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Aberrant Processing of Plasma Vitronectin and High-Molecular-Weight Kininogen Precedes the Onset of Preeclampsia

Marion Blumenstein, PhD, Roneel Prakash, BSc,
Garth J. S. Cooper, MD, PhD, and
Robyn A. North, MD, PhD; for the SCOPE Consortium

To date, there is no reliable test to identify women in early pregnancy at risk of developing preeclampsia. Difference gel electrophoresis (DIGE) identified the plasma proteins vitronectin (VN) and high-molecular-weight kininogen (HK) in association with preeclampsia. In a longitudinal proteomics study, the plasma of preeclamptic patients (n = 6) was compared to healthy control participants (n = 6) before the onset of preeclampsia (week 20) and at the time of presentation with clinical disease (weeks 33-36). The 75-kd single-chain VN molecule increased 1.6- to 1.9-fold in preeclampsia, whereas the 65-kd moiety of the 2-chain VN molecule decreased 1.5- to 1.7-fold compared to healthy controls (P < .05). Immunoblots revealed differences in proteolytic processing of VN and/or HK in women who develop preeclampsia or preeclampsia further complicated by small-for-gestational-age. Vitronectin and HK may prove to be useful as early markers of fibrinolytic activity and neutrophil activation, which are known to be associated with preeclampsia.

Key words: Preeclampsia, small-for-gestational-age, difference in gel electrophoresis, plasma, vitronectin, high molecular weight kininogen.

INTRODUCTION

Preeclampsia is a serious multisystem disorder, unique to human pregnancy. The rapidly progressing syndrome is characterized by the development of maternal hypertension and proteinuria, but in severe cases may result in seizures, kidney failure, and liver dysfunction.[1] The underlying pathophysiology of preeclampsia is complex. Inadequate trophoblast invasion and abnormal remodeling of spiral arteries during early placentation are believed to be initiating events.[2] As a consequence of reduced uteroplacental blood flow and placental hypoxia, factors are released by the placenta into the maternal circulation.[3] These factors trigger maternal vascular dysfunction, an inflammatory response and enhanced coagulation, culminating in this multisystem disorder.[3] Furthermore, preeclampsia is a recognized risk factor for the later development of cardiovascular disease and type 2 diabetes, suggesting there is an underlying maternal metabolic and vascular predisposition.[4]

Advances in proteomic technologies have enabled unbiased mapping of the plasma proteome to elucidate novel biological pathways that may contribute to disease pathogenesis and toward a clinical marker set for preeclampsia.[5,6] Here, we conducted a longitudinal proteomic study to identify preeclampsia-specific proteins in the maternal circulation. The study design was aimed at identifying plasma proteins that were differentially

From the School of Biological Sciences, Faculty of Science, University of Auckland, Auckland, New Zealand (MB, GJSC); HortResearch, Auckland, New Zealand (RP); Medical Research Council Immunochemistry Unit, Department of Biochemistry, University of Oxford, United Kingdom (GJSC); and Discipline of Obstetrics and Gynecology, School of Pediatrics and Reproductive Health, University of Adelaide, Australia (RAN).

Address correspondence to: Marion Blumenstein, PhD, School of Biological Sciences, The University of Auckland, 3a Symonds Street, Private Bag 92019, Auckland, New Zealand. E-mail: m.blumenstein@auckland.ac.nz.

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expressed both in early pregnancy (week 20 of gestation) when the women were apparently healthy and once preeclampsia became clinically manifested (33-36 weeks' gestation). Difference gel electrophoresis (DIGE) was used to analyze EDTA plasma, depleted of the 6 most abundant proteins, from women who developed preeclampsia and from matched healthy pregnant controls who did not go on to develop the disease.

Difference gel electrophoresis analysis of pregnancy plasma identified the glycoproteins vitronectin (VN) and high-molecular-weight kininogen (HK) as being implicated in the pathogenesis of preeclampsia. Follow-up immunoblots showed that women who developed preeclampsia alone appear to have a different expression profile of VN compared to women with preeclampsia complicated by small-for-gestational-age (SGA). Both patterns differed from that seen in early pregnancy plasma from normal pregnancy. Moreover, proteolytic processing of HK appears to be different in women who later developed preeclampsia and SGA but not in women with preeclampsia alone.

PATIENTS AND METHODS

Study Groups and Sample Collection

A longitudinal study was performed using DIGE analysis of plasma from nulliparous women recruited into the SCOPE study (SCreening fOr Pregnancy Endpoints, Australian and New Zealand Clinical Trials Registry ACTRN12607000551493). For each woman (n = 6), 2 time points were studied: (1) at week 20 of gestation prior to disease onset and (2) in the third trimester (weeks 33-36) after preeclampsia was diagnosed. Control participants had a healthy pregnancy outcome and were sampled at week 20 and at weeks 33 to 36 (n = 6) to provide matched controls for gestation at the time of disease onset. Study protocols were approved by the Auckland Regional Ethics Committee (2000/157 and AKX/02/00/364) and written informed consent was obtained from each woman. For immunoblot analysis, native plasma from the women included in the DIGE study was used. Additional plasma at 20 + 1 weeks' gestation was obtained from women with subsequent preeclampsia and an SGA baby (n = 4) and healthy controls (n = 4) for western blot analysis. Blood was collected into EDTA-tubes, centrifuged (2400*g*, 10 minutes at 4 C), and the plasma stored at −80°C. The mean time between specimen collection and storage at −80°C was 1.9 (SD 1.2) hours for cases and 1.7 (SD 0.6) hours for controls, $P = .74$.

Preeclampsia was defined as systolic blood pressure (BP) ≥140 mm Hg and/or diastolic BP ≥90 mm Hg on 2 or more occasions after 20 weeks' gestation but prior to the onset of labor, or postpartum systolic BP ≥140 mm Hg and/or diastolic BP ≥90 mm Hg postpartum on at least 2 occasions 4 hours apart, combined with either proteinuria (spot protein to creatinine ratio ≥30 mg/mmol, or 24-hour urinary protein ≥0.3 g/24 h, or dipstick proteinuria ≥2+), or any multiorgan complication.[7] Severe preeclampsia was defined by the presence of 1 or more of the following additional findings: coagulopathy, hemolysis, hepatic impairment, acute renal insufficiency, imminent eclampsia, or eclampsia. Small-for-gestational-age was defined as a birth weight less than the tenth customized centile (adjusted for infant sex and maternal ethnicity, height, and weight).[8]

Top 6 Depletion of Plasma

To remove the 6 most abundant proteins (albumin, transferrin, immunoglobulin G [IgG], IgA, haptoglobin, and α-1-antitrypsin), plasma was immunodepleted using the Multiple Affinity Removal System (MARS; Agilent, Santa Clara, California) according to the manufacturer's instructions. Before depletion, complete protease inhibitor cocktail was added (Roche Applied Science, Auckland, New Zealand). Depleted samples were buffer exchanged using 5 kd molecular weight cut-off centrifugal filters into 7 mol/L urea, 2 mol/L thiourea, and 1% C7BzO detergent. Protein content was determined using the 2D Quant protein assay (GE Healthcare, Auckland, New Zealand).

Difference Gel Electrophoresis Analysis of Plasma

Depleted and buffer-exchanged plasma samples were labeled with 200 pmol per 50 μg of protein each with CyDye minimal dyes (GE Healthcare) according to the manufacturer's protocol. For the pooled internal standard, equal amounts of all preeclamptic cases and healthy controls (n = 6 each group) included in the experiment were combined. To minimize dye bias samples were labeled with Cy3 or Cy5 as follows: within each group half of the controls were labeled with Cy3 and run on the same gels with cases labeled with Cy5. The other half of the controls were labeled with Cy5 and run with Cy3 labeled cases. All gels included the internal standard, labeled with Cy2.

Immobilized pH gradient (IPG) strips (11 cm, pH 4-7) were rehydrated with a multiplexed plasma sample comprising 50 μg protein for each CyDye-labeled case, control, and the pooled internal standard in rehydration buffer (7 mol/L urea, 2 mol/L thiourea, 1% C7BzO detergent, 1% IPG Buffer pH 4-7, 65 mmol/L DTT, and 0.002% bromophenol blue). First dimension separation was performed on a Multiphor II flatbed (GE Healthcare) followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using Criterion 8% to 16% Tris-HCl midigels run in a Dodeca cell (Bio-Rad, Bio-Rad Laboratories, Gladesville, NSW, Australia) for 30 minutes at 15 mA per gel, and 90 minutes at 30 mA per gel. All 12 DIGE gels were run simultaneously. Typhoon 9410 (GE Healthcare) digitized images were imported into DeCyder 2-D Differential Analysis software v6.5 (GE Healthcare) for spot detection and matched to an automatically chosen master gel using an estimated number of spots of 10 000 and applying a spot volume filter of greater than 26 000.

Statistical Analysis

Clinical data comparisons were performed using the Student *t* test for continuous clinical data and Fisher exact test for categorical data (SAS system 9). A P value $<.05$ was considered significant. Difference gel electrophoresis data were analyzed within DeCyder software using 2-way analysis of variance (ANOVA) of spot volumes normalized to the internal pooled standard. To eliminate false positive findings, standardized volume abundances of protein spots that were upregulated or downregulated between cases and controls, we implemented a false discovery rate (FDR) correction.[9] An FDR-corrected P value $<.05$ was considered significant. The resulting spots were submitted to liquid chromatography mass spectrometry (LC-MS/MS) analysis for protein identification. For quantitative analysis of Western blots, Student *t* test (1-tailed) and Mann Whitney *U* test (1-tailed) was used to compare volume intensities of bands between controls and cases. A P value $<.05$ was considered to be statistically significant.

Protein Identification by LC-MS/MS

Spots were excised from gels and digested with trypsin according to published methods[10] and submitted for LC-MS/MS analysis on a QSTAR XL ESI-qTOF (Applied Biosystems, Foster City, California) at the Maurice Wilkins Centre for Molecular Biodiscovery, the University of Auckland. Tandem MS/MS data were extracted from raw spectra using Mascot Distiller (Matrix Science, London, UK). Data were searched against the Swiss-Prot database (version 52.2, dated April 14, 2007), using the Mascot search engine v2.2.0 with the following parameters—taxonomy: human, semitrypsin cleavage with up to 1 missed cleavage allowed; fixed modification: propionamidation; variable modification: oxidation (M), mass tolerances $\pm$ 0.1 d, peptide charges 2+ and 3+. Positive identifications reported here had at least 2 unique peptides matched to the relevant database entry.

Western Blot Analysis

Native plasma (20 μg per lane) from women with subsequent preeclampsia and preeclampsia with SGA in comparison to healthy controls was separated on 4% to 12% NuPAGE gradient gels (Invitrogen) according to the manufacturer's instructions. (NUPAGE Technical Guide, IM-1001, 2003) Proteins were transferred to Immobilon FL transfer membrane (Millipore Corporation) and blocked overnight at 4°C with phosphate-buffered saline containing 0.1% Tween, 5% fatty acid- and IgG-free bovine serum albumin (Invitrogen). Detection of VN, HK, and cleaved HKa heavy chain and HKa light chain (domain 5, kininostatin) was accomplished with anti-human VN (Abcam #11591 at 1:5000), anti-human HK/HKa heavy chain (R&D Systems # MAB15692 at 1:1000), and anti-human HK/HKa light chain D5 region (Kininostatin, R&D Systems # AF1396 at 1:1000) antibodies, respectively. Respective Q655 nanodot conjugated IgGs (Invitrogen, 1:1000) were used as secondary antibodies. Signals were detected with a FujiLAS-3000 CCD camera with UV light for excitation and a 655 nmWB20 filter. Densitometric analysis of total signal intensity of Western blot bands was performed with Quantity-One analysis software v4.6.2 (Bio-Rad) using local background subtraction.

RESULTS

Maternal characteristics and pregnancy outcome are shown in Table 1. None of the women with preeclampsia had an SGA baby. Severe preeclampsia was seen in 3 women, 1 of whom developed HELLP syndrome.

Table 1. Maternal Characteristics and Pregnancy Outcome[a]

	Preeclampsia, n = 6	Controls, n = 6	*P* Value
Age (years)	31.7 (1.8)	30.7 (2.9)	.28
Caucasian	5 (83%)	6 (100%)	1.0
Early pregnancy blood sampling			
Gestation (weeks)	20.2 (1.0)	19.9 (0.5)	.51
Systolic blood pressure (mm Hg) at 20 weeks	114 (7)	108 (8)	.2
Diastolic blood pressure (mm Hg) at 20 weeks	69 (11)	63 (8)	.36
Blood sampling at preeclampsia			
Gestation (weeks)	34.7 (2.3)	34.7 (2.1)	1.0
Systolic blood pressure (mm Hg)	144 (13)	105 (9)	.0001
Diastolic blood pressure (mm Hg)	96 (6)	65 (6)	<.0001
Uterine artery resistance index (mean)	0.52 (0.14)	0.52 (0.08)	.94
Pregnancy outcome			
Max systolic blood pressure (mm Hg)	156 (8)	122 (11)	<.0001
Max diastolic blood pressure (mm Hg)	99 (5)	74 (9)	<.0001
Protein Creatinine Ratio (mg/mmol)	63 (50-246)	-	
24 hours proteinuria (g) N = 5	0.8 (0.5-4.5)	-	
Gestation at delivery (weeks)	36.2 (1.8)	40.8 (0.8)	.0002
Preterm delivery (<37 weeks)	4 (66%)	0 (0%)	.06
Birthweight (g)	2728 (734)	3760 (441)	.01
Customized birthweight centile	47.2 (36.5)	51.3 (21.7)	.81

[a] Values are mean (SD), median (interquartile range), or number (%).

Difference Gel Electrophoresis Analysis of Plasma

Spot detection and matching in DeCyder revealed on average 787 (SD 51) protein spots per gel using a 26k spot volume filter to exclude artifacts. The average spot matching rate was consistently at 83% (SD 3%) across all 12 gels. Comparison between control and healthy plasma samples revealed 19 protein spots that were significantly different in preeclampsia both before and at the time of disease compared to matched, healthy-gestation controls. Of these 19 spots, 9 remained significant ($P < .05$) after applying FDR correction to eliminate false positives. One spot was subsequently excluded as a technical artifact. Intergel variation using the DIGE method was negligent as evident by a low coefficient of variation (CV) for the Cy2 values (internal standard) of the 8 spots identified as being significantly different, with a mean (range) CV of 0.3% (0.19-0.4). The 8 protein spots were present in all cases and controls. Seven were located to the upper left region of the gel as part of 2 distinct spot trains indicative of protein isoforms due to posttranslational modifications (Figure 1). Three spots in the upper chain (spots 1-3) were upregulated (1.6-, 1.9-, and 1.7-fold) in preeclampsia whereas the 4 spots in the lower chain (spots 4-7) were downregulated (1.5-, 1.7-, 1.7-, and 1.5-fold) in comparison to healthy controls (Figure 2, Table 2). In addition to being differentially expressed in preeclampsia, only 1 spot (#8) showed gestation-related differences ($P < .05$).

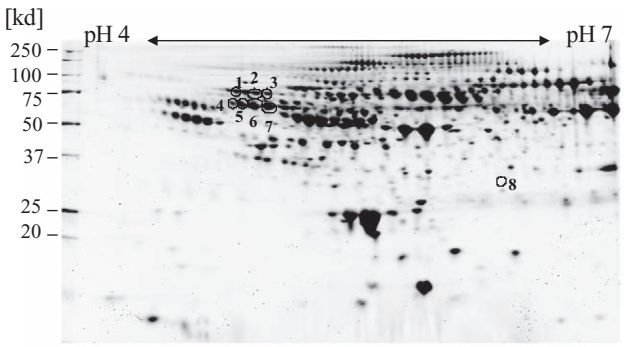

Figure 1. Digitized image of a 2D gel loaded with 150 μg of plasma depleted of the 6 most abundant proteins using the MARS system (Agilent, Santa Clara, CA). Protein spots of interest that were differentially regulated in preeclampsia are annotated by numbers and their protein IDs are listed in Table 2.

LC-MS/MS analysis revealed VN as the predominant protein in 5 of the spots (1 and 2; 5 to 7) identified in the spot trains (Figure 1, Table 2). As spot 3 was part of the same train as spots 1 and 2, its identity was assumed to be VN and it was not submitted for LC-MS/MS analysis. Vitronectin circulates in blood as both single-chain and double-chain molecules. Under reducing SDS-PAGE

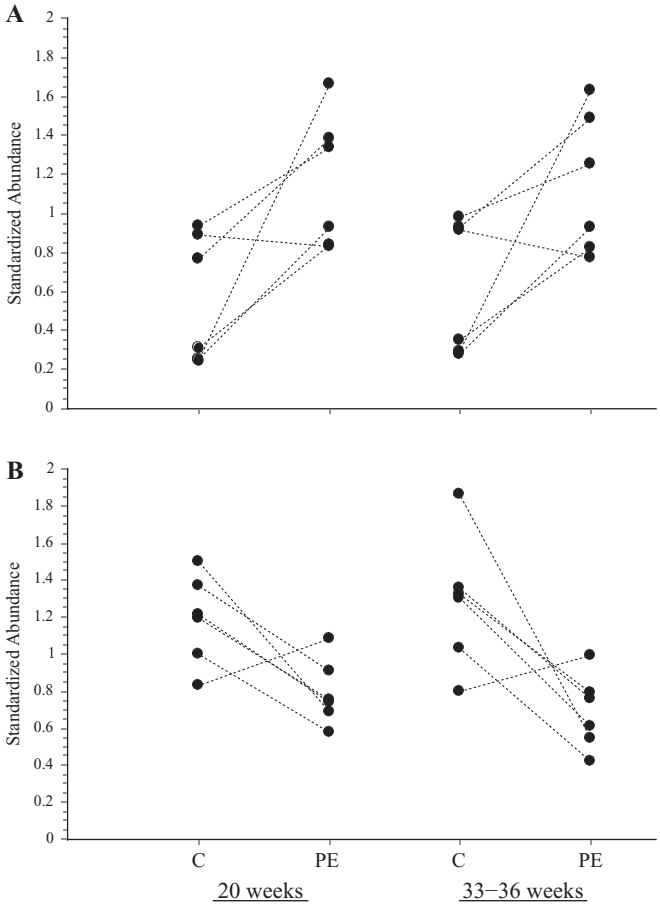

Figure 2. Spot plots of Cy2-standardized spot volumes for spot 2 (A) and spot 6 (B) identified by LC-MS/MS as human vitronectin. Single-chain vitronectin with a molecular weight of 75 kd was increased 1.9-fold in plasma prior to preeclampsia (PE) and once disease is clinically manifested, $P < .05$ (A), whereas the 65 kd component of the double-chain vitronectin molecule was decreased 1.7-fold in the preeclamptic group, $P < .05$.

conditions, 2 forms of VN were observed, at 75 kd (single-chain) and at 65 kd and 10 kd (2-chain VN). Figure 2A shows increased levels of spot 2 in preeclampsia, representing the single-chain VN molecule (75 kd) whereas the 65 kd moiety of the double-chain VN molecule (spot 6) was decreased in preeclamptic plasma (Figure 2B). Kininogen 1 was identified in the downregulated spots 4, 5, and 6, as was antichymotrypsin (SERPINA3) in spots 4 and 6 (Table 2). For spot 6, several proteins were identified by LC-MS/MS. Vitronectin was the predominant protein identified (11 unique peptides) in spot 6, but angiotensinogen, kininogen, and SERPINA3 were also identified with 5, 6, and 2 unique peptides, respectively (Table 2). The phenomenon of spot overlapping in 2-dimensional (2D) gels due to comigration of proteins has been reported previously.[11] Ultimately, other methods such as immunoassays or mass spectroscopy-based methods such as multiple reaction monitoring are required to assess the contribution of each protein to the observed differential expression. Spot 8 could not be identified due to the low peptide signal it generated.

Immunoblots Confirm Aberrant Plasma Levels of VN in Preeclampsia

Densitometric analysis of Western blots is in agreement with our proteomic findings (Table 2), confirming a significant 1.8-fold increase ($P < .05$) of the 75 kd VN band from plasma in women with preeclampsia alone ($n = 3$) compared to healthy controls ($n = 3$). The 65 kd moiety of the 2-chain VN molecule appeared to be downregulated in 2 women with preeclampsia alone (P2 and 3; Figure 3A) but this was not significant by densitometry ($n = 3$ per group). Plasma immunoblots from women with preeclampsia at 33 to 36 weeks showed a similar trend in densitometry values of VN isoforms to early pregnancy but this was not significant, $P = 0.08$ (Figure 3A). Contrary to preeclampsia alone, the immunoblot of plasma from women with subsequent preeclampsia and SGA shown in Figure 3 revealed significantly reduced levels of both the 75 kd and the 65 kd VN isoforms (1.5- and 1.2-fold, respectively; $P < .05$).

The mean uterine artery resistance index (RI; a measure of impedance to flow in the uterine arteries that is used to identify those at risk for subsequent development of pregnancy-related hypertension) at 20 ± 1 weeks' gestation was significantly elevated in women who subsequently developed preeclampsia with an SGA baby (mean RI 0.74, SD 0.07) compared to healthy controls (mean RI 0.51, SD 0.08), $P = .005$. This contrasts with the normal uterine artery RI values measured in the preeclampsia-alone group (Table 1).

Differential Processing of HK in Preeclampsia

Kininogens are thiol proteases that circulate as 2 separate isoforms produced by alternative splicing, HK, and low molecular weight kininogen (LK). In our Western blot analysis, we focused on HK because it is involved in coagulation, and thrombin- and plasmin-induced aggregation of platelets, processes that are commonly known to be altered in preeclampsia. High-molecular-weight kininogen contains 6 domains (D1-6) which are cleaved by plasma kallikrein, releasing the blood pressure–lowering

Table 2. Liquid Chromatography Mass Spectrometry (LC-MS/MS) Analysis of Protein Spots Differentially Regulated in Plasma of Women With Subsequent Preeclampsia and at Clinical Manifestation of Disease

Spot #	Fold Change	Accession	Description	Mascot Score	Coverage (%)	Peptides (unique)	MW (kd)	Isoelectric point (pI)
1	↑1.6	AAH05046	Vitronectin	275	14	6	55.2	5.55
2	↑1.9	NP_000629	Vitronectin precursor	679	29	17	55.2	5.55
4	↓1.5	AAA51543	α-1-antichymotrypsin (SERPINA3)	200	8	4	48.8	5.79
		NP_000884	Kininogen 1	124	6	3	48.9	6.29
5	↓ 1.7	NP_000629	Vitronectin precursor	248	12	5	55.2	5.55
		NP_000884	kininogen 1	73	6	3	49.2	6.29
6	↓ 1.7	NP_000629	Vitronectin precursor	538	20	11	55.2	5.55
		AAA51679	Angiotensinogen	232	13	5	53.4	5.78
		NP_000884	Kininogen 1	209	15	6	49.2	6.29
		AAA51543	α-1-antichymotrypsin (SERPINA3)	105	4	2	48.8	5.79
7	↓1.5	AAH05046	Vitronectin	166	9	4	55.2	5.55

Abbreviation: MW, molecular weight.

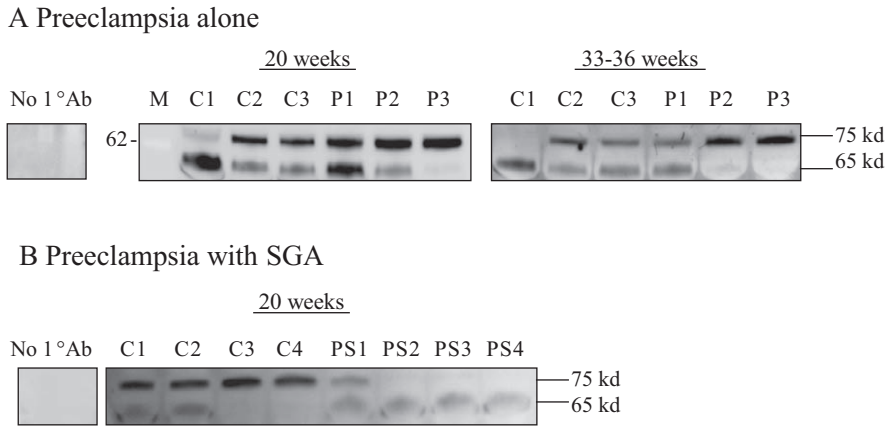

Figure 3. Aberrant levels of plasma vitronectin in preeclampsia. Immunoblot analysis of vitronectin in native plasma from (A) women with a healthy pregnancy outcome (C1-C3) compared to women at week 20 of gestation who subsequently developed preeclampsia (P1-P3) and the same women in late pregnancy (weeks 33 to 36) with clinically manifested disease; higher levels of the 75 kd single-chain vitronectin with lower levels of the 65 kd moiety of 2-chain vitronectin in preeclampsia; (B) decreased vitronectin levels in women at week 20 of gestation with subsequent preeclampsia complicated by small-for-gestational-age (SGA; PS1-4) compared to controls (C1-4). No-primary antibody control (panel to the left) showed no unspecific binding. M indicates molecular weight marker, band size at 62 kd.

peptide bradykinin from D4. Cleavage of HK results in the formation of HKa comprising a heavy chain (D1-3) which is linked by disulfide bond to a light chain (D5-6).[12] Domain 5 of HKa light chain inhibits endothelial cell migration and proliferation and has been termed kininostatin.[13] Western blot analysis of native plasma at week 20 of gestation revealed aberrant processing of HK in women with preeclampsia further complicated by SGA (PS1 to 4, Figure 4) but not in women with preeclampsia alone (data not shown). The anti-human kininogen antibody used here is specific for the detection of the mature chain of HK as well as the heavy chain, but not the light chain, of HKa. This coincides with approximate apparent molecular masses of 110 kd for mature HK and 60 kd for the cleaved form, HKa, under reducing SDS-PAGE conditions (Figure 4). Densitometry of immunoblots showed that HK (110 kd) was significantly reduced in 20 weeks plasma prior to preeclampsia with SGA compared to healthy controls, ($P < .05$, $n = 4$ per group). In 2 women with subsequent preeclampsia and SGA, a 56 kd band appeared to be overexpressed, with an associated reduction in the 110 kd and 60 kd bands

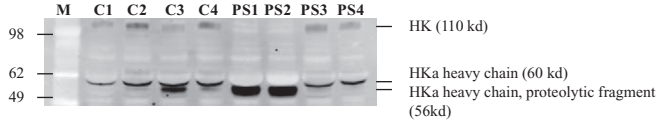

Figure 4. Differential proteolytic processing of high–molecular-weight kininogen (HK) in early pregnancy plasma prior to the onset of preeclampsia (PE) with small-for-gestational-age (SGA). Immunoblot analysis of HK and cleaved products (HKa) in plasma obtained at week 20 of gestation prior to clinically-manifest preeclampsia with SGA (PS1-PS4) and controls (C1-C4). Detection of the mature chain of HK (110 kd) as well as the heavy chain, but not the light chain, of HKa (60 kd) was achieved with anti-human kininogen specific to the heavy chain. Another proteolytic fragment of HKa (56 kd) was overexpressed in 2 women with subsequent preeclampsia and SGA (PS1 and PS2) accompanied by a loss of both mature HK and HKa heavy chain. M indicates molecular weight marker.

(Figure 4, PS1 and PS2). Densitometric analysis of the 60 kd and 56 kd bands, however, was not significant, ($P = .07$ and $P = .14$).

There was no difference in plasma levels of the anti-angiogenic kininostatin in preeclampsia, with or without SGA, compared to healthy controls (Western blot data not shown).

DISCUSSION

In the current study, maternal VN was differentially expressed in plasma both at 20 weeks' gestation prior to development of preeclampsia, and in the third trimester once preeclampsia was manifested clinically. Moreover, HK appears to be aberrantly processed in early pregnancy plasma from women who later developed preeclampsia with SGA but not in women with preeclampsia alone. This is the first report of aberrant processing of plasma VN and HK in preeclampsia.

Vitronectin is a multifunctional glycoprotein that modulates cell adhesion and migration, hemostasis, fibrinolysis, and complement-induced apoptosis and cell death.[14,15] It is found in plasma, platelets, and extracellular matrix.[16] Vitronectin is predominantly present in plasma in 2 molecular forms: a single chain (75 kd) molecule and a cleaved form consisting of 2 chains (65 and 10 kd) linked by an intrachain disulfide bond.[16] In addition, VN circulates as a high molecular weight (324 kd) complex with plasminogen activator inhibitor-1 (PAI-1) and is stored in the α-granules of platelets.[17,18] Vitronectin is heavily glycosylated with a carbohydrate moiety contributing approximately 30% of its actual molecular weight. This explains the discrepancy between the estimated molecular weight as shown in Table 2 derived from database searches of peptide sequences and the actual molecular weight observed in 2D gel maps of plasma (Figure 1).

In both DIGE analysis and Western blots, we found increased levels of the single-chain 75 kd VN in women who later developed preeclampsia alone and a relative reduction in the 65 kd VN. In contrast, we found decreased plasma levels of both VN isoforms prior to preeclampsia with SGA. Differences in the prevalence of a single nucleotide polymorphism near the cleavage site of the 75 kd VN (residue 381) can alter the ratio of circulating 75- and 65-kd V.[19,20] No association with preeclampsia has been investigated to date for polymorphisms of the *VN* gene.

Changes in circulating 75 kd and 65 kd VN prior to the development of preeclampsia may reflect binding of VN to other molecules, and/or altered production or movement of VN from the plasma into the vessel wall in response to damage to the endothelium.[16] Following platelet activation, VN is released from α-granules.[17] Vitronectin harbors functional binding domains for several ligands including PAI-1, urokinase-type plasminogen activator receptor (uPAR), integrins, thrombin-antithrombin III complex (TAT), activated protein C, collagen, plasminogen, and heparin.[14] Binding of VN to any of these molecules may modify its circulating levels and, possibly, its molecular forms, by modulating its susceptibility to proteolytic processing. With increasing disease severity, as seen in preeclampsia with an SGA baby, depletion of 75 kd VN may reflect consumption of VN through excessive binding to ligands.

A number of biological processes known to be involved in the pathogenesis of preeclampsia are modulated by VN. Elevated circulating VN could contribute to the hypercoagulable state associated with preeclampsia by complexing with and modifying the action of PAI-1. Of particular relevance to preeclampsia is the role of VN in cell adhesion and migration. This is of importance in trophoblast invasion to establish the uteroplacental vascular bed.[21,22] Fisher and coworkers showed that integrin switching occurs during human trophoblast invasion and that VN's main integrin receptor, $\alpha v\beta_3$, has an integral role during this process.[21] Aberrant attachment to VN-coated surfaces has been observed in trophoblast cells from preeclamptic pregnancies.[23] Vitronectin may also have a role in the repair of maternal endothelium following endothelial cell damage that is characteristic of preeclampsia.[24,25] It binds to $\alpha v\beta_3$, uPAR, and caveolin in the membrane of endothelial cells, triggering an

intracellular signaling cascade that mediates focal adhesion and migration.[12]

In the current study, we have shown differential processing of HK in women with subsequent preeclampsia further complicated by SGA. In the Difference Gel electrophoresis (DIGE) study of 20-week plasma, women who later developed preeclampsia showed reduced levels of HK at approximately 60 to 65 kd, but given the small sample size, we failed to confirm this by Western blots. In PE with SGA, we observed a loss of the mature HK (110 kd) band on Western blots that was not observed in controls. HK is a plasma protein that is cleaved by kallikrein resulting in the release of the nonapepetide bradykinin and HKa, a derivative of HK. High-Molecular-Weight Kininogen, together with prekallikrein and factor XII, comprise the contact system of coagulation. Like VN, HK plays an important role in cellular adhesion and vascular permeability.[12] Our results suggest differential cleavage of HK in early pregnancy plasma prior to the onset of preeclampsia with SGA.

More than three decades ago, Stark proposed that preeclampsia may result from an imbalance in proteolytic control.[26] At the same time, Folkman proposed that proteolytic fragments of proangiogenic proteins could be potent inhibitors of angiogenesis.[27] Examples of this paradigm are angiostatin derived from plasminogen and kininostatin derived from domain 5 of HK or HKa.[13] Of interest, it has recently been shown that kininostatin can disrupt the VN-$\alpha v\beta_3$-uPAR-caveolin complex on endothelial cells, thereby inhibiting cell signaling that mediates endothelial cell adhesion and spreading.[28] Although we identified a 56-kd fragment, which coincides with the molecular weight of the HKa light chain that contains the anti-angiogenic D5 domain (kininostatin), the anti-human HK/HKa heavy chain monoclonal antibody used here is reported not to bind to the light chain of HKa. Using a polyclonal to HK/HKa light chain (domain 5), we were unable to confirm differences in kininostatin levels in 20-week gestation plasma, prior to the onset of clinically-apparent preeclampsia. Besides kallikrein, kininogens are also subject to proteolysis by enzymes such as plasmin[29] and elastase released from neutrophils and granulocytes.[30,31] Plasma elastase levels are known to be elevated in preeclampsia as a result of neutrophil activation.[32]

In summary, our results are suggestive of aberrant proteolytic processing of both VN and HK before the onset of preeclampsia and at the time of presentation with clinical disease. These proteins, in combination with other biomarkers, may prove to be useful in the early detection of preeclampsia subgroups. Further studies are necessary to elucidate the potential roles of VN and HK in the biological processes related to trophoblast invasion and vascular remodeling in response to endothelial damage associated with preeclampsia.

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