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Low molecular weight heparin therapy during pregnancy is associated with elevated circulatory levels of placental growth factor

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ABSTRACT

Introduction: Low molecular weight heparin (LMWH) has been shown to be effective in decreasing the recurrence of placenta-mediated complications of pregnant women. The aim of this study was to determine the effect of LMWH on circulating levels of soluble fms-like tyrosine kinase-1 (sFlt-1), soluble endoglin (sEng) and placental growth factor (PLGF) in pregnant women who required anticoagulation therapy.

Methods: A longitudinal prospective cohort study was performed including pregnant women in whom anticoagulation therapy by LMWH during pregnancy was clinically indicated ($n = 33$). Healthy pregnant women, matched for gestational age, who did not require thromboprophylaxis served as controls ($n = 29$). Maternal plasma samples were obtained throughout gestation every 4 weeks and stored at −70 °C. Maternal plasma concentrations of sFlt-1, sEng and PLGF were determined by ELISA and compared between the two groups.

Results: Patients treated with LMWH had significantly increased circulatory levels of PLGF during the third trimester compared with controls (28–34 weeks: 719.2 pg/ml vs 558.6 pg/ml at, $p < 0.01$; 35–40 weeks: 975.6 pg/ml vs 511.2 pg/ml, $p < 0.01$, respectively). In contrast, circulatory levels of sFlt-1 and sEng were similar between the LMWH treatment group and controls throughout gestation. Consistent with these findings, the ratio of sFlt-1/PLGF was lower in patients treated with LMWH compared to controls (28–34 weeks: 1.9 vs 7.2, $p < 0.05$; 35–40 weeks: 5 vs 12.9, $p < 0.05$, respectively).

Discussion: Anticoagulation treatment of pregnant women with LMWH is associated with a pro-angiogenic state. These findings may explain the effectiveness of LMWH in the prevention of placenta-mediated complications of pregnancy.

1. Introduction

Preeclampsia is a clinical syndrome defined as the new onset of hypertension and proteinuria during the second half of pregnancy [1] affecting 3–5% of pregnant women worldwide and is a major cause of maternal and neonatal morbidity and mortality [2,3]. Recent studies suggest that imbalance of endogenous angiogenic factors plays a key role in the pathogenesis of preeclampsia. Placental expression of anti-angiogenic factors soluble fms-like tyrosine kinase- 1 (sFlt-1), a soluble splice variant of the VEGF-receptor Flt-1, and soluble endoglin (sEng), a soluble form of TGF-β receptor Endoglin, are increased in preeclampsia and are associated with a marked increase in the circulatory levels of these peptides [4–7]. sFlt-1 antagonizes the pro-angiogenic factors vascular endothelia growth factor (VEGF) and placental growth factor (PLGF) by binding them in the circulation thus preventing interaction with their endogenous receptors. Notably, clinical studies have confirmed that the increase in maternal circulating sFlt-1 precedes the clinical manifestation of preeclampsia by 5–6 weeks and is correlated with disease severity [4,5,8,9]. Moreover, circulatory PLGF levels are decreased in preeclampsia as early as the first trimester, before the sFlt-1 rise, suggesting that an imbalance of antiangiogenic and proangiogenic factors rather than the level of either sFlt-1 or PLGF alone plays a role in the pathophysiology of preeclampsia [5,10]. These findings, and others, have led to the notion that treatment strategies aiming at restoring the normal angiogenic balance in the maternal circulation could potentially prevent or attenuate the preeclamptic phenotype [11].

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Since thrombosis in the uteroplacental circulation is frequently observed in placental-mediated complications of pregnancy, including preeclampsia, intra-uterine growth restriction (IUGR), placental abruption and intra-uterine fetal death (IUFD), anti-coagulation seemed to be a promising therapeutic option [12]. However, Kupferminc et al. have shown that 50% of women treated by low molecular weight heparin (LMWH) had placental infarcts, which was similar to their control arm [13]. In addition, Rey et al. reported that LMWH was effective in decreasing the recurrence of placental-mediated complications even in women without a known thrombophilia [14]. These findings suggest that LMWH may exert potentially beneficial effects on placental function via other, non anti-thrombotic pathways.

Heparin induces cytotrophoblast proliferation and attenuates trophoblast apoptosis [15,16]. Recently, in-vitro studies have showed that LMWH stimulated sFlt-1 release from placental villous explants [17,18]. Consistent with the in-vitro data, heparin treatment was associated with increased circulatory levels of sFlt-1 [18]. This paradox between heparin's protective effect and its up regulation of circulating sFl-1 has not been settled.

In the present study, we investigated the effect of LMWH on circulating levels of sFlt-1, sEng and PLGF in pregnant women who required anticoagulation therapy. Serial measurements of plasma levels of sFlt-1, sEng and PLGF were performed throughout pregnancy in LMWH treated women, as well as in pregnant women who did not require thromboprophylaxis.

2. Materials and methods

This was a prospective cohort study of patients in whom anticoagulation therapy with LMWH during pregnancy was clinically indicated. Patients were enrolled between June 2012 and December 2013 at a single tertiary center. The study was approved by the local institutional ethics committee, and all patients provided written informed consent. The study group included 33 patients who were treated by LMWH throughout gestation and were matched by gestational age to 29 healthy pregnant women with uncomplicated pregnancies who did not require thromboprophylaxis and served as controls. The indications for LMWH administration in the study group included previous thromboembolism and thrombophilia ($n = 11$), bad obstetric history and thrombophilia ($n = 7$), antiphospholipid syndrome ($n = 5$), inherited thrombophilia without previous thromboembolism or bad obstetric history ($n = 4$), previous thromboembolism without thrombophilia ($n = 4$), and bad obstetric history without thrombophilia ($n = 2$). Bad obstetric history was defined as previous early-onset preeclampsia or IUGR requiring delivery prior to 34 weeks of gestation, placental abruption, IUFD or preterm birth less than 34 weeks. All patients were already undergoing LMWH treatment upon recruitment to the study. Most patients (28 of 33) received prophylactic dose of 1 mg enoxaparin per kg body weight daily, while 5 patients with previous thromboembolism received a treatment dose of 1 mg enoxaparin per kg body weight twice daily. All patients with antiphospholipid syndrome ($n = 9$) were also treated by low dose aspirin (100 mg/d). Patients with chronic hypertension, pre-gestational diabetes, chronic renal disease, as well smokers, were excluded. Gestational age was determined based on menstrual history and first trimester ultrasound. Demographic and clinical data, ultrasound findings and perinatal outcomes were entered prospectively into a computerized database. Preeclampsia was defined as blood pressure $\geq$140/90 mmHg measured on two occasions at least 4 h apart, accompanied by proteinuria ($\geq$300 mg/24 h or 2 + dipstick) occurring after 20 weeks of gestation in a previously normotensive woman [19]. Intra-uterine growth restriction (IUGR) was defined as birthweight below the 5th percentile.

Serial samples of peripheral blood were obtained throughout pregnancy starting at the patient's first visit to our high risk clinic and thereafter every 4 weeks. Blood samples were collected in tubes containing EDTA, centrifuged at 4 °C for 10 min and stored at −70 °C until further analysis. Maternal plasma levels of sFlt-1, sEng and PLGF were determined by enzyme-linked immunoassays (R&D Systems, Minneapolis, MN). All samples were assayed in duplicate at the same time using the same standard curve to minimize interassay variation. The calculated interassay coefficients of variation for sFlt-1, sEng and PLGF were 3.8%, 6.1% and 7.2% respectively. The calculated intraassay coefficients of variation for sFlt-1, sEng and PLGF were 2.3%, 2.8% and 4.4% respectively.

Normality of the data was tested using Kolmogorov–Smirnov test. Comparison of continuous variables between the groups was conducted using Mann–Whitney U-test or student *t*-test as appropriate. Chi-square or Fisher exact test were used for comparison of categorical variables. Data are presented as mean ± standard error of the mean (SEM). Significance was accepted at $P < 0.05$. Statistical analyses were conducted using the IBM Statistical Package for the Social Sciences (IBM SPSS v.19; IBM Corporation Inc, Armonk, NY, USA).

3. Results

Demographic and clinical characteristics of the patients are shown in Table 1. Maternal age, rate of primigravidity and pre-pregnancy BMI were similar between the two groups. The rate of preeclampsia as well as IUGR did not differ between the two groups. As expected, in patients treated by LMWH there was a trend towards earlier delivery (38.3 vs 38.8 weeks, $p = 0.053$) and consequently their neonates were smaller than controls (2805 vs 3182 g, $p = 0.037$). For the purpose of analysis of the data, we grouped obtained blood samples into three pregnancy/time categories: second trimester (16–27 weeks of gestation), early third trimester (28–34 weeks) and late third trimester (35–40 weeks of gestation). The samples were grouped in this manner based on previous data showing that in normal pregnancies sFlt-1 concentrations remain constant until 34 weeks of gestation and then increase until delivery, while PLGF levels peak at 28 weeks of gestation, and decrease after 34 weeks [5]. The plasma levels of sFlt-1 were similar between study group and controls throughout gestation (Table 2). In contrast, LMWH treatment was associated with significantly increased circulatory levels of PLGF in the early third trimester starting at 28 weeks of gestation ($p < 0.01$, Fig. 1 and Table 2). Similarly, circulatory levels of PLGF were higher in patients receiving LMWH compared with controls in the late third trimester ($p < 0.01$, Fig. 1 and Table 2). During the second trimester serum levels of PLGF did not differ between the two groups ($p = 0.9$, Fig. 1 and Table 2). The increased circulatory PLGF levels in patients treated with LMWH without significant change in sFlt-1 levels resulted in decreased sFlt-1/PLGF ratio in that group compared to controls both at 28–34 weeks of gestation and at 35–40 weeks of gestation (Fig. 2 and Table 2, $p < 0.05$). LMWH treatment was not associated with alterations in circulatory levels of sEng throughout gestation (Table 2). No difference in circulatory PLGF levels was found between the 5 patients who received treatment dose of LMWH and the patients who received prophylactic dose. Likewise, there were no differences in circulatory levels of these peptides between patients who were treated with aspirin and patients who were not and between patients with ($n = 27$) or without thrombophilia ($n = 6$) who received LMWH.

4. Discussion

The evidence that LMWH treatment may promote improved perinatal outcomes [14,20,21] has prompted us to investigate its effect on circulating angiogenic and anti-angiogenic factors during pregnancy, as these play a key role in the pathogenesis of preeclampsia. Herein, we demonstrated for the first time that LMWH treatment was associated with increased circulating levels of PLGF during the third trimester without alteration in the circulating

Table 1
Demographic and clinical characteristics.

	LMWH ($n = 33$)	Control ($N = 29$)	P
Maternal age (years)	33 (30.5–35.5)	32.5 (29–34)	0.94
Primigravida (%)	30.3	28	0.8
Pre-pregnancy BMI (kg/m^2)	25.8 (23.2–30.7)	25.4 (22.5–29.2)	0.93
Preeclampsia (%)	6	0	0.5
IUGR (%)	9	7	1
Gestational age at delivery (wks)	38.3 (36.7–38.7)	38.8 (36.7–40.2)	0.053
Birth weight (grams)	2805 (2525–3345)	3182 (2875–3678)	0.037

Values are expressed as median (interquartile range) or as percentage.

Table 2
Maternal plasma levels of sFlt-1, PLGF, sFlt-1/PLGF ratio and sEng in LMWH-treated and untreated pregnant women.

Peptide	LMWH group	Control group	*p* Value
sFlt-1 pg/ml			
16–27 weeks	1309 ± 323	1507 ± 306	0.68
28–34 weeks	2223 ± 359	2687 ± 581	0.5
35–40 weeks	5134 ± 908	4992 ± 1253	0.9
PLGF pg/ml			
16–27 weeks	342 ± 53	348 ± 47	0.9
28–34 weeks	719 ± 38	558 ± 40	<0.01
35–40 weeks	975 ± 110	511 ± 59	<0.01
sFlt-1/PLGF ratio			
16–27 weeks	6.5 ± 1.5	5.3 ± 1.4	0.6
28–34 weeks	2.7 ± 0.4	5.8 ± 1.4	<0.05
35–40 weeks	5 ± 1.3	12.9 ± 3.7	<0.05
sEng pg/ml			
16–27 weeks	3525 ± 157	3574 ± 170	0.8
28–34 weeks	7731 ± 802	8113 ± 947	0.75
35–40 weeks	9483 ± 1072	10,023 ± 1102	0.7

Data are presented as means ± SEM.

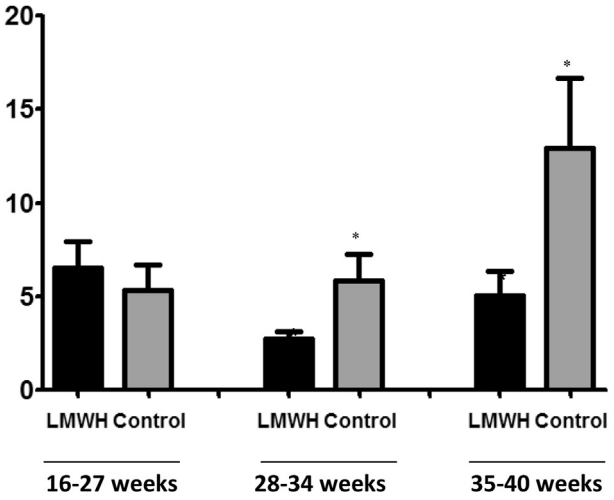

Fig. 2. The ratio sFlt-1/PLGF throughout gestation in LMWH-treated and untreated pregnant women. Data are presented as means ± SEM.*$p < 0.05$.

levels of sFlt-1. This resulted in a decreased ratio of sFlt-1/PLGF. In contrast to our findings, Rosenberg et al. have showed that heparin treatment was associated with increased circulating levels of sFlt-1 in the third trimester in 21 pregnant women, who received prophylactic heparin anticoagulation, with no effect on the levels of PLGF. In vitro, LMWH stimulated sFlt-1 release form placental villous explants, in a dose and time-dependent manner [18]. Similarly, Drewlo et al. has showed that first trimester placental villi exposed to LMWH significantly increased the expression and release of sFlt-1 by the syncytiotrophoblast into culture media [17]. It appears that heparanase, an endogenous enzyme that cleaves heparin, facilitates the release of sFlt-1, as its inhibition resulted in reduced amounts of sFlt-1 released from the placental villi [22]. How can the conflicting findings of our study and previous reports regarding the effect of heparin on circulating levels of sFlt-1 be reconciled?

While a relatively large body of in-vitro data indicates that heparin promotes sFt-1 release from placental villi [17,18,22], in-vivo studies have been limited to very small cohorts [18,22]. Furthermore, alternative splicing and posttranslational processing produce multiple isoforms of sFlt-1 in the circulation of women with preeclampsia, as well as in uncomplicated pregnancies [23,24]. One of these variants of sFlt-1 is designated sFlt-14, which is the predominant VEGF inhibitor produced by the placenta in preeclamptic women [25]. Thus, it is possible that the alleged inconsistency in sFlt-1 levels following heparin treatment reflects methodological variance in the determination of different isoforms.

Despite lack of effect of LMWH on sFlt-1 and sEng circulating levels, we found that LMWH treatment was associated with elevated of PLGF levels starting at the third trimester. In accordance with these findings, Drewlo et al. found increased release of PLGF from villous explants to the media in response to LMWH, which was dose-dependent [17]. We did not find a dose-dependent effect of LMWH on PLGF circulatory levels, most probably since only 5 patients in our cohort received treatment dose of 2 mg per kg body weight while the rest received prophylactic dose. The sFlt-1 to PLGF ratio is an index of antiangiogenic activity that reflects changes in the balance between sFlt-1 and PLGF, and has been shown to be more strongly associated with preeclampsia than either measure alone [4]. Moreover, the ratio of sFlt-1/PLGF in the second trimester can accurately predict severe preeclampsia in high risk women [26]. In the current study, LMWH treatment was associated with decreased ratio of sFlt-1/PLGF, which might explain the beneficial effect of LMWH in the prevention of several placenta-mediated complications of pregnancy reported in clinical studies [14,20,21].

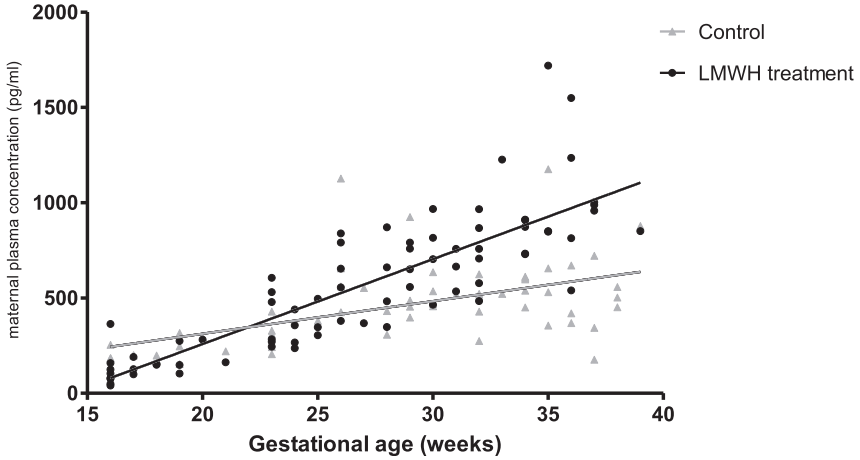

Fig. 1. Maternal plasma levels of PLGF as a function of gestational age in LMWH-treated and untreated pregnant women. The slopes of best-fit lines of LMWH treatment group ($r^2 = 0.7$) and controls ($r^2 = 0.28$) are significantly different: 44.5 (95% CI 37.9–51.2) vs 17.1 (95% CI 9.8–24.4), $p < 0.001$.

Interestingly, two of our patients treated by LMWH developed preeclampsia at 30 and 34 weeks of gestation, and both of them had decreased plasma levels of PLGF and increased sFlt-1/PLGF ratio at 28 weeks despite the anticoagulation treatment.

Our findings support the view that heparin exerts its beneficial effect on placental function not through its anticoagulant action but possibly by promoting angiogenesis. This is further supported by the findings reported by Sobel et al., who showed that exposure of first and second trimester placenta-conditioned media to unfractionated or LMWH significantly promoted angiogenesis whereas placenta −conditioned media alone from normal first and second trimester explants inhibited angiogenesis [27]. As expected, placenta-conditioned media from pregnancies with severe preeclampsia arrested angiogenesis regardless of exposure to LMWH [27]. In contrast, Rosenberg et al. demonstrated that serum of heparin-treated women inhibited both basal and VEGF-induced capillary-like tube formation, suggesting that heparin actually inhibits angiogenesis [18]. Hence, the mechanism underlying the protective effect of heparin against pregnancy complications still needs to be elucidated.

The patients in our study were already on LMWH treatment when enrolled and therefore we could not assess the change in the circulating levels of sFlt-1, PLGF and sEng following initiation of treatment. Nevertheless, the study groups were well-matched and had serial samples collected at similar time points throughout gestation. Therefore, we believe that the significantly elevated circulatory levels of PLGF observed in LMWH treated women compared to controls represent a true biological effect of heparin.

In summary, we conclude that LMWH treatment during pregnancy is associated with increased maternal circulatory levels of PLGF and decreased sFt-1/PLGF ratio. This effect may explain the protective role heparin might have in preventing placenta-mediated complications. Further investigation is required in order to determine whether the altered balance of sFlt-1 and PLGF during LMWH treatment is the key factor underlying the beneficial effect of heparin treatment during pregnancy.

Disclosure

None of the authors have a conflict of interest.

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