus of alternatively spliced sFlt-1) detected most noticeably a band at 130 kDa (Figure 5C), which did not change consistently with heparin treatment (Figure 5D). The Zymed antibody also recognized additional bands at 50 and 36 kDa, but these were also unaffected by heparin. A significant correlation was seen between sFlt-1 immunoreactivity by ELISA and optical density of the Sigma-Aldrich antibody 100- to 112-kDa band complex (r=0.914, P<0.001), but there was no correlation with Zymed antibody’s 130-kDa band (r=0.019, P=0.945).

Next, we sought to investigate whether similar sFlt-1 isoform variants could be identified in vitro. As shown in Figure 6A through 6C, in conditioned medium of placental explants, the Sigma-Aldrich antibody primarily recognized 2 bands at 100–112 kDa. However, when more protein was

Figure 4. A, Representative DNA ladder in placental explants incubated for 3 hours with either 5 or 50 IU/mL unfractionated (UFH, lanes 3 and 4) or low molecular-weight heparins (LMWH, lanes 6 and 7). Control tissues (CRL, lanes 2 and 5) received an equivalent volume of water (diluent). Lane 8 (T0) was loaded with DNA extracted from the same placental tissue that had not been incubated ex vivo. B, Lactate dehydrogenase (LDH) activity in placental explant medium normalized for total protein in incubated tissue with UFH (0.5, 5, and 50 IU/mL) or LMWH (0.5, 5, and 50 IU/mL). Error bars depict SEM (n=7 placental explants). Results analyzed by 2-way repeated-measures ANOVA.

Figure 5. Representative Western-blot gels (SDS-PAGE, 4–15%, reducing conditions) of serum samples (2 μL/lane) from women treated with heparin (HEP: lanes 3–5) and controls (CRL: lanes 1, 2, and 6). Primary antibody (ab.) was omitted on lanes 5 and 6 (negative). A, Western blotting performed with the anti-Flt-1 Sigma-Aldrich ab. B, Densitometric analysis of the 100- to 112-kDa complex from serum samples of HEP-treated women (n=8) and CRL (n=8). All women were term (>37 weeks). *P<0.001 versus CRL (Student t test). C, Western blotting performed with the polyclonal anti-sFlt-1 Zymed ab. D, Densitometric analysis of the 130-kDa sFlt-1 isoform observed in the serum samples of the same HEP-treated women (n=8) and CRL (n=8) presented in A and B. Error bars depict SEM.
loaded (5 μg/lane), the same antibody recognized additional lower molecular-weight bands (85, 67, 57, 47 kDa) suggestive of nonrandom cleavage of the extracellular domain of Flt-1. Quantification of the 100- to 112-kDa band complex demonstrated upregulation in a heparin-type and dose-dependent fashion that confirmed the ELISA pattern (P<0.017). As for the Zymed antibody, when 0.5 μg of protein/lane was loaded, the antibody did not recognize any immunoreactive sFlt-1 bands (Figure 6D). Yet, at 5 μg of protein/lane, the Zymed antibody detected several additional peptides of a lower than expected molecular weight (Figure 6E). Bands at 85 and 50 kDa were most noticeable, and no band was detected at 130 kDa. Densitometric analysis demonstrated that heparin altered the immunoreactivity of the 85-kDa band (ANOVA P=0.003), but not that of the 50-kDa band (P>0.05) (Figure 6F). The dose of 5 IU/mL UFH increased the intensity of the 85-kDa band (P=0.015 versus control), whereas the dose of 50 IU/mL UFH decreased its level (50 versus 5 IU/mL UFH, P<0.001). A similar pattern was observed with the LMWH, for which the 50-IU/mL dose rendered the 50-kDa band almost undetectable (P<0.001 versus CRL and P=0.003 versus 5 IU/mL LMWH).

Effect of Serum From Heparin-Treated Women on Angiogenesis

In Figure 7A, we show representative images of capillary tube formation, which indicate that sera of heparin-treated women have antiangiogenic activity both under basal conditons.

Figure 6. Western blot analysis of conditioned medium from placental explants treated in vitro for 18 hours with either unfractionated heparin (UFH: 5 mg/mL, lanes 5 and 6 or 50 mg/mL, lanes 3 and 4) or low molecular-weight heparin (LMWH: 5 mg/mL, lanes 9 and 10 or 50 mg/mL, lanes 7 and 8). Lanes 1 and 2 are from control (CRL) wells. The four gels were run using conditional medium retrieved from the same explant and were electrophoresed and blotted under different conditions. Electrophoresis was performed under reducing conditions on either 4–15% (A and D) or 10% SDS-PAGE gels (B and E). Lanes on each gel were loaded with equal amounts of total protein of either 0.5 μg/lane (A and D) or 5 μg/lane (B and E). Western blotting was performed with either the Sigma-Aldrich anti-Flt-1 antibody (ab.) (A and B) or the Zymed anti-alternatively spliced sFlt-1 ab. (D and E). Primary abs. were omitted from lanes 11 and 12 (B and E). C, Densitometric analysis of the 100- to 112-kDa bands detected by the Sigma-Aldrich antibody. F, Densitometric analysis of the 50- and 85-kDa bands detected by the Zymed antibody. Data represented as fold changes from the average of the CRL wells calculated for each explant. Error bars depict SEM (n=4 placental explants). Means marked with different symbols are statistically different from their CRLs and from each other (P<0.05). Results analyzed by 2-way repeated-measures ANOVA.

Figure 7. The angiogenic potential of serum from women who delivered at term (gestational age ≥37 weeks) and received heparin (HEP) throughout gestation versus controls (CRL). A, Representative micrographs of HUVECs exposed to serum of a HEP-treated woman and a gestation age-matched CRL under basal and VEGF-stimulated conditions. B, Average tube length from n=5 independent observations for each group. Error bars depict SEM. *P<0.001 for HEP versus CRL. †P<0.001 for VEGF-stimulated versus basal angiogenesis. Results analyzed by 2-way ANOVA.
tions and in the presence of additional exogenous VEGF. This inhibition was statistically significant, as shown in Figure 7B (P<0.001, for both).

**Discussion**

Two molecular events are proposed as responsible for sFlt-1 biosynthesis: alternative splicing and proteolytic cleavage of Flt-1’s ectodomain.28,29 The Flt-1 gene codes for a large premRNA transcript, which is translated into an \( \approx 150 \)-kDa protein.30 This represents the full length of the Flt-1 receptor consisting of an N-terminal extracellular ligand-binding region, a transmembrane region, and a C-terminal intracellular region that carries the tyrosine-kinase domain.31 Posttranslational glycosylation processes result in a \( \approx 185 \)-kDa or higher molecular-weight membrane-bound proteins. As previously outlined, the Sigma-Aldrich anti-Flt-1 antibody recognizes an N-terminus epitope present in the full-length Flt-1 receptor. Further studies should clarify if some of the high molecular-weight bands on Western blots could be the result of posttranslational processing of the full length Flt-1 receptor.

The sFlt-1 molecule (\( \approx 80 \) kDa) can be generated by alternative splicing of the same premRNA that encodes Flt-1.28 The alternatively spliced sFlt-1 retains the first 6 alternatively spliced premRNA transcript, which is translated into an \( \approx 150 \)-kDa protein. This represents the full length of the Flt-1 receptor posttranslational processing of the full length Flt-1 receptor. However, the Sigma-Aldrich antibody recognizes an N-terminus epitope present in the full-length Flt-1 receptor. Further studies should clarify if some of the high molecular-weight bands on Western blots could be the result of posttranslational processing of the full length Flt-1 receptor.

The mechanism by which heparin and its natural inhibitor, heparanase, regulate angiogenesis remains incompletely understood.18 Our comments regarding the possible role of heparanase in modulating the circulatory levels of sFlt-1 in women receiving anticoagulation therapy is presented in the Discussion section of the online-only Data Supplement.

Polyproteolytic cleavage and ectodomain shedding of Flt-1 receptor could be responsible for the observed upregulation in the maternal systemic levels of sFlt-1.29 In support of a nongenomic mechanism, in vitro, heparin had the ability to rapidly elevate sFlt-1. We observed a significant stimulatory effect for both heparins at 1.5 hours. Data presented by Denizot et al suggest that a similar phenomenon occurs in vivo.20 Kinetic experiments showed that circulating sFlt-1 peaked within the first hour after the beginning of cardiopulmonary bypass surgery, a procedure that requires full anticoagulation therapy.21 Using an antibody that recognizes an epitope within the first 251 amino acids of the extracellular domain of Flt-1 (Sigma-Aldrich), we found by Western blotting that 2 sFlt-1 variants (\( \approx 100 \) and 112 kDa) were upregulated by heparin both in vivo and in vitro. With the aid of an antibody with similar properties, Rahimi et al noted that Flt-1 of leukemic cells undergoes ligand-induced proteolytic fragmentation.23 Similar to our findings, a mixture of sFlt-1 variants was identified including sFlt-1 proteins with molecular weight of \( \approx 100 \) kDa.

In vitro, the Sigma-Aldrich antibody recognized several specific sFlt-1 isoforms (85, 67, 57, 47 kDa). Although heparin seems to preferentially target the 100- to 112-kDa complex, our data suggest that fragmentation of the extracellular domain of Flt-1 may occur at several nonrandom cleavage sites. For now, we can only propose that the 112-kDa sFlt-1 variant is the result of a posttranslational processing of the 100-kDa fragment, such as acetylation or oxidation. How and why heparin upregulates release of the 100- to 112-kDa sFlt-1 complex remains unknown. Pre-eclamptic women also display upregulation of the 100-kDa sFlt-1 variant.27 It is possible that heparin promotes cleavage of the ectodomain of the Flt-1 receptor at specific sites by activation of a variety of “sheddase” enzymes (ie, ADAM,
tumor necrosis factor-α—converting enzyme). Biological factors that are relevant in preeclampsia may act via similar mechanisms. It is also conceivable that heparin may not instigate the cleavage process. The fourth immunoglobulin-like loop in the ectodomain of Flt-1 includes a major heparin-binding site. If the 100- to 112-kDa complex retains this binding site, heparin may simply trap and enrich serum of anticoagulated women with this specific 100- to 112-kDa sFlt-1 variant even though the cleavage mechanisms per se are not hyperactivated.

In vitro, serum of heparin-treated women inhibited both basal and VEGF-induced capillary-like tube formation. If this antiangiogenic activity is due to sFlt-1, our results imply that circulating sFlt-1 of heparin-treated women likely retains its antiangiogenic properties. In a recent study, Sobel et al showed that in vitro heparin promoted angiogenesis. In our study, direct interference of heparin was excluded because none of the tested samples had detectable anti-Factor Xa activity. Equally intriguing is that in vivo heparin augmented the circulatory concentrations of sFlt-1 to levels similar to those reached in preeclamptic women. Yet, none of the women receiving anticoagulation developed preeclampsia. It is known that heparin regulates VEGF binding to its receptors in a receptor type- and dose-dependent manner. In vitro, at lower concentrations, heparin facilitates HUVEC proliferation by promoting VEGF-receptor interactions. If this phenomenon is functionally active in vivo, prophylactic doses of heparin may paradoxically promote angiogenesis by facilitating the interaction of VEGF and its receptors in a competitive fashion with sFlt-1. Lastly, sFlt-1 contains a heparin-binding domain. Thus, in certain patients, heparin may render a protective effect against pregnancy-specific complications, such as preeclampsia, through its direct interaction with sFlt-1.

The functional relevance of our results needs further evaluation. Clinical evidence indicates that patients with cancer treated with heparin survived longer, and data from various animal models demonstrate the ability of heparin to attenuate metastasis. In cancer research, heparin is viewed as an inhibitor of angiogenesis and of the protumorigenic heparanase. Additionally, increased systemic levels of sFlt-1 were associated with improved ventricular function of patients with myocardial infarction. Hence, during pregnancy, heparin may augment the maternal systemic levels of sFlt-1 to actually protect against abortion, fetal growth restriction, and stillbirth through mechanisms that remain to be further investigated.

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**Disclosures**

None. Contribution to Authorship: V.A.R., I.A.B., A.A., C.J.L., and C.S.B. formulated the hypothesis, designed the study, analyzed and interpreted the data and drafted the manuscript. M.J.P., V.A.R., C.J.L., and C.S.B. identified, recruited, and, together with A.T.D. and S.S.A., collected the biological specimens and followed the patients prospectively to the point of delivery. A.T.D., S.S.A., V.A.R., and I.A.B. performed the placental explant experiments. G.Z., W.R., C.S.B., and I.A.B. conducted and analyzed the ELISA assays, Western blot, and PCR experiments. S.A. and A.A. performed the endothelial tube formation assays at University of Edinburgh, and analyzed the data derived from these experiments. All the coauthors participated with aspects of study design and critical interpretation of the data, contributed to writing of the article, and reviewed and approved the final version.

**References**

In pregnancy, disarray of highly coordinated hemostatic processes at the maternal-fetal interface may lead to fetal growth restriction, preeclampsia, placental abruption, and/or fetal death. Although the link between inherited thrombophilia and these adverse pregnancy outcomes remains controversial, heparin, a recognized modulator of angiogenesis, is commonly used as prophylaxis for maternal thrombosis and as therapy for antiphospholipid antibody syndrome to prevent these complications. Herein, we investigated whether heparin altered the maternal circulatory profile of pro- and antiangiogenic factors (VEGF, PlGF, sFlt-1, and soluble endoglin) in women who received anticoagulation during pregnancy. We found that heparin upregulated maternal circulating sFlt-1 levels in the absence of preeclampsia, fetal growth restriction, or abortion. Our in vitro data showed that placental apoptosis, necrosis, increased transcription, or alterations in protein secretion could not explain these results. Western blotting confirmed that heparin induced shedding of the N-terminus of Flt-1 receptor. Using an angiogenesis assay, we noted that serum of heparin-treated patients inhibited VEGF-induced capillary-like tube formation. These findings imply that, in the setting of heparin treatment, increased sFlt-1 immunoactivity concurs with enhanced antiangiogenic potential. This study provides new insights into the mechanism responsible for the rise in circulating sFlt-1 associated with heparin treatment that may lead to improved therapeutic strategies aimed to prevent adverse pregnancy outcomes.
Heparin Elevates Circulating Soluble fms-Like Tyrosine Kinase-1 Immunoreactivity in Pregnant Women Receiving Anticoagulation Therapy
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to
Heparin Elevates Circulating Soluble Fms-Like Tyrosine Kinase-1 Immunoreactivity in Pregnant Women Receiving Anticoagulation Therapy

Supplemental Methods

1. Assessment of the human plasma and placental explant medium anti-Factor Xa activity.

Anti-Factor Xa activity was measured in citrated plasma from the blood used to measure levels of placental angiogenic factors. Heparin standards were prepared from pooled normal plasma spiked with known low molecular weight heparin (LMWH) concentrations (Sigma-Aldrich, St Louis, MO). Heparin levels in all research samples were then interpreted as chromogenic levels read at 405 nm against a prepared blank. This assay was also used to indirectly measure the residual heparin in placental explants following incubation with either LMWH or with unfractionated heparin (UFH), as previously described.1

2. Immunoassays procedures for sFlt-1, sEng, VEGF, PlGF and heparanase. Samples were assayed in duplicate in a 96-well plate pre-coated with a capture antibody directed against free sFlt-1, sEng, VEGF, PlGF or heparanase. Incubation protocols were performed followed by washings and reading at 450 nm in accordance with the procedure summary. The inter-assay and intra-assay coefficients of variation varied from 3 to 10%. Plates were read at 450 nm with 570 nm wavelength correction. The Softmax Pro 3.1.1 software (Molecular Devices, Sunnyvale, CA) reports a positive value if the optical density of the sample wells is above that of the zero standard (blank wells). If the optical density of a sample well is below that of the zero standard, a negative value is reported and automatically converted to zero. VEGF was the only analyte that
had instances of undetectable levels (values lower than zero standards) in any of the assays. Preliminary spike and recovery experiments validated the use of sFlt-1, PlGF and VEGF assays in biological matrices (serum and explant medium) containing UFH and LMWH and found no significant interference.

3. **Western blotting.** Samples were mixed 1:2 with reducing sample buffer (Bio-Rad), boiled and applied to SDS-PAGE gels. After electrophoretic transfer, polyvinylidene fluoride (PVDF) membranes were blocked with 5% BSA and then incubated overnight at 4°C with two primary antibodies (“Sigma” mouse monoclonal anti-Flt-1; “Zymed” rabbit polyclonal anti-sFlt-1). Detection was performed using appropriate biotinylated secondary antibodies (1:5,000, Jackson Immunoresearch, West Grove, PA) followed by streptavidin-linked horseradish peroxidise, (1:8000, Amersham Biosystems, Piscataway, NJ) and chemiluminescence (ECL-Plus, Amersham). Optical density of the bands of interest was analyzed with Image J software (NIH, http://rsb.info.nih.gov).

4. **Quantitative real time RT-PCR procedures and primer sequences.** Total RNA was isolated using Trizol Reagent (Sigma-Aldrich, St Louis, MO). Reverse transcription was carried out with avian myeloblastosis virus reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) using oligo (deoxythymidine) primers to synthesize first strand complementary DNA (cDNA). sFlt-1 primers were designed using the Light Cycler Probe Design Software (Roche, Indianapolis, IN), synthesized and gel-purified at the Yale DNA Synthesis Laboratory (Critical Technologies, New Haven, CT): *sFlt-1* forward primer 5’–TGGGACTGTGGGAAGAAAC–3’; reverse primer 5’–TTTTTGTGCTGCTACCT–3’ (366 bp). This primer pair amplifies soluble, alternatively spliced variants of sFlt-1 (*sFlt-1*: NM_00159920.1 and U01134) but not the transmembrane form of Flt-1 (*Flt-1*: NM_002019). sFlt-1 mRNA levels were normalized to
18S rRNA amplified using the following primers 5’–GATATGCTCATGTGGTGTTG–3’; reverse primer 5’–AATCTTCTTCAGTCGCTCCA–3’ (236 bp). cDNA (500 ng) was amplified using the FastStart Taq DNA Polymerase master mix (Roche, Indianapolis, IN) in the presence of the specific primer pair for each target using the LightCycler® 2.0 System (Roche). To verify that the correct targets were amplified, PCR products were run on an agarose gel and bands of interest cut out and sequenced to confirm their identity.

5. Placental villous explant culture. Placental cotyledons from the central portion of the placenta were removed randomly from the placental disk. Villi were dissected free and then washed thoroughly to remove the blood, all within 30 minutes of delivery. Equal amounts of tissue (~100 mg wet weight) were suspended into each well of a 24-well plate containing 1.5 mL RPMI (1640 medium Gibco, Grand Island, NY), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). Cultures were maintained at 37°C in a humidified gas mixture of 5% CO2-95% air. Following incubation, with UFN, LMWH, monensin and amiloride the supernatants were collected, centrifuged to remove cellular debris and stored at –80°C until the investigated analytes were measured by ELISA. Duplicate experiments were conducted for each condition. The incubated tissue was blotted on filter paper and homogenized in 1-mL extraction buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton X-100 and complete protease inhibitor cocktail (Roche, Indianapolis, IN). Homogenates were spun at 1,000g at 4°C for 15 min. and total protein of the incubated tissue was quantified using Bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL). The concentration of the analytes in the explant medium was normalized to total protein in tissue extract, to correct for variations in amount of tissue incubated per well. For each experimental condition, values were derived by averaging normalized values from duplicate wells either without (untreated controls) or with the
various heparin treatments. Tissue viability during in vitro incubations, was assessed through the release of lactate dehydrogenase (LDH) into the incubation medium (LDH Liqui-UV® Assay; Stanbio, Boerne, TX). All drugs and chemicals were from Sigma Chemical Co. (St Louis, MO) unless otherwise specified.

6. Evaluation of placental apoptotic activity. Tissues were homogenized in 70% ethanol. Following extraction the DNA purity and yield were determined by spectrophotometry at 260 and 280 nm. After addition of 6 µl of loading buffer [2.5 g/L Bromophenol Blue and 30% (v/v) glycerol in water], samples were loaded on a 2.2% (w/v) agarose gel in 0.5× TBE (45 mmol/L Tris base, 45 mmol/L borate, 1 mmol/L EDTA) containing 300 µg/L ethidium bromide. Agarose-gel electrophoresis was performed at 10 V/cm for 2.5 h. The ethidium bromide-stained characteristic DNA laddering patterns in the gel were visualized under UV light and photographed. Densitometry was performed on the negative image using the NIH ImageJ software (http://rsbweb.nih.gov/ij/). For each lane, a predetermined area was analyzed between the positions corresponding to 1198 and 179 bp of the pGem markers (Promega, Madison, WI) run on the same gel. Apoptotic activity was also assessed using the Cell Death Detection ELISA (Roche) which measures the release of histone-associated DNA fragments (mono- and oligo-nucleosomes). Both the incubation medium and homogenate supernatant were analyzed.

7. In-vitro angiogenesis. The tube formation assay was performed as previously described.² Briefly, HUVECs (10⁴ cells/well), were plated on growth factor-reduced Matrigel precoated 96-well plates (BD Biosciences, Oxford, UK), and incubated at 37°C in a CO2 incubator for 4-6 hours containing either 20% human serum samples from heparin-treated women (n=5) or gestational-age matched healthy controls (n=5) in the presence or absence of 20ng/mL VEGF
(PeproTech, Rocky Hill, NJ). Total tube length was quantified by analysing 10 fields/well using Image Pro Plus (Media Cybernetics, Bethesda, MD).

8. **Statistical analysis.** Comparisons between 2 groups were performed using Student *t* tests or Mann-Whitney rank-sum tests as appropriate. Multiple comparison procedures were performed using one-way or Kruskal-Wallis analysis of variance (ANOVA). *In-vivo* data, time course and dose response results were analyzed by two-way repeated measures ANOVA and Holm-Sidak method for post-hoc comparisons. Multiple stepwise regression analysis was used to explore concurrent relationships between circulating sFlt-1 level as dependent variable and gestational age, factor Xa activity, heparanase, sEng, PlGF as independent variables. Variables were entered in the model based on *P*<0.05 and removed if *P*>0.1. Proportions were compared with chi-square of Fischer exact tests. Relationships between variables (correlations) were explored using Pearson’s product moment rank-order correlations. Med-Calc (Broekstraat, Belgium) and SigmaPlot statistical softwares (v.11, Systat software, San Jose, CA) were used for analysis. We used TableCurve 2D (Systat software) for linear and non-linear curve fitting. We compared the evolution of curves in Cartesian spaces by using the F-test.

**Supplemental Figures and Figure Legends**

Heparin and heparan-sulfate have a high affinity for pro-angiogenic factors such as VEGF, as well as for angiogenic inhibitors such as thrombospondin, platelet factor-4 and sFlt-1. Key points of control may include capture of VEGF on heparin sulfate proteoglycans or modulation of the interaction between VEGF and its soluble or cell surface receptors in a dose-dependent manner. In preliminary experiments we found that incubation *ex-vivo* of the serum of heparin-treated women with heparinase I (from *Flavobacterium heparinum*, Sigma-Aldrich), left
unchanged the basal levels of sFlt-1 and VEGF immunoreactivity. This suggests that circulatory heparanase may play a limited role in up-regulating the systemic levels of sFlt-1 through a displacement mechanism in human pregnancy (Figure 1 Supplemental Material). Furthermore, in heparin-treated women, the maternal circulatory concentration of heparanase remained unchanged. This phenomenon appears to be in contrast with observations in mice and does not exclude a possible role for local tissue heparanase. Further studies are necessary, given that in multivariate analysis, heparanase impacted on the maternal circulating level of sFlt-1.

Figure 1 Supplemental Material. Sera from control and heparin-treated women (n=6-9) were treated in-vitro with heparinase I. Free sFlt-1 (A) and VEGF (B) levels were measured by ELISA before and after incubation; ns: P>0.05 vs before incubation (paired t-test). Error bars depict SEM.

Supplemental References


