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Alteration of endothelial function markers in women with gestational diabetes and their fetuses

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Abstract

Objective—We tested the hypothesis that women with gestational diabetes mellitus (GDM) and their fetuses would demonstrate alterations in markers of endothelial nitric oxide synthase (eNOS) uncoupling, oxidative stress, and endothelial dysfunction and these changes would correlate with the levels of hyperglycemia through a pilot observational case-control study of women with GDM and their fetuses.

Methods—Levels of soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), C-reactive protein (CRP), nitric oxide (NO), eNOS, p22-phox, and SOD gene expression, and endothelial progenitor cells (EPC) counts in both maternal and cord blood were measured at the time of delivery in women with and without GDM.

Results—We demonstrated the presence of decreased maternal circulating EPC counts, increased soluble adhesion molecules in maternal blood, decreased SOD expression in both maternal and cord blood and increased eNOS expression in both maternal and cord blood in women with GDM.

Conclusions—These data suggest that the molecular mechanisms behind oxidative stress in women with GDM and their fetuses appear similar to those hypothesized for non-pregnant adults with type 2 diabetes mellitus (DM).

Keywords

Endothelial dysfunction; gestational diabetes mellitus; oxidative stress

Introduction

Cardiovascular disease (CVD) is the main cause of death in the adult population in the United States and many countries [1]. Endothelial dysfunction is now considered a key element in the development of CVD, and has also been strongly associated with obesity and insulin resistance, which are key features of type 2 diabetes mellitus (DM) [2]. Endothelial dysfunction has been broadly defined as an imbalance between various vasodilating and vasoconstricting substances produced by or acting upon endothelial cells [3]. Impairment of

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endothelial function often occurs prior to diagnosis of DM [4], and endothelial dysfunction is often present years before any signs of microangiopathy are apparent [5]. Such findings have generated the hypothesis that, in type 2 DM, endothelial dysfunction may precede the development of chronic hyperglycemia, prompting the suggestion that the next generation of therapeutic agents should also focus on the mechanisms of endothelial dysfunction, and not solely on glycemia [6].

In this regard, it is critical to identify biomarkers that may appear in diabetes prior to irreversible damage to tissues. Hyperglycemia has been shown to lead to increased production of reactive oxygen species (ROS) and reactive nitrogen species via a number of enzymatic pathways, resulting in a state of imbalance between the production of these ROS and the body's biological antioxidant defenses, commonly referred to as oxidative stress [7,8]. The mechanisms associated with these pathways are now being elucidated and show early indicators of heightened oxidative stress [9]. Specifically, these mechanisms are associated with a decreased availability of nitric oxide (NO) secondary to endothelial nitric oxide synthase (eNOS) uncoupling [10]. NO is a potent vasodilator, and appears essential for the function of endothelial progenitor cells (EPC), which can migrate to and differentiate at sites of injury to promote angiogenesis and vasculogenesis in healthy individuals [11]. However, both the number and function of EPC have been shown to be decreased in diabetes, where EPC proliferation is impaired in hyperglycemic states (Figure 1) [12,13].

Two standard molecules associated with endothelial dysfunction resulting from inflammation are soluble intercellular adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1). Although adhesion molecules are vital for the normal development and function of the heart and blood vessels, they have been implicated in the pathogenesis of CVD. Elevations in the levels of these biomarkers as well as decreases in EPC have been a consistent finding in studies of patients with type 2 DM and people at increased risk for DM, however the mechanisms of endothelial dysfunction have not been thoroughly investigated in women with gestational DM (GDM) [14,15]. Such evidence would have implications not only for the diagnosis and treatment of affected mothers and their offspring, but also for modeling early vascular changes associated with type 2 DM. The purpose of this study was to test whether biomarkers of oxidative stress, eNOS uncoupling, and endothelial dysfunction may be altered in women with GDM and their fetuses.

Materials and methods

Study setting, participants, and design

Patients were enrolled at two hospitals affiliated with the Keck School of Medicine of the University of Southern California. We conducted a case-control observational study of women with GDM and their neonates using pregnant patients without a diagnosis of GDM as controls. Pregnant women with preterm and multiple gestations, pre-gestational diabetes, autoimmune disorders, maternal history of smoking, or chronic hypertension were excluded from the study. Case-patients were eligible for study enrollment if they had GDM as diagnosed by having two of the following: a fasting blood glucose >95 mg/dL, a 1-h oral glucose tolerance test (OGTT) >180 mg/dL, or a 3-h OGTT >140 mg/dL [16]. Eligible case-patients were identified from outpatient clinics in their last month of pregnancy and enrolled upon hospital arrival prior to delivery. Eligible control-patients were identified upon hospital arrival for delivery. Maternal and venous cord blood was drawn to quantify markers of endothelial dysfunction. Maternal hemoglobin A1c (HbA_{1c}) was tested to determine the level of glycemic control in both case and control patients. The study was approved by the University of Southern California Institutional Review Board, and complied with all patient protection criteria set forth therein.

Maternal venous blood was collected 3–12 h before delivery. Venous cord blood was collected from the placental side of the cord after the delivery and cord clamping. All blood samples were centrifuged within 30 min after collection and processed immediately, or stored at the appropriate temperature until analyzed.

Flow cytometry

One hundred microliter of whole blood was added to culture tubes with 1 μ g of isotype control or fluorophore-conjugated mouse antihuman antibodies against cell-surface markers expressed on human EPC (CD34, CD309 (KDR), and CD133) in phosphate-buffered saline (PBS) and incubated at 4°C in the dark for 30 min. Following incubation, 2 mL of RBC Lysis Buffer, prewarmed to room temperature, was added to each tube, vortexed gently, and incubated in the dark for 10 min. Blood samples were centrifuged at 400g at room temperature, supernatant aspirated, and washed with 500 μ L of staining buffer (PBS with 2% fetal calf serum). Samples were then centrifuged at 400g at room temperature, supernatant aspirated, resuspended in 500 μ L of 2% paraformaldehyde fixation buffer, and stored at 4°C in the dark until analysis. Flow cytometric analysis was performed on a LSR II flow cytometer using FACSDiva software (Becton Dickinson, Franklin Lakes, NJ, USA) at the University of Southern California School of Pharmacy.

Analysis of mRNA expression

For each sample, 1 mL of whole blood was transferred to a 15 mL polypropylene conical centrifuge tube and brought to volume with 5 mL of RBC Lysis Buffer immediately upon receipt at the laboratory. The samples were incubated at 4°C for 30 min, and then centrifuged at 600g for 10 min at 4°C. The supernatant was decanted, pellet resuspended in 1 mL of RBC Lysis Buffer, and transferred to a 1.5 mL microcentrifuge tube. After 5 min, the sample was centrifuged at 1000g for 5 min at room temperature. The supernatant was aspirated, washed with 1 mL of sterile PBS, centrifuged for 5 min at 1000g at room temperature, and supernatant aspirated again. Total RNA was extracted from the resultant cells using TRIzol by following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). For each sample, ~100 ng of RNA was reverse-transcribed using Maxima Reverse Transcriptase (Fermentas, Glen Burnie, MD, USA). Real-time PCR was conducted to examine expression of eNOS, superoxide dismutase isoforms (SOD2 and SOD3), and p22-phox, a subunit of NADPH oxidase in maternal and cord blood. Quantitative amplification of the cDNA was performed using SYBR Green PCR Master Mix (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) for 40 cycles using an ABI 7300 (Applied Biosystems). Expression of eNOS, SOD2, SOD3, and p22-phox mRNA were normalized against 18S mRNA and reported as fold-change compared to nondiabetic women.

Nitrite, HbA_{1c}, sVCAM-1, sICAM-1, and CRP

Nitrite was determined using the Griess reaction, Griess Reagent System (Promega, Madison, WI, USA). The remaining parameters were measured using commercial kits according to manufacturer's protocols (HbA_{1c}: BioQuant, San Diego, CA, USA; sVCAM-1, sICAM-1, and C-reactive protein (CRP): R&D Systems, Minneapolis, MN, USA).

Data collection and statistical analysis

Relevant patient information was retrieved from medical records using standardized tools. Data were analyzed using SAS statistical software (Cary, NC, USA). Laboratory data were combined with the patient data for analysis. Univariate analyses were performed, and means are expressed $\pm$ the SD, with median and range. Analyses of continuous variables were performed with Kruskal–Wallis testing. For each marker, and for maternal and cord blood, data were examined comparing case- and control-patients. Correlations were determined

using linear regression in GraphPad Prism version 5.0d for Mac OS X (GraphPad Software, San Diego, CA, USA).

Results

Sixteen patients were enrolled in this study, where nine patients met criteria classifying them as GDM, and seven were classified as non-GDM controls. Three control patients and one GDM patient were nulliparous. Only one GDM patient had a previous history of GDM, whereas no other patients had a previous history of any glucose abnormalities. In the GDM group, one had a cesarean in early labor, and one had an elective repeat cesarean (without labor); three of the non-GDM group had elective cesarean deliveries. All other patients delivered vaginally, with one control patient delivered by vacuum because of maternal exhaustion. There was no difference in maternal BMI or fetal birth weight between the two groups. The supplementary table provides additional details on the patients enrolled in this study. (Supplementary Table S1). While a statistically significant difference in maternal age was detected between the two groups ($p < 0.05$), when we analyzed the association between maternal age and percentage circulating EPC, there was no significant correlation detected (data not shown). Mothers diagnosed with GDM had significantly higher HbA_{1c} as compared to the control subjects (Supplementary Table S1; 7.6% vs. 5.2%, respectively; $p < 0.01$).

Maternal and cord blood EPC counts

EPC play an important role in the neovascularization process following injury, and have been shown to be decreased in diabetes [11,12]. We therefore evaluated mean circulating EPC counts from maternal blood. A statistically significant reduction in the percentage of circulating EPC was seen in GDM vs. nondiabetic patients (Figure 2A; 0.26% vs. 0.41%, respectively; $p < 0.05$). Additionally, we measured EPC counts from cord blood of fetuses delivered. Umbilical cord EPC were higher from GDM patients than control patients, however this difference did not reach statistical significance (1.76% vs. 1.46%, respectively).

Plasma nitrite levels and blood eNOS expression

In diabetes, decreased circulating NO levels are often accompanied by a paradoxical increase in eNOS expression, a molecular mechanism referred to as eNOS uncoupling. To determine circulating NO levels, the stable metabolite, nitrite, was measured using the Griess reaction. Mean nitrite levels from maternal plasma were significantly lower in women diagnosed with GDM compared to nondiabetic women (54 vs. 28 μM , respectively; $p < 0.05$; Figure 2B). Mean nitrite levels from umbilical cord plasma of fetuses delivered from women diagnosed with GDM were higher compared to those of nondiabetic women, however this difference did not reach statistical significance. In addition, maternal and cord blood eNOS mRNA expression were significantly higher in women diagnosed with GDM compared to nondiabetic women (Figure 2C; $p < 0.01$).

Blood SOD and p22-phox mRNA expression

An imbalance in SOD isoform and NADPH oxidase expression is also observed in diabetes, which may explain increases in oxidative stress and concomitantly decreasing antioxidant mechanisms. SOD2 (mitochondrial SOD) and SOD3 (extracellular SOD) mRNA expression in the blood was measured using reverse transcription-PCR (RT-PCR), where maternal SOD2 and SOD3 expression were significantly decreased in women with GDM compared to nondiabetic women (Figures 3A, B; $p < 0.01$). In cord blood, SOD3 mRNA expression was significantly lower in women diagnosed with GDM compared to nondiabetic women, while there was no significant change in cord blood SOD2. Maternal blood p22-phox expression

was increased in women with GDM, however, this difference did not reach statistical significance (Figure 3C). Surprisingly, cord blood p22-phox mRNA expression was significantly decreased from women with GDM compared to nondiabetic women ($p < 0.05$).

Plasma sVCAM-1, sICAM-1, and CRP levels

Levels of adhesion molecules such as sVCAM-1, sICAM-1, and CRP are associated with the inflammatory process, and can occur as a result of damage to tissue such as the endothelium. Increased levels of these adhesion molecules are observed in diabetes, and have been correlated with severity of disease. We evaluated maternal plasma for sVCAM-1 and sICAM-1, where both levels were significantly higher in GDM patients as compared to nondiabetic women (Figures 4A, B; $p < 0.05$). There was a significant increase in cord blood sVCAM-1 levels from patients with GDM ($p < 0.01$), however there was no significant difference in cord sICAM-1 levels. In addition, there were no significant differences between any groups in regards to plasma CRP levels, a marker of the overall inflammatory state (data not shown).

Correlation with HbA_{1c}

To determine whether a correlation exists between HbA_{1c} as a measure of glycemic control and markers of oxidative stress or endothelial dysfunction exist, linear regression was performed on all parameters measured (Supplementary Figure S1A–E). Maternal blood EPC correlated negatively with HbA_{1c}, where an increase in HbA_{1c} was associated with a decrease in circulating EPC numbers ($R^2 = 0.55$; $p < 0.01$). In addition, maternal plasma sVCAM-1 and sICAM-1 levels correlated positively with maternal HbA_{1c}, where increased HbA_{1c} was associated with increased sVCAM-1 and sICAM-1 levels ($R^2 = 0.79$, $p < 0.01$; $R^2 = 0.33$, $p < 0.05$, respectively). Maternal blood eNOS mRNA expression also correlated positively with maternal HbA_{1c}, where increased HbA_{1c} was associated with increased eNOS mRNA expression ($R^2 = 0.25$; $p < 0.05$). Lastly, cord blood SOD3 mRNA expression correlated inversely with maternal HbA_{1c} ($R^2 = 0.60$; $p < 0.01$).

Discussion

GDM affects 3–8% of pregnancies in the United States and up to 12–14% in some high-risk populations [17]. The majority of women with GDM appear to have β -cell dysfunction that occurs on a background of chronic insulin resistance [18]. Hyperglycemia, as determined by glucose tolerance testing at mid-pregnancy, has been the mainstay of GDM diagnosis for decades. Maternal hyperglycemia leads to fetal hyperinsulinemia and can lead to adverse long-term maternal outcomes (e.g. development of type 2 DM and atherosclerotic CVD), increased perinatal morbidity (e.g. macrosomia, birth trauma, pre-eclampsia), and long-term sequelae in offspring (e.g. childhood overweight, and metabolic factors that may increase risk of CVD).

In this case-control study, we have demonstrated an increase in circulating adhesion molecules in patients with GDM. Moreover, GDM subjects had significantly decreased circulating EPC counts in maternal blood, SOD isoform mRNA expression in both maternal and cord blood, and increased eNOS mRNA expression in both maternal and cord blood. These findings are consistent with the hypothesized mechanisms where hyperglycemia can lead to increased oxidative stress and endothelial dysfunction in diabetes. Importantly, our results suggest that alterations in endothelial function are present in both mothers and their fetuses in GDM pregnancies. If these measures remain elevated, it is possible that these alterations can confer an increased risk for the development of CVD and type 2 DM. Furthermore, if confirmed in ongoing larger studies with longitudinal measurements, the identification of disturbances at the molecular level may not only enable the identification of

potential new treatments (e.g. pharmaceuticals to prevent eNOS uncoupling and oxidative stress), but also should provide earlier and more specific and sensitive targets for monitoring the effectiveness of current treatment strategies, including dietary changes, exercise regimens, and pharmacological management.

In spite of the historical use of hyperglycemia as the diagnostic criterion for GDM, it is now evident that hyperglycemia may be a late consequence of the disease process, becoming detectable only after the progressive loss of insulin secretion due to longstanding demands imposed by chronic insulin resistance [18]. Even with strict glycemic control, women diagnosed with GDM are still at risk for adverse pregnancy outcomes. Therapeutic strategies that focus on tight glucose control often have limited success in avoiding accelerated fetal growth, particularly in pregnancies characterized by maternal obesity [19,20]. Furthermore, adverse pregnancy outcomes have also been documented in women with mild glucose intolerance who do not meet current diagnostic criteria for GDM [21]. In 2010, the International Association of Diabetes and Pregnancy Study Groups created a consensus panel to arrive at diagnostic criteria because there have been no obvious glucose thresholds at which risks of key outcomes are increased. Controversies regarding diagnostic criteria remain active, impeding the generation of new approaches to prevention and treatment.

The current literature provides limited evidence of endothelial dysfunction among women with GDM. A few studies have suggested that normoglycemic women with a history of GDM demonstrate aspects of endothelial dysfunction, such as impaired endothelium-dependent vasodilatation and increased artery stiffness. Multiple biomarkers have also been implicated in the connection between endothelial dysfunction and GDM. Krauss and colleagues found evidence for potentially elevated sICAM-1 levels in women with GDM, and Kautsky-Willer and colleagues demonstrated increased levels of sVCAM-1 in women with GDM and pregnant women with normal glucose tolerance compared with non-pregnant controls [22,23]. In women with GDM, these levels remained elevated 12 weeks postpartum, while correcting in women with normal glucose tolerance. In an evaluation conducted years after delivery, Bo and colleagues studied women with and without a history of GDM, and confirmed these findings [24]. Our studies support these findings. In addition, we found a significant inverse correlation of HbA_{1c} with EPC. Hyperglycemia promotes an environment prone to oxidative stress, which can result in tissue damage and also prevent the ability to repair existing damage. Interestingly, a correlation between HbA_{1c} and sVCAM-1 as well as sICAM-1 has been demonstrated in this study, suggesting that high glucose increases the adhesiveness of the circulating cells, which could explain the endothelial dysfunction often observed in diabetic patients.

For offspring, *in utero* exposure to a diabetic environment has been associated with obesity, type 2 DM, and dyslipidemia in the offspring, consistent with the concept of “fetal programming,” wherein adult disease may find its origins in fetal development [25]. Elevated levels of sICAM-1 and sVCAM-1 were found in obese, hypertensive, and diabetic children with a mean age of 15 years, suggesting that endothelial activation appears early in life, and that adhesion molecules are related to the earliest stages of atherosclerosis [26]. Although the mechanisms by which exposure to a diabetic environment *in utero* causes metabolic abnormalities in the offspring are unknown, it is hypothesized that disturbed endothelial function may play a role [27].

In summary, our results have demonstrated the presence of maternal and fetal molecular markers of endothelial dysfunction and oxidative stress in women with GDM and their fetuses. We submit that continued advances in the understanding of the molecular mechanisms associated with endothelial dysfunction will have a substantial impact in the field of GDM, where the dynamics of the perinatal environment are remarkably complex.

Given that current standards for strict glycemic control during pregnancy do not appear to eliminate the maternal and fetal repercussions of GDM, new treatment avenues are needed. As we have demonstrated in this study, the ability to identify and track specific and sensitive molecular markers of vasculopathy in women with GDM may provide a foundation for an improved understanding of the pathophysiology of the disease process. In addition, these biomarkers may help to better monitor the long-term effects of GDM in both the mother and fetus, and allow for pharmacological therapies and other interventions to be introduced at an earlier stage in the disease process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Mechanisms involved in endothelial dysfunction in gestational diabetes.

Figure 2.
(A–C) Maternal and cord blood endothelial progenitor cells % (EPC%), blood nitrite levels, and endothelial nitric oxide synthase (eNOS) mRNA expression in nondiabetic and gestational diabetes mellitus (GDM) patients.

Figure 3.
(A–C) Maternal and cord blood SOD and p22-phox mRNA expression in nondiabetic and gestational diabetes mellitus (GDM) patients.

Figure 4.
(A, B) Maternal and cord plasmas VCAM-1 and sICAM-1 in nondiabetic and gestational diabetes mellitus (GDM) patients.