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Tissue factor dependent thrombin generation across pregnancy

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Abstract

Objectives—Normal pregnancy results in a pro-thrombotic state. Studies investigating the capacity of pregnant women to generate thrombin are limited. Our aim was to longitudinally evaluate thrombin generation from the pre-conception period, through pregnancy, and post-pregnancy.

Study Design—We evaluated young, healthy nulligravid women, n= 20, at 4 time points and compared them to 10 control women at 2 time points. Coagulation was initiated in contact pathway inhibited plasma, and thrombin generation was determined in the presence of a fluorogenic substrate.

Results—The maximum level and rate of thrombin generation increased during pregnancy, with the highest level and rate occurring in late pregnancy compared to pre-pregnancy ($p < 0.001$). Subsequently, thrombin generation decreased in the post-pregnancy samples, including maximum level, rate, and area under the curve (AUC) ($p < 0.001$).

Conclusions—Our data provide evidence for an increase in tissue factor-dependent thrombin generation with pregnancy progression, followed by a return to pre-pregnancy thrombin levels.

Keywords

Pregnancy; Thrombin Generation; Longitudinal

Introduction

Pregnancy presents a hematologic paradox. Despite hemorrhage being the leading cause of maternal mortality worldwide, pregnancy is a well-described hypercoagulable state, conferring significantly increased thrombotic risk.^{1–3} In more developed areas of the world, where hemorrhage is better-treated, and/or prevented, thromboembolic disease is the leading cause of maternal death.^{4,5} Indeed, pregnant women have a 4 to 5-fold increased risk of venous thromboembolism (VTE), with the third trimester being the period of greatest risk.⁶ Pregnancy-related hormonal changes- particularly increases in estrogen levels, are thought

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to result in this shift to a more procoagulant state by resulting in increases in most clotting factors, a decrease in physiologic anticoagulants, and a decrease in fibrinolytic activity.^{7,8}

Empirical investigations into the mechanisms of enhanced coagulation in pregnancy are lacking. Most studies investigating the hemostatic system in pregnancy have described blood coagulation factors, fibrinolytic factors, and platelet function separately.^{7,9} More global tests of the hemostatic system, such as thrombin generating capacity, have only recently been utilized.^{10–13} Thrombin generation captures the end result of a complex array of enzymatic reactions and interactions. Because of this it has been hypothesized that measurement of an individual's capacity to generate thrombin, using blood or plasma subjected to a well-defined initiator, may be a better indicator of a thrombotic or hemorrhagic tendency than clot based assays or comparative analyses of potential biomarkers.^{14,15} Determination of the tissue factor (Tf) initiated thrombin generation during pregnancy is therefore essential to a better understanding of the characteristic procoagulant state of normal pregnancy.

Data on thrombin generation in pregnancy are both limited and conflicting.^{11,12,16} There are few longitudinal studies evaluating thrombin generation at different time points over the course of pregnancy. Further, to our knowledge, there have been no studies performed to evaluate the relationship between a woman's capacity to generate thrombin outside of pregnancy, and her ability to do so once pregnant. Likewise, and perhaps most importantly, there are no studies investigating whether pregnancy results in a persistent change in a woman's capacity to generate thrombin. There have been multiple published studies which suggest long-term, and even permanent cardiovascular changes resulting from pregnancy.^{17–21} Whether or not pregnancy has a long-term effect on thrombin generating capacity, however, is unknown.

In this study, we investigated a woman's individual capacity to generate thrombin pre-pregnancy, in early and late pregnancy, and approximately 1 year post-pregnancy (n=20). Additionally, control studies (n=10) were performed in non-pregnant women over a 2–3 year time period (30 months on average). Plasma samples from each time point in all 30 women were evaluated for thrombin generating capacity via the Tf pathway.

Subjects, Materials, and Methods

Subjects and Study Procedures

Subjects—Plasma samples used in this study were collected by Bernstein *et al.*, as previously described.^{1,22} In addition to plasma samples, a large body of clinical, physiologic, laboratory, and birth outcome data was collected and analyzed by Bernstein *et al.*^{1,22}, and was made available to us. Briefly, 60 nulligravid women intending conception were enrolled through an open advertisement. All participants were young (18–40), healthy, and non-smoking, with no history of hypertension, diabetes, autoimmune disease, clotting or bleeding disorders.

Study Procedures—Study participants were placed on sodium and total calorie balanced diets for 3 days prior to each blood draw, and asked to abstain from alcohol and caffeine for at least 24 hours. Additionally, women were asked to avoid the use of decongestants and nonsteroidal medications for at least 48 hours before the study. All women had regular menstrual cycles at the time of study enrollment. Thirty women conceived, however 8 women conceived before baseline pre-pregnancy studies were performed, 1 participant had a first trimester miscarriage, and one participant was lost to follow-up. The remaining 20 participants comprising the primary study population all conceived singleton pregnancies, had complete pre-pregnancy assessments, and delivered full-term live born infants. Three of

the pregnant study subjects went on to develop complicated hypertension, with 2 of these 3 women meeting strict criteria for preeclampsia, as previously reported by Bernstein *et al.*²² All 4 time point plasma samples were available for only 1 of these 3 women, while 3 time point samples were available for the other 2 women.

All pre-pregnancy assessments were performed during the follicular phase of the menstrual cycle (pre pregnancy sample). Assessments during pregnancy were performed between 11 and 15 menstrual weeks (early pregnancy sample), and again in the third trimester between 30 and 34 weeks (late pregnancy sample). Ovulation detection and early pregnancy ultrasound assessments were used to calculate gestational age. Post pregnancy blood draws were performed between 6 months and 2 years after delivery, once breast-feeding had been discontinued (post pregnancy sample). As with the pre-pregnancy draws, the post-pregnancy blood draws were performed in the follicular phase of the menstrual cycle. All 4 plasma samples were available for 14 women, 3 samples were available for 4 women, and two women had only two blood draws performed. Women originally enrolled in the Bernstein *et al.* study who did not become pregnant were continued in the study as controls (control time 1).^{1,22} Most of these women (n=27) had a second blood draw performed an average of 2.5 years after the initial blood draw (control time 2). Ten of these subjects served as the control population. Blood samples were obtained without the use of a tourniquet and after at least 30 minutes of supine rest. Citrated platelet poor plasma was separated immediately via centrifugation, and the samples frozen at -80°C. The research protocols were approved by the University of Vermont Human Investigational Committees. All women provided written informed consent.

Materials

Recombinant Tf (Tf₁₋₂₄₂) was provided as a gift from Drs. Shu Len Liu and Roger Lundblad (Baxter Healthcare Corp). Corn trypsin inhibitor (CTI) was isolated from corn as described elsewhere.⁹ 1,2-Dioleoyl-*sn*-Glycero-3-Phospho-L-Serine (PS) and 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (PC) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Phospholipid vesicles (PCPS) composed of 25% PS and 75% PC were prepared as described, and used to relipidate Tf.²³ The fluorogenic substrate benzyloxycarbonyl-Gly-Gly-Arg-7-amido-4methylcoumarin•HCl (Z-GGR-AMC) was purchased from Bachem (Torrance, CA) and prepared as previously described.^{24,25}

Methods

Thrombin generation assay—Previously frozen citrated platelet poor plasma samples were thawed at 37°C in the presence of 0.1mg/ml CTI to inhibit the contact activation pathway (*e.g.* intrinsic pathway). The thawed samples were then incubated with Ca⁺² (15mM) and the slow reacting fluorogenic substrate Z-GGR-AMC (416 μM) for 3 minutes. Blood coagulation was initiated with 5 pM Tf, relipidated in 20 μM PCPS, and thrombin generation was monitored continuously in a Synergy4 plate reader, powered by Gen5 data analysis software (BioTek, Winooski, VT).^{24,25} Experiments were done in duplicate or in triplicate, depending upon the volume of plasma available to us. The final volume added to each well included 80μL of plasma, 20μL substrate, 10μL Tf. For each sample, Tf-dependent thrombin generation was evaluated using a thrombin generation curve. Each curve was analyzed for the peak rate of thrombin formation (Peak Rate), the maximum level of thrombin generated (Max Level), and the total thrombin generated (area under the curve (AUC)).

Statistical Analysis—Data are presented as the mean +/- SD unless otherwise stated. Repeated measures analysis of variance (ANOVA) was used to evaluate thrombin generation parameters across 4 time points. Pearson correlation coefficients were examined

to test for correlations between study subject lab/physiologic data and thrombin generation. A p-value <.05 was considered significant.

Results

Clinical characteristics of subjects

Demographic data and clinical characteristics for the 20 study subjects and the 10 controls are presented in Table 1. As shown, baseline demographic and /or clinical characteristics were not significantly different. Additionally, as part of Bernstein et al.'s primary study, a wide array of physiologic and lab data were collected pre-pregnancy, in early and late pregnancy, and post-pregnancy. These data included measurement of hemoglobin, D-dimer, fibrinogen, and platelet count. As expected in normal pregnancy, average hemoglobin levels decreased from 12.0 ± 0.8 g/dL pre-pregnancy, to 11.2 ± 1 g/dL by late pregnancy, while platelet levels decreased to a lesser extent, from $225 \pm 49 \times 10^3/\mu\text{L}$ to $212 \pm 30 \times 10^3/\mu\text{L}$. Fibrinogen levels increased, from 219 ± 46 mg/dL pre-pregnancy, to 449 ± 133 mg/dL in late pregnancy. Similarly, D-Dimer levels increased from 0.26 ± 0.15 mg/L pre-pregnancy to 0.97 ± 0.4 mg/dL post-pregnancy. Overall, findings were in agreement with expected physiologic and laboratory changes reported in normal pregnancy.²⁶

Thrombin generation

Figure 1 and Table 2 show Tf-initiated thrombin generation parameters, including Max Level, Peak Rate, and AUC (analogous to 'endogenous thrombin potential', or ETP). The results in Figure 1 and Table 2 span the pre-pregnancy to the post-pregnancy period for all 20 subjects. Maximum level of thrombin generation, peak rate, and AUC all significantly increased from the pre-pregnancy state to the early pregnancy setting ($p < .001$). As pregnancy progressed, the maximum level and peak rate of thrombin generation continued to significantly increase. The AUC showed a trend toward increased thrombin generation in the late pregnancy sample vs. the early pregnancy sample, but this was not a statistically significant increase. As can be seen in Figure 1, in the post-pregnancy samples, thrombin generation in all parameters measured returned to levels consistent with pre-pregnancy values. This decrease in thrombin generation to baseline values was significant ($p < .001$) when compared both to early pregnancy and late pregnancy thrombin generation.

There were no significant differences in thrombin generation parameters of the 3 women with complicated hypertension and the other healthy 17 women whose pregnancies were without such complications (data not shown). The thrombin generation data presented therefore reflect inclusion of all 20 women, including the 3 women who developed complicated hypertension.

There were no statistically significant correlations between any of the clinical characteristics presented in Table 1 and Tf dependent thrombin generation parameters. Further, there was no statistically significant correlation between thrombin generation and any of the laboratory and clinical data collected by Bernstein et al., nor was there any significant relationship between neonatal birth weight and thrombin generation parameters.

Comment

In this study we show a longitudinal evaluation of thrombin generation over time in pregnancy, spanning the preconception to the post-pregnancy state. Thrombin generation significantly increased during pregnancy, but by 1-year post-pregnancy, returned to a level consistent with preconception. These findings provide greater insight into the evaluation of hemostatic changes occurring over the course of normal pregnancy.

Pregnant women are relatively prothrombotic, with risk of venous thromboembolism increasing as pregnancy progresses.⁸ As such, one would expect to see a quantifiable increase in thrombin generating capacity over the course of pregnancy. Previous published data by other groups has been inconsistent, however, with some studies showing no change, others showing a decrease, and still others showing an increase in thrombin generation over the course of normal pregnancy.^{10–12,16} Our findings of increased thrombin generation are in agreement with Dargaud *et al.* and Rosenkranz *et al.*^{12,13} However, the study by Dargaud *et al.* was not a longitudinal study, and Rosenkranz *et al.* followed a subset of women longitudinally only during pregnancy. The current study is the first longitudinal evaluation of thrombin generation spanning the pre-conception to the post-pregnancy state. Interestingly, most previous studies have reported solely on ETP, and not on other thrombin generation parameters, such as maximum thrombin level and/or maximum rate of production, which may also be of clinical significance. Our study showed an increase in all thrombin parameters measured in the pregnant samples versus the non-pregnant samples, but only the maximum thrombin level and peak rate significantly increased with pregnancy progression. The area under the curve (AUC), which is analogous to the ETP measured in previous studies, showed a non-significant increased trend between early and late pregnancy, though was significantly increased in pregnancy (early and late) versus the non-pregnant state (pre-pregnancy and post-pregnancy).

Previous work has shown that an individual's capacity to generate thrombin- or their coagulation phenotype, is relatively constant over time.²⁷ It is likely that such a coagulation phenotype changes with changing circumstances, such as pregnancy. The current study demonstrates a relative consistency in thrombin generation parameters over time in our non-pregnant controls, versus significantly increased parameters in pregnancy. Further, we showed thrombin generation parameters to be similar in the pre-pregnancy samples and those collected one year after pregnancy. Pregnancy results in marked cardiovascular and hematologic alterations, including increases in cardiac output, blood volume, and a decrease in systemic vascular resistance. Several studies have investigated the presence of persistent cardiovascular changes after pregnancy. Gunderson *et al.* examined longitudinal blood pressure changes in women before and after pregnancy, showing a persistent decline in blood pressure from preconception to years after delivery.¹⁷ Inquiries such as Gunderson's point to the possibility of persistent changes in a woman's physiology as a result of pregnancy-associated physiologic changes. In light of data pointing to such long-term pregnancy-related cardiovascular changes, and because of the magnitude of hematologic alterations in pregnancy, questions around whether such alterations persist are worthy of careful investigation.^{17–20} Our data are unique in that we were able to assess post pregnancy thrombin generation parameters *remote* from pregnancy in the context of pre-pregnancy thrombin generation. Our data revealed no persistent effect of pregnancy on thrombin generating capacity.

Several methods that profile thrombin generation (either directly or indirectly) have potential utility in the realm of clinical testing.^{24,28–35} In this study, we chose to use previously collected citrated plasma, and measured thrombin generation using a thrombography assay system with a fluorogenic substrate directly in the reacting plasma mixture.²⁹ This assay was modified to include CTI to block factor XIIIa (contact pathway activation) and a recalcification step prior to initiation with Tf.³⁰ The goal of these modifications was to better approximate Tf-initiated coagulation in samples collected in citrate. Since these samples were collected into a chelator (Na-citrate) and had to be re-calcified, estimates of qualitative and quantitative biological functions during Tf-induced blood coagulation should still be evaluated with caution, even in the presence of CTI. Sodium citrate chelation has been a benefit to the development of transportable blood products, for tests of coagulant function, and has provided stable sources of both blood and

plasma. However, the addition of chelators can influence cellular metabolism and plasma protein functions including the vitamin K-dependent zymogens, fXIII activation, and the crosslinking of fibrinogen.³⁰ Regardless of the fact that our samples were collected in citrated plasma, they nevertheless provide a significant improvement in the quantity and quality of information collected relative to that available with conventional tests for the evaluation of coagulation disorders.

The small study size is a limitation of our study. The nature of our longitudinal data collection (which required recruitment prior to conception, spanned the course of pregnancy, and was completed remote from pregnancy), makes larger studies less logistically feasible. However, despite the small sample size, our findings were highly statistically significant, particularly when considered in light of the substantial individual variation in thrombin generation parameters (as illustrated in Figure 1). It is certainly possible, though, that a much larger data set might demonstrate the presence of specific associations between thrombin generation parameters and pregnancy outcome. As previously discussed, the plasma samples used in this study were collected by Bernstein *et al.*^{1,22} as part of a longitudinal study to investigate how pre-pregnancy physiologic characteristics might predict pregnancy course and outcome, and included a large collection of biochemical, clinical, and physiologic data. Our data was analyzed in the context of this substantial quantity of data, including birth outcome. We did not find an association between thrombin generation and any of these data. Because studies in women with certain acquired or genetic hypercoagulable conditions reveal an association with pregnancy complications, such as pregnancy loss, intrauterine growth restriction, placental abruption, and VTE, it is possible that a larger study might demonstrate the presence of specific associations between thrombin generation parameters and pregnancy outcome.³⁶ Indeed, because our data showed considerable variation in thrombin generation parameters at baseline, and in the magnitude of thrombin generation increases in pregnancy between individuals, an appropriately powered study might evaluate such pregnancy complications as postpartum hemorrhage in women with lower thrombin generation, and venous thromboembolism in women who generate large amounts of thrombin.

In summary, our data show that while individuals vary considerably in their ability to generate thrombin, thrombin generation increases in pregnancy, regardless of individual baseline thrombin generation levels. Further, the majority of women generate increasing levels of thrombin as pregnancy progresses, and return to their pre-pregnancy values 1–2 years post-pregnancy (Figure 2).

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Figure 1. Thrombin Generation Parameters

Box plot shows thrombin parameters for 20 pregnant women over 4 time points and 10 control subjects at 2 time points (C1, C2): A, max level; B, peak rate; C, area under the curve (AUC). The boundary of each box closest to zero indicates the 25th percentile, the boundary furthest from zero indicates the 75th percentile. The *line within each box* represents the median. *Error bars* represent the 90th and 10th percentiles; samples outside of these parameters are represented as outlying points (*closed circles*).

NS, not statistically significant; *Pre*, prepregnancy; *Post*, after pregnancy; *RFU/s*, relative fluorescence units.

Figure 2. Longitudinal Thrombogram

Thrombograms for 1 individual: **A)** before conception, **B)** in early pregnancy, **C)** in late pregnancy, **D)** 1 year after delivery. In all panels, *closed circles* represent reactions initiated with 5pmol/L Tf in the presence of Ca²⁺ and 20-μmol/L phospholipid vesicles.

Tf, tissue factor.

Table 1

Subject and control demographic characteristics

Characteristic	Subjects: Pre-Preg	Controls	P-value
Age (yrs)	29.8±3.2	29.4±5.4	0.82
Weight (kg)	65.2±10	63.5±5.0	0.62
BMI (kg/m ²)	23.3±3.2	23.3±2.4	0.97
Cycle Day #	8.3±3.8	9.6±3.1	0.39
Hemoglobin (g/dL)	12.9±.8	12.7±.7	0.66

Table 2

Thrombin generation parameters for pregnant and control subjects

Thrombin Parameters	PRE (N=19)	EARLY (N=19)	LATE (N=19)	POST (N=15)	Control Time 1 (N=10)	Control Time 2 (N=10)
Peak Rate (RFU/s)	35±18	97±53	146±77	50±45	68±43	57±40
Max Level (nM)	81±41	219±117	336±178	101±81	159±100	133±93
AUC (nM*min)	1162±446	2157±466	2410±543	1392±718	1553±567	1480±653

MaxRate: Maximum rate of thrombin generation; **MaxLevel:** Maximum Level of thrombin generation; **AUC:** Area under the curve