Aberrant Glycosylation of Plasma Proteins in Severe Preeclampsia Promotes Monocyte Adhesion

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Abstract

Glycosylation of plasma proteins increases during pregnancy. Our objectives were to investigate an anti-inflammatory role of these proteins in normal pregnancies and determine whether aberrant protein glycosylation promotes monocyte adhesion in preeclampsia. Plasma was prospectively collected from nonpregnant controls and nulliparous patients in all 3 trimesters. Patients were divided into cohorts based on the applicable postpartum diagnosis. U937 monocytes were preconditioned with enzymatically deglycosylated plasma, and monocyte adhesion to endothelial cell monolayers was quantified by spectrophotometry. Plasma from nonpregnant controls, first trimester normotensives, and first trimester patients with mild preeclampsia inhibited monocyte–endothelial cell adhesion ($P < .05$), but plasma from first trimester patients with severe preeclampsia and second and third trimester normotensives did not. Deglycosylating plasma proteins significantly increased adhesion in all the cohorts. These results support a role of plasma glycoprotein interaction in monocyte–endothelial cell adhesion and could suggest a novel therapeutic target for severe preeclampsia.

Keywords

adhesion, glycosylation, monocyte, pregnancy, severe preeclampsia

Introduction

Glycosylation is the addition of carbohydrate chains to proteins. It is one of the most abundant posttranslational modifications in humans and is associated with many physiologic and pathologic events, including cell adhesion, migration, cell growth, cell differentiation, and tumor invasion.¹ Glycosylated proteins also modulate many events in the acute inflammatory response, including the regulation of immunocyte adhesion to endothelial cells during inflammation.^{2–4} Several acute phase proteins in pregnancy are heavily glycosylated, and their numbers increase exponentially throughout gestation.^{4–6} This increase in glycosylated proteins may be important in regulating the immune response during pregnancy.⁵

In response to inflammatory stimuli, monocytes adhere to endothelial cells, migrate into the intima, and ultimately transform into activated macrophages and foam cells with cytokine release.⁷ It is well known that glycans on the surface of endothelial cells contribute to the monocyte adhesion and the development of vascular inflammatory disease.³ More recent evidence suggests a role of circulating plasma glycoproteins in attenuating this inflammatory response by competitively inhibiting endothelial–monocyte interaction.⁸ However, it is unknown how the

simultaneous increase in glycoproteins and acute phase proteins in the second and third trimesters of pregnancy affects monocyte adhesion and/or immunomodulation of pregnancy.

Severe preeclampsia is an inflammatory condition characterized by both aberrant plasma protein glycosylation patterns and activated immunocytes in the maternal circulation.^{5–7} Although the exact etiology of preeclampsia is unknown, the syndrome is associated with the genetic susceptibility locus PREG1, which encodes the gene for N-acetylgalactosamine

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transferease (GalNAcT), the enzyme catalyzing the addition of O-linked glycosyl chains to plasma proteins.⁹⁻¹¹ However, the role of O-linked glycosylation in severe preeclampsia and its influence on monocyte–endothelial cell adhesion remains to be determined.

Since circulating glycoproteins are elevated in pregnant women and glycoproteins are known to inhibit endothelial–monocyte interactions, we hypothesized that removing glycosylation chains from maternal plasma proteins would increase monocyte adhesion to endothelial cells. The objectives of this study were to determine (1) whether deglycosylation of maternal plasma proteins from nulliparous normotensive women in the first, second, and third trimesters increases monocyte adhesion to endothelial cells and (2) whether plasma from patients with severe preeclampsia modulates monocyte–endothelial cell adhesion.

Patients and Methods

Participants

The Madigan Army Medical Center Institutional Review Board approved the protocol (protocol #205031). Healthy nulliparous women with no history of heart disease, cardiovascular abnormalities, preexisting diabetes, or other chronic medical conditions were invited to participate. Patients were excluded from study participation if they were under 18 years of age or had a history of infertility treatment. Maternal blood was collected by venipuncture at 8 to 12, 16 to 22, and 34 to 38 weeks gestation in BD vacutainer EDTA-containing tubes (Becton, Dickinson, and Company, Franklin Lakes, New Jersey) and centrifuged at 1500g at 4°C in a Sorvall RC3C centrifuge (Sorvall Instruments, ThermoFisher Scientific, West Palm Beach, Florida). Plasma supernatant was supplemented with protease inhibitor cocktail (catalog number 1-836-145; Roche Diagnostics, Mannheim, Germany) and stored at –140°C. Following parturition, antenatal vitals, laboratory values, outpatient records, and clinical notes at delivery were reviewed. Review of antepartum records confirmed diagnoses of normotensive, mild preeclampsia, or severe preeclampsia according to the current American College of Obstetricians and Gynecologists (ACOG) guidelines.^{12,13} Patients with mild preeclampsia were diagnosed by more than 300 mg of protein in a 24-hour urine culture and blood pressures ≥ 140 mm Hg (systolic) or 90 mm Hg (diastolic) on 2 independent measurements spaced at least 6 hours apart after 20 weeks gestation. Severe preeclampsia hypertensive and proteinuria thresholds were 160 mm Hg (systolic) or 110 mm Hg (diastolic), respectively, and more than 5 g of protein was collected in 2 separate specimens more than 4 hours apart. In this study, patients who were diagnosed with mild preeclampsia were all diagnosed and delivered beyond 37 weeks gestation compared to patients with severe preeclampsia who were diagnosed and delivered before 37 weeks gestation. Venous blood was collected from nonpregnant women during the menstrual phase of the ovulatory cycle and processed as described for the gravidas.

Adhesion Assays

Human umbilical cord endothelial cells (HUVEC, CRL-1730) and human histiocytic lymphoma monocytes (U937, CRL-1593.2) were cultured in 96-well format as recommended by the supplier (American Tissue Type Collection, Manassas, Virginia). U937 cell lines have been used in established in vitro models of endothelial cell–monocyte adhesion in the studies of pregnancy and other inflammatory conditions.¹⁴⁻¹⁶ Culture media was supplemented with 100 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) to preserve physiological pH.

Plasma total protein content was determined by bicinchoninic acid (BCA) assay (ThermoFisher Scientific) and confirmed by gel electrophoresis with either Sypro Ruby or colloidal blue according to manufacturer's instructions (Invitrogen, Carlsbad, California). Plasma was pretreated overnight in either enzyme reaction buffer (2 mmol/L CaCl₂, 200 mmol/L sodium acetate pH 5.4) or combination of glycosidases in reaction buffer (O-glycosidase from *Diplococcus pneumonia* [11 347 101 001] and neuraminidase [sialidase, 11 080 725 001], Roche Diagnostics) in accordance with the previously published methods (see Figure 1A).¹⁴

The adhesion assays were performed as described (see Figure 1B).^{8,16} Monocytes were fluorescently labeled with 1 μ mol/L 2',7'-bis-(carboxyethyl)-5(6')-carboxyfluorescein acetoxymethyl ester (Calbiochem/EMD, San Diego, California) in assay buffer (Hanks balanced salt solution, 0.1% bovine serum albumin [pH 7.4]; Sigma Chemicals, St Louis, Missouri) for 25 minutes at 37°C, then resuspended in assay buffer supplemented with 5% glycosidase-treated plasma (v/v total protein) or buffer-treated control plasma. Since plasma volume expands and plasma protein concentration decreases as pregnancy progresses, we determined total plasma protein concentration of all plasma specimens by bicinchoninic acid (BCA) assay (Biorad Laboratories, Hercules, California). Plasma volumes were adjusted to achieve equal protein concentrations among all the cohorts. Equal plasma protein concentrations among trimesters were confirmed by Sypro Ruby or colloidal blue staining (Life Technologies, Carlsbad, California) performed in parallel with adhesion assay experiments. To ensure that maternal plasma did not alter HUVECs directly, excess maternal plasma was washed from monocytes prior to the application to HUVEC monolayers (plated at 100 000 cells/well). Monocytes were incubated at 37°C for 1 hour or overnight with 5% deglycosylated plasma (or buffer-treated control plasma) in the presence of glycosidases. In direct competition experiments, unconditioned monocytes and deglycosylated plasma were immediately added to HUVECs such that the total HUVEC/monocyte exposure to glycosidases was 10 minutes (see Figure 1B). Cells were washed, resuspended in assay buffer, and coincubated with HUVECs for 10 minutes at 37°C. Unattached monocytes were aspirated from the HUVEC monolayers. Adherent monocytes were measured at excitation wavelength 485 nm and emission wavelength 530 nm by Synergy HT microplate spectrophotometer (Biotek Instruments, Inc,

Figure 1. Schematic of the sites of action of glycosidase enzymes and adhesion assay. A, Neuraminidase cleaves sialic acids, and O-glycosidase cleaves sialic acid-free GalNAc (N-acetylgalactosamine). B, Adhesion assay protocol: fluorescently labeled U937 monocytes were conditioned with deglycosylated maternal plasma and O-glycosidases/neuraminidase for: (a) 0 hour (ie, monocytes and plasma were simultaneously added to endothelial cells); (b) 1 hour; or (c) overnight (16 hours). Preconditioned monocytes were coincubated with human umbilical cord endothelial cell (HUVEC) monolayers for 10 minutes. Unlabeled monocytes were washed away, and residual fluorescence was measured.

Winooski, Vermont). Adherent monocytes were visualized at 4× magnification using an Olympus IX-71 reflected fluorescence microscope (Olympus, Melville, New York) equipped with an inline charge-coupled device camera. Images were digitally captured with uniform exposure time between wells. Mean pixel intensity (integrated density) was measured using ImageJ software (National Institutes of Health, Bethesda, Maryland). As a positive assay control, HUVECs were pretreated with 10 µg/mL lipopolysaccharide (Sigma Chemical). To determine whether glycosidases affected monocyte adhesion to endothelial cells in the absence of plasma, negative controls were included in each microtiter plate in which monocytes and/or endothelial cells were pretreated with enzyme cocktail or reaction buffer alone. Pilot time course experiments confirmed that 1-hour incubation with deglycosylated plasma and monocytes was sufficient to modulate monocyte–endothelial cell interaction without disrupting monocyte cell surface glycans. Negative controls confirmed that monocyte adhesion was not affected by 1 hour glycosidase treatment in the absence of maternal plasma.

Digoxigenin Glycan Immunoassays

Hydroxyl moieties on glycoconjugate sugars were oxidized to aldehydes by metaperiodate treatment and covalently labeled to the hydrazide moiety of digoxigenin (DIG) using a Roche

Applied Biosciences DIG-glycan detection system. Protein concentration was determined by BCA as described earlier. Equal concentrations of the labeled proteins were electrophoresed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% to 20% gradient tricine gels (Invitrogen) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corporation, Bedford, Massachusetts). The membranes were incubated with alkaline phosphatase-conjugated antibodies detecting the DIG-labeled glycoproteins. Incubation with nitro blue tetrazolium chloride initiated a colorimetric reaction with the alkaline phosphatase-conjugated DIG antibody in the region of glycoprotein mobility on the gel. Parallel SDS-PAGE gels were loaded with maternal plasma and stained with Sypro Ruby total protein dye according to manufacturer's instructions to confirm equal loading of proteins on the gels (Life Technologies). Molecular weights were calculated by interpolation to a molecular weight standard (30–460 kDa; Hi Mark, Life Technologies) run concomitantly with the plasma using imaging software (Kodak Molecular Imaging Software; Carestream Health, Inc, Rochester, New York).

Western Blot Analysis

Equal protein concentrations were loaded onto 10% to 20% tricine gradient gels, and proteins were electrophoretically

separated according to manufacturer's recommendations (Invitrogen). Proteins were electrophoretically transferred to PVDF membranes (Millipore) in glycine buffer containing 20% methanol using an Invitrogen transfer apparatus. Membranes were blocked overnight in Tris-buffered saline containing 10% nonfat dry milk. Membranes were incubated in primary antibodies for 4 hours in blocking buffer supplemented with 0.1% Tween-20 (Sigma Chemical). Membranes were washed and incubated in secondary antibody for 1 hour. Immunolabeled proteins were visualized and quantified using a Kodak In Vivo imaging system (Carestream Health). The following primary/secondary antibody combinations were used (1) apolipoprotein E (ApoE, #7620; Abcam, Cambridge, Massachusetts)/Qdot 655 (#Q11821MP; Life Technologies), (2) C1-inhibitor (C1INH, #105316; GeneTex, San Antonio, Texas)/horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (#Q11821MP; GeneTex), and (3) ApoC-III (raised in goat, Genway Biotech, Inc, San Diego, California (#18-732-29228-3)/HRP conjugated anti-goat secondary antibody; GeneTex). After antibody binding, the bands were visualized by ECL reagent (GE Healthcare). Molecular weights were determined as described earlier.

In-Gel Trypsin Digestion

Colloidal blue-stained fragments were excised and analyzed at the Fred Hutchinson Cancer Research Center Proteomics Core Facility (Seattle, Washington). The gel slices were destained with 25 mmol/L ammonium bicarbonate in 50% acetonitrile and subsequently dehydrated using acetonitrile. The gel slices were rehydrated in a solution of 5 ng/ μ L trypsin (Promega Corporation Fitchburg, Wisconsin) in 50 mmol/L ammonium bicarbonate and digested overnight at 37°C. The peptides were extracted using 5% (v/v) formic acid in water after 30 minutes incubation and then with acetonitrile. The extracts were dried in a SpeedVac and concentrated/desalted using a ZipTip C18 (Millipore Corporation) before the subsequent mass spectrometry (MS) analysis.

Liquid Chromatography/Tandem Mass Spectrometry Analysis

Liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis was performed with an Easy-nLC 1000 (Thermo Scientific) coupled to an Orbitrap Elite mass spectrometer (Thermo Scientific). The LC system configured in a vented format consisted of a fused-silica nanospray needle (PicoTip emitter, 75 μ m ID; New Objective) packed in house with Magic C18 AQ 100 Å reverse-phase media (25 cm; Michrom Biore-sources Inc), and a trap (IntegraFrit Capillary, 100 μ m ID; New Objective, Woburn, Massachusetts) containing Magic C18 AQ 200 Å (2 cm).¹⁷ The peptide sample was diluted in 10 μ L of 2% acetonitrile and 0.1% formic acid in water, and 8 μ L was loaded onto the column and separated using a 2-mobile-phase system consisting of 0.1% formic acid in water (1) and 0.1% acetic acid in acetonitrile (2). A 60-minute gradient from 2% to 35% acetonitrile in 0.1% formic acid at a flow rate of 400

nL/min was used for chromatographic separations. The mass spectrometer was operated in a data-dependent MS/MS mode over the m/z range of 400 to 1800. For each cycle, the 20 most abundant ions from the precursor scan were selected for MS/MS analysis using 35% normalized collision energy. Selected ions were dynamically excluded for 30 seconds.

Mass Spectrometry Data Analysis

Raw MS/MS data were submitted to the Computational Proteomics Analysis System,¹⁸ a Web-based system built on the LabKey Server v11.2 (LabKey Corporation, Seattle, Washington) that utilizes Trans-Proteomic Pipeline v4.3,¹⁹ and searched using the X! Tandem search engine (2009.10.01.1)²⁰ against IPI human protein database (v3.87), which included additional common contaminants. Oxidation of methionine was set as a variable modification. The mass tolerances were set ± 2 and ± 0.5 Da for precursor and fragment ions, respectively. The enzyme was set to trypsin, and up to 2 missed cleavages were permitted. The search output files were analyzed and validated by ProteinProphet (Seattle Proteome Center, Seattle, Washington).²¹ Peptides were filtered with PeptideProphet error rate of 0.01, and proteins with a probability score of were accepted.²²

Statistical Evaluation

Statistical significance among cohorts was determined by Kruskal Wallis analysis of variance (ANOVA) with post hoc Dunnett t test, with significance set a priori at $P < .05$. Repeated measures ANOVAs were used to determine the statistical differences among metrics across trimesters. The Wilcoxon signed-rank test was used to analyze statistical significance among enzyme-treated and control pairs of data. Treatment groups were considered statistically different from the hypothetical value of 100% (ie, no change after treatment with enzyme mixture) if $P < .05$.

Results

Maternal age at obstetric intake was 22 ± 3 years. The average gestational age of delivery was 39.1 ± 1.2 weeks in uncomplicated pregnancies, 38.7 ± 0.9 weeks in patients with mild preeclampsia, and 33.4 ± 6.2 weeks in patients with severe preeclampsia ($P < .05$ for severe preeclampsia relative to other cohorts).

Assessment of Plasma Protein Glycosylation in Normal Pregnancy

To determine the effect of maternal plasma glycoproteins on monocyte adhesion, maternal plasma was enzymatically deglycosylated with O-glycosidase and neuraminidase. Enzymes were selected to simulate the net result of aberrant GalNAc transferase activity in preeclampsia.^{9,10} To confirm that the enzyme treatment was effective, we evaluated plasma before and after glycosidase treatment by modified DIG glycan Western blot analysis. Sypro Ruby total protein staining confirmed

Figure 2. Decrease in maternal plasma protein glycan immunoreactivity after treatment with O-glycosidase and neuraminidase in proteins comigrating with apolipoprotein E (ApoE), C1-inhibitor (C1INH) protein, and lipoprotein B-associated ApoC-III. A, Sypro Ruby staining for total protein content demonstrates approximately equal loading among proteins in all lanes. Sypro Ruby stains all the glycosylated or unglycosylated proteins. B, DIG glycan immunoreactivity for carbohydrate in maternal plasma from $n = 10$ donors per trimester (pooled plasma) in the presence (+) or absence (–) of 48-hour incubation with neuraminidase and O-glycosidase. Immunoreactive bands indicate the presence of glycan groups. Decrease in immunoreactivity postglycosidase treatment (+) denotes O-linked glycosylation at the corresponding molecular weight. C, Immunoreactivity for ApoE was evident at 37 kDa in maternal plasma. D, Immunoreactivity for C1INH protein was detected at 104 kDa by Western Blot. Immunoreactivity was higher in third trimester than first trimester for both the proteins. Molecular weights comigrated with the DIG glycan gel proteins at identical molecular weights. First and third trimesters from a single representative normotensive patient are shown for each protein. Results are representative of 5 normotensive patients in each trimester. E, Immunoreactivity for ApoC-III was detected at >460kDa and approximately 75kDa by Western blot. DIG indicates digoxigenin.

equal protein loading in each lane (Figure 2A), and deglycosylation was confirmed by a qualitative reduction in DIG glycan immunoreactivity after enzymatic treatment (Figure 2B). Enzymatic treatment reduced DIG glycan immunoreactivity most visibly in protein bands that mapped to >460, 104, and 37 kDa by interpolation to molecular weight standards and increased abundance of a protein at approximately 90 kDa (Figure 2B). The highest molecular weight protein was outside the range of the molecular weight standards used (30–460 kDa). O-glycosylated, high abundance plasma proteins known to migrate at molecular weights corresponding to 104 and 37 kDa include C1INH protein and ApoE, respectively.^{8,9,14} Western blots probed with antisera for ApoE immunostained a single band at 37 kDa, consistent with the molecular weight of glycosylated ApoE (Figure 2C). Blots probed with C1INH antisera immunostained a single band at 104 kDa, consistent with the molecular weight of glycosylated C1INH (Figure 2D).⁸ The molecular weights of the highly immunoreactive bands at 37 and 104 kDa in the DIG glycan immunoblots (Figure 2B)

corresponded to the predicted molecular weights of the proteins identified by Western blot as ApoE (37 kDa, Figure 2C) and C1INH (104 kDa, Figure 2D).

The band at >460 kDa did not correspond to any known high-abundance, O-glycosylated protein in maternal plasma. To identify the potential targets for Western blot analysis, we subjected the excised band to MS analysis. The MS analysis identified both ApoC-III and ApoB in excised gel slices from the heavily glycosylated band at >460 kDa (Table 1; Figure 3). The ApoB-containing lipoprotein (513 kDa) binds to ApoC-III (8.8–9.7 kDa), and the complex migrates at 513 kDa in gel electrophoresis experiments.²³ The ApoC-III is O-glycosylated at threonine 74, but ApoB does not contain consensus sites for O-glycosyl moieties.^{24,25} To determine whether ApoC-III migrated at a molecular weight commensurate with the DIG glycan immunoreactive band at >460 kDa, we performed Western blot analysis. Probing with ApoC-III antisera identified 2 bands, >460 and 75 kDa (Figure 2E). The 75-kDa band immunoreactive for ApoC-III antisera corresponded to the

Table 1. Mass Spectrometry Identification of ApoB and ApoC-III in the >460-kDa Gel Fragment.

Protein	Probability	Peptides	Unique Peptides	Amino Acid Coverage
ApoC-III	1	3	2	27.3
ApoB	1	1981	230	56.8

Abbreviations: ApoC-III, apolipoprotein C-III.

molecular weight of serum albumin (67 kDa) complexed to glycosylated ApoC-III (8.8-9.7 kDa).²⁶ In addition, a doublet at 45 to 50 kDa was immunoreactive for ApoC-III antisera in some patients.

Assessing the Effect of Plasma Protein Glycosylation on Monocyte Adhesion in Normal Pregnancies

To determine the optimal incubation time for monocytes with maternal plasma prior to the adhesion assays, we compared 3 protocols: 0 hour (ie, simultaneous application of monocytes and plasma to endothelial monolayers), 1 hour, or overnight treatment of monocytes with 5% maternal plasma (see Figure 1B for experimental paradigm). Results of these experiments are shown in Figure 4. When maternal plasma was added to the monocytes and then immediately coincubated with endothelial monolayers (0 hour, left set of bars in Figure 4), plasma from all 3 trimesters inhibited monocyte adhesion to endothelial cell monolayers. In contrast, only first trimester plasma inhibited monocyte adhesion after 1 hour of incubation with monocytes (middle set of bars in Figure 4). Overnight treatment of monocytes with maternal plasma resulted in no significant difference in adhesion after monocytes were incubated with first trimester plasma; second and third trimester plasma were not investigated in this study (right set of bars in Figure 4). In pilot time course experiments, longer incubation times disrupted monocyte surface glycans as evidenced by increased monocyte–endothelial cell adhesion in the absence of plasma (data not shown). Based on these results, 1 hour of incubation was selected for all further experiments for the following reasons: (1) coculture incubation times longer than 1 hour nonspecifically increased monocyte–endothelial cell adhesion and (2) shorter incubation times resulted in the same degree of inhibition in all 3 trimesters, suggesting that the effects at this time point were an effect of plasma proteins in general rather than an effect specific to gestation.

Preconditioning monocytes with glycosidases for 1 hour in the absence of maternal plasma had no effect on monocyte adhesion to endothelial cell monolayers as assessed by microscopy (Figure 5A, left panels) and spectrophotometry (Figure 5B, first set of bars; 36.6 ± 5.8 fluorescence units without enzymes and 40 ± 14.8 fluorescence units with enzymes [adherent monocytes]). In pilot time course experiments, longer incubation times disrupted monocyte surface glycans as evidenced by increased monocyte–endothelial cell adhesion in the absence of plasma (data not shown). However, when

monocytes were pretreated with both glycosidase enzymes and deglycosylated plasma for 1 hour, all plasma-containing groups treated with enzymes showed an increase in adhesion relative to no-enzyme controls (Figure 5B). Conditioning monocytes with a combination of glycosidases and glycosidase-treated plasma potentiated monocyte adhesion to HUVECs in all cohorts in the fluorescence spectrophotometry experiments (from 19.2 ± 5.2 to 43.25 ± 5.2 mean pixel intensity units after glycosidase treatment of trimester 1 plasma; 38.6 ± 4.3 to 58.7 ± 11.7 units after glycosidase treatment of trimester 2 plasma; and 41 ± 4.2 to 66.0 ± 12.8 units after glycosidase treatment of trimester 3 plasma; Figure 5B; $P < .05$). Monocyte treatment with first trimester plasma resulted in significantly lower monocyte adhesion than no-plasma controls (from 36.6 ± 5.8 fluorescence units in no-plasma controls to 19.2 ± 5.2 units in first trimester plasma, $P < .05$). Monocyte clustering was also elevated in second and third trimester relative to the other cohorts (Figure 5A). Results are representative of 2 independent experiments ($n = 6$ gravidas per cohort). Comparable results were obtained when adherent monocytes were evaluated by quantitative fluorescence microscopy. Coincubation of recombinant C1INH protein with monocytes and trimester 1 plasma resulted in a 1.96-fold reduction in monocyte adhesion (replicate experiments with $n = 4$ patients plated in quadruplicate; standard error of ± 0.25).

Since we used a malignant cell line in this study, we wanted to confirm our results in a more physiologically relevant system.^{8,14-16} Therefore, we repeated the adhesion assays with peripheral blood mononuclear cells (PBMCs) harvested from second trimester maternal blood. We incubated the PBMCs with maternal plasma ($n = 3$). Incubation with first trimester plasma decreased PBMC adhesion to endothelial monolayers to approximately $75\% \pm 10\%$ of no plasma controls ($n = 3$), whereas trimester 3 plasma caused a negligible decrease in PBMC–endothelial cell adhesion ($95\% \pm 3\%$). Incubation of PBMCs for 1 hour with deglycosylated first or third trimester plasma increased PBMC–monocyte adhesion by $163\% \pm 6\%$ and $124\% \pm 19\%$ over untreated controls ($n = 3$).

The Effect of First Trimester Plasma Protein Glycosylation on Monocyte Adhesion in Preeclampsia

Because only first trimester plasma significantly attenuated monocyte adhesion to endothelial cells in the 1-hour protocol, we used this protocol to compare monocyte–endothelial cell adhesion after conditioning with first trimester plasma from uncomplicated pregnancies, nonpregnant controls, mild preeclampsia cases, and severe preeclampsia cases (Figure 6). Monocyte–endothelial cell adhesion was significantly lower than no-plasma controls after preconditioning with plasma from nonpregnant controls, normotensive individuals, and patients with mild preeclampsia (32.2 ± 5.3 , 13.6 ± 3.7 , 13.2 ± 0.9 , and 12.8 ± 0.6 mean pixel intensity units for no plasma, nonpregnant, normotensive, and mild preeclampsia, respectively). However, severe preeclampsia plasma did not significantly attenuate adhesion (compare no plasma controls

Figure 3. Mass spectrometry identification of highest abundance peptides in >300-kDa band excised from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. Tandem mass spectrometry analysis identified 2 unique peptides for ApoC-III and 230 unique peptides for ApoB with high confidence (Peptide Prophet probability 1.0). Spectral matches of 2 representative peptides are shown for (A) ApoC-III and (B) ApoB. ApoB indicates apolipoprotein B.

at 32.2 ± 5.3 and severe preeclampsia at 21.9 ± 4.1 in Figure 6). Furthermore, the difference in adhesion between patients with severe preeclampsia and normotensive individuals was statistically significant, with more adherent monocytes in the patients with severe preeclampsia (21.9 ± 4.1 units) than normotensive controls (13.2 ± 0.9 units; $P < .05$).

To determine whether removal of O-glycosylation linkages from plasma proteins could reverse the attenuation of monocyte adhesion observed in normotensive and mild preeclampsia cohorts, we enzymatically removed glycosylated residues from maternal plasma proteins by treatment with O-glycosidase and neuraminidase for 48 hours prior to adhesion assays. Preconditioning monocytes with deglycosylated maternal plasma

significantly increased adhesion to endothelial cells in all cohorts (Figure 7). Notably, preconditioning monocytes for 1 hour with glycosidases only (no maternal plasma) negligibly increased monocyte adhesion to $110\% \pm 1\%$ of no-enzyme treatment; see “no plasma controls” in Figure 7, left bar. Glycosidase treatment reversed the plasma-associated attenuation observed in Figure 6. Conditioning monocytes with deglycosylated plasma increased adhesion by $164\% \pm 31\%$ (in nonpregnant controls), $153\% \pm 17\%$ (normotensive individuals), $137\% \pm 23\%$ (mild preeclampsia cases), and $195\% \pm 49\%$ (severe preeclampsia) over no-plasma controls. Although there was a trend toward greater potentiation of monocyte adhesion in the patients with severe preeclampsia than the other cohorts,

Figure 4. Optimization of adhesion protocol: incubation of monocytes with maternal plasma results in differences in adhesion among trimesters at 1 hour. Fluorescently labeled monocytes were preconditioned 0, 1, or 16 hours (overnight) with maternal plasma at various trimesters or vehicle (no plasma control) and then applied to human umbilical cord endothelial cell (HUVEC) monolayers. Nonadherent cells were washed away, and the remaining adherent cells were quantified by fluorescence spectrophotometry and compared with the no plasma controls in each protocol variation (*, $P < .05$ relative to no-enzyme treatment; TM, trimester; plasma from $n = 6$ individuals at each trimester; results representative of $n = 2$ independent experiments).

the difference was not statistically significant owing to greater patient-to-patient variability.

Discussion

This study has 3 major findings (1) plasma proteins from non-pregnant and first trimester patients attenuate PBMC and monocyte–endothelial cell adhesion; (2) plasma from first trimester patients with severe preeclampsia and second and third trimester normotensive individuals does not significantly attenuate monocyte–endothelial cell adhesion; and (3) removal of O-linked glycosylation chains from plasma proteins reverses the attenuation of monocyte–endothelial cell adhesion in all cohorts, regardless of the disease status.

It is well established that the addition of glycosyl (carbohydrate) chains to proteins affects how monocytes interact with endothelial cells, but the composition of plasma proteins affecting this interaction remains less understood. This question is especially important in pregnancy, where a dynamic change in the plasma protein content is accompanied by a rise in acute phase proteins, glycosylation, and leukocytosis. The published literature supports the hypothesis that plasma protein glycosylation impairs monocyte adhesion. In a longitudinal study of immunocyte endothelial adhesion molecule expression in maternal plasma (eg, intercellular adhesion molecule [ICAM], vascular adhesion molecule [VCAM], and the selectins), soluble forms of E-selectin, P-selectin, and ICAM were elevated more in patients with preeclampsia at 20 weeks gestation

than normotensive controls, whereas soluble L-selectin and VCAM-1 were reduced.²⁷ These results support the differential regulation of adhesion by discrete inflammatory mediators. Carbohydrate facets of glycoproteins also serve as adhesion receptors for leukocytes.²⁸ For example, glycans of the heavily glycosylated protein C1INH inhibit endothelial leukocyte adhesion during inflammatory responses.⁸ MUC 16, a heavily glycosylated mucin, has increased binding affinity to natural killer cells and monocytes of patients with preeclampsia than healthy controls.²⁹

The ApoE, C1INH, and ApoC-III are all glycosylated O-proteins, and all increase carbohydrate content during pregnancy. The ApoE is glycosylated at threonine 194, increasing its apparent molecular weight from 34 kDa to approximately 37 kDa.³⁰ The C1INH is the most heavily glycosylated protein in human plasma. Of the apparent molecular mass (104 kDa), the unmodified protein is only 53 kDa. Carbohydrate constitutes the remainder, with 7 O-linked and 6 N-linked glycosylation sites. In accordance with the published literature, O-glycosidase and neuraminidase treatment reduces the apparent molecular weight of C1INH to approximately 90 kDa on SDS-PAGE gels.⁸ The appearance of an approximately 90-kDa band immunoreactive for carbohydrate on our DIG glycan gels after O-glycosidase/neuraminidase is consistent with residual N-glycosylated protein.⁸ ApoC-III exists in plasma as predominantly singly or doubly sialylated at residue 74 (threonine), increasing its apparent molecular mass from 8.8, 9.4 to 9.7 kDa.³¹ In plasma, ApoC-III can associate with apoB-containing lipoproteins.²³ The ApoC-III and ApoE glycosylation patterns are differentially expressed in patients who develop preeclampsia, and different physiological functions have been proposed for various glycosylation subtypes.^{2,3,9,32-34} Further, O-glycosyl chains of C1INH protein competitively inhibit monocyte adhesion to endothelial cells in nonpregnant individuals.⁸ However, competitive inhibition is only 1 possible mechanism; receptor-mediated signaling could also affect adhesion by interaction with integrins and/or adhesion molecules such as granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, or monocyte chemoattractant protein. Our time course data (Figure 4) support multiple mechanisms, but our study was not designed to identify discrete signaling pathways leading to adhesion. The reason for the ineffectiveness of trimesters 2 and 3 plasma in inhibiting monocyte–endothelial cell adhesion after 1 hour is probably due to a change in plasma protein expression or posttranslational modifications during gestation, which could affect adhesion. For example, immunoreactive factors such as acute phase proteins are known to increase in pregnancy^{26,35} and could play a role in modifying adhesion in trimesters 2 and 3.^{36,37} Blood volume also increases in pregnancy; although we controlled for total protein load, changes in blood volume might differentially affect specific proteins.³⁵ However, further experimentation is needed to fully elucidate the molecular mechanisms involved in this differential response.

Acute phase proteins and glycoproteins increase exponentially in uncomplicated deliveries. Since glycoproteins have demonstrated anti-inflammatory and immunomodulatory properties in response to tissue injury and infection, it is likely that

Figure 5. Enzymatic removal of maternal plasma protein glycans potentiates monocyte adhesion to HUVEC monolayers. **A**, Fluorescently labeled monocytes were mixed with vehicle (no plasma) or plasma from gravid donors (trimester [TM] 1, 2, or 3) or nonpregnant controls, incubated for 1 hour, and applied to HUVEC monolayers for 10 minutes. Nonadherent monocytes were washed away, and adherent monocytes were visualized by epifluorescence microscopy (**A**) and quantified by fluorescence spectrophotometry (**B**). Results are representative of 2 independent adhesion assays with $n = 6$ individual patient plasma specimens in each trimester; *, $P < .05$ relative to vehicle (no enzyme) controls; #, $P < .05$ relative to TM 1. HUVEC indicates human umbilical cord endothelial cell.

glycosylation plays a similar role in pregnancy. Several acute phase proteins, such as α 1-antichymotrypsin (ACHY) and α 1-acid glycoprotein (AGP), are heavily glycosylated in plasma. The ACHY neutralizes proteases released by leukocytes in response to trauma and inflammatory stimuli, thereby affecting monocyte adhesion.³³ The AGP may elicit anti-inflammatory properties by binding E- or P-selectin and interfering with leukocyte–endothelial interactions.³⁸ The AGP concentrations and glycan-branching complexity were recently shown to increase in healthy pregnancies, while α 3-fucosylation decreased. The AGP levels and glycan branching complexity were higher in patients with pregnancy-associated remission of rheumatoid arthritis than in patients with unchanged or worsening disease. Recent results from our laboratory demonstrate a correlation between increased monocyte adhesion and abnormal ratios of glycosylated variants of ApoC-III in first trimester plasma from women who later developed severe preeclampsia.² Collectively, these data support a role of glycosylated proteins in modulating the inflammatory response in normal and complicated pregnancies.

Recent studies implicate glycosylated proteins in inflammatory disease states and, more importantly, support the role of these proteins as a therapeutic target. In a mouse model of experimental autoimmune encephalitis, for example, oral N-acetylglucosamine regimens altered the glycosylation-branching patterns of leukocytes and resulted in symptom alleviation.³⁹ Studies with GalNAc transferase 1-deficient mice indicate an essential role of O-glycosylation in leukocyte trafficking. Deficits in O-glycan-mediated adhesion result in elevated levels of blood lymphocytes and neutrophils in these animals.⁴⁰ Published results from our laboratory and others suggest that patients with preeclampsia have abnormal sialylation on O-GalNAc-glycosylated proteins.^{2,9} Taken together, these observations support the potential for glycotherapeutics in inflammatory disorders, including preeclampsia.

Conclusion

The relationship between glycosylated plasma proteins and monocyte adhesion has not been well described in pregnancy. Our study describes an interaction between maternal plasma

Figure 6. Severe preeclampsia—but not mild preeclampsia—increases monocyte adhesion to endothelial cell monolayers. Fluorescent monocytes were incubated for 1 hour with 5% plasma from $n = 3$ nonpregnant individuals in the menstrual phase of their ovulatory cycle or 5% first trimester plasma (8–12 weeks) from $n = 6$ patients with confirmed diagnoses of normotensive, mild preeclampsia, or severe preeclampsia. Adhesion of monocytes to endothelial cells was compared to vehicle (no plasma) controls. Plasma from all but the patients with severe preeclampsia significantly attenuated monocyte–endothelial cell adhesion relative to controls, with significantly lower adhesion in normotensive individuals than in patients with severe preeclampsia. *, $P < .05$ relative to no plasma controls; NP, nonpregnant; NT, normotensive; MPE, mild preeclampsia; SPE, severe preeclampsia.

glycoproteins and monocyte surface glycoproteins, which results in decreased monocyte adhesion to endothelial cells. Our experimental model provides the basis for testing the significance of plasma protein glycosylation in normal and complicated pregnancies. Our results support further investigation into whether glycosylation status of proteins plays a causative role in the endothelial dysfunction characteristic of the maternal syndrome in preeclampsia. Identifying these plasma proteins and the subsequent development of a glycoprotein profile could be useful in the early diagnosis and treatment of preeclampsia.

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Authors' Note

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Figure 7. Deglycosylation of first trimester maternal plasma proteins increases monocyte adhesion to endothelial cells in nonpregnant controls, normotensive individuals, and patients with mild or severe preeclampsia. Preconditioning monocytes for 1 hour with plasma treated for 48 hours with glycosidase enzymes to remove O-glycosylated linkages potentiated monocyte adhesion to endothelial cells. The degree of potentiation was not affected by the disease state. Pretreatment of monocytes (1 hour) with glycosidases in the absence of maternal plasma negligibly increased monocyte–endothelial cell adhesion (left bar). *, $P < .05$; $n = 3$ nonpregnant patients and $n = 6$ individuals per disease state; NP, nonpregnant; NT, normotensive; MPE, mild preeclampsia; SPE, severe preeclampsia.

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