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GESTATIONAL DIABETES ALTERS MATERNAL AND NEONATAL CIRCULATING ENDOTHELIAL PROGENITOR CELL SUBSETS

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Abstract

Objective—To examine whether women with gestational diabetes (GDM) and their offspring have reduced endothelial progenitor cell (EPC) subsets and vascular reactivity.

Study Design—Women with GDM, healthy controls, and their infants participated. Maternal blood and cord blood were assessed for colony forming unit-endothelial cells (CFU-ECs) and EPC subsets using polychromatic flow cytometry. Cord blood endothelial colony forming cells (ECFCs) were enumerated. Vascular reactivity was tested by laser Doppler imaging.

Results—Women with GDM had fewer CD34+CD133+CD45+CD31+ cells (circulating progenitor cells, CPCs) at 24–32 weeks gestation and 1–2 days post-partum compared to controls. No differences were detected in CFU-ECs or ECFCs. In controls, CPCs were higher in the third trimester compared to post-partum. Cord blood from GDM pregnancies had reduced CPCs. Vascular reactivity was not different between GDM and control subjects.

Conclusion—The normal physiologic increase in CPCs during pregnancy is impaired in women with GDM, which may contribute to endothelial dysfunction and GDM-associated morbidities.

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Keywords

endothelial progenitor cell; gestational diabetes; pregnancy

INTRODUCTION

Maternal vascular remodeling occurs throughout pregnancy to support increasing fetal metabolic demands. The endothelium has a major role in sustaining vascular health. Cellular and molecular processes that maintain endothelial function require a complex interplay between endothelial cells and subpopulations of circulating cells, termed endothelial progenitor cells (EPCs). In non-pregnant adults, reduced EPCs correlate with increased vascular disease risk.^{1–3} EPC dysfunction is speculated to contribute to the pathogenesis of several gestational disorders including pre-eclampsia and gestational diabetes (GDM).^{4–5} However, the few studies that assess EPCs during pregnancy report conflicting data due to the variety of assays used to quantitate EPCs.^{4–10} Moreover, since these studies were conducted, the definition of an EPC has evolved because the majority of methods that were previously used identify hematopoietic progenitors, not endothelial progenitors.¹¹

The most common techniques to quantitate EPCs are colony assays and flow cytometry. Colony forming unit-endothelial cells (CFU-ECs) are hematopoietic progenitors that facilitate angiogenesis while endothelial colony forming cells (ECFCs) are progenitors with vessel forming capacity.¹¹ Flow cytometry detects the simultaneous expression of several antigens on individual cells and is used to examine cell subsets involved in vascular homeostasis.¹² Specifically, circulating progenitor cells (CPCs) co-express CD34, CD133, CD45, and CD31 (CD34+CD133+CD45+CD31+). CPCs are hematopoietic progenitors with robust angiogenesis facilitating functions.^{13–14} Importantly, CPCs are being used as markers of vascular disease risk and to evaluate antiangiogenic therapies for cancer.¹² Therefore, we questioned whether pregnant women with GDM and their offspring have reduced CFU-ECs and CPCs.

MATERIALS AND METHODS

Women with GDM and healthy pregnant controls were recruited via obstetric clinics and advertisements. Women were studied between 24–32 weeks gestation using the obstetrician's best estimate of gestation, 1–2 days post-partum, and 6–8 weeks post-partum. Newborns were studied at 1–2 days of age. GDM was defined per American College of Obstetrics and Gynecology guidelines. Exclusion criteria included type 1 or type 2 diabetes, illnesses known to affect glucose metabolism (i.e. Cushing syndrome, polycystic ovarian syndrome), use of medications that affect glucose metabolism (i.e. dexamethasone), multiple gestation, cardiovascular disease, history of pre-eclampsia, and women carrying fetuses with chromosomal abnormalities. Additionally, delivery <35 weeks gestation was an exclusion criterion for mother and infant post-partum visits and cord blood studies, though data obtained during the third trimester visit was included in all analyses. Historical and clinical data were obtained at each visit. Maternal blood was collected for glycosylated hemoglobin (HbA1C), fructosamine, CFU-EC assays, and polychromatic flow cytometry (PFC). Cord blood was used for CFU-EC assays, ECFC assays, and PFC. The Institutional Review Board at the Indiana University School of Medicine approved all protocols, and informed consent was obtained from all women.

Mononuclear cell preparation

Blood was collected in citrate cell preparation tubes (BD Biosciences, Franklin Lakes, NJ) and processed in our Angiogenesis and Endothelial Progenitor Cell Core (AEPCC). Tubes were centrifuged at 1600g for 30 minutes, and mononuclear cells (MNCs) were collected.¹⁵

CFU-EC assay

CFU-ECs were assessed using the EndoCult Kit (StemCell Technologies, Vancouver, BC, Canada) as described.¹⁵ Briefly, MNCs were plated in complete EndoCult media (Cambrex, Walkersville, MD) on fibronectin-coated tissue culture plates (BD Biosciences). After 48 hours, nonadherent cells were replated on fibronectin-coated tissue culture plates for 3 days, and colonies scored in a blinded fashion.

ECFC assay

ECFCs were enumerated as described.¹⁶ Briefly, tissue culture plates pre-coated with collagen (BD Biosciences) were seeded with MNCs. Medium was changed daily. Colonies appeared between 5–8 days and were identified as circumscribed monolayers of cobblestone-appearing cells. All samples were scored in a blinded fashion.

Polychromatic flow cytometry

PFC utilizes advanced analytical methodologies to enumerate rare cell subpopulations and represents a superior technique compared to conventional flow cytometric approaches.^{13–14} Accurate measurement of infrequent cell populations using PFC is accomplished by including critical controls to enhance data acquisition, reproducibility, and analysis.¹³ MNCs were stained with directly conjugated monoclonal antibodies, including CD31 fluorescein isothiocyanate (FITC, BD Pharmingen, San Diego, CA), CD34 phycoerythrin (PE, BD Pharmingen), CD133 allophycocyanin (APC, Miltenyi Biotec, Auburn, CA), CD45 APC-AlexaFluor (AF) 750 (Invitrogen), the viability marker ViViD (Invitrogen) and CD41a (BD Pharmingen) and CD235a (glyA, R&D Systems, Minneapolis, MN) both conjugated to Pacific Blue (PacB, Invitrogen) for platelet and red blood cell exclusion, respectively. Fluorescence minus one controls were prepared as negative gating controls, and anti-mouse Ig CompBeads (BD Biosciences, Bedford, MA) were stained with antibodies as single-color compensation controls as described.^{13–14} CPCs that co-express CD34, CD133, CD45, and CD31 (CD34+CD133+CD45+CD31+), and circulating endothelial cells (CECs) that express CD34 and CD31, but not CD133 and CD45 (CD34+CD133–CD45–CD31+) were analyzed on a Becton Dickinson LSR II cytometer with FlowJo software 8.7.3 (Tree Star, Inc., Ashland, OR).^{13–14} CD133 negative non-angiogenic progenitors were enumerated (CD34+CD133–CD45+CD31+) to calculate a ratio of CPCs to non-angiogenic progenitors (nonCPCs) as described.¹⁴ At least 500,000 events were collected. CPC data are reported as percent live cells in a low side scatter and low forward scatter gate. No differences were detected in total events evaluated between control and GDM samples. Analyses were conducted by the AEPCC personnel, who were blinded to all clinical data.

Vascular Reactivity

Endothelial function in women (24–32 weeks gestation) and newborns (1–2 days of age) was evaluated by iontophoresis of acetylcholine (Ach) to the skin and measuring blood flow by laser Doppler as described.^{17–18} A probe, connected to a laser Doppler (Periflux 5001, Wavelength 780nm), was attached to the forearm. The probe temperature was 32°C during all tests. Basal skin perfusion was measured for 2 minutes, then Ach was introduced by iontophoresis for 20 seconds at 60 second intervals and repeated 6 times. The laser Doppler output is expressed in perfusion units (PU) of output voltage (1 PU = 10mV). The % change

in perfusion was calculated by dividing PU after each Ach stimulation by basal PU X 100. Subjects were fasting for 2–3 hours.

Statistical Methods

Unless otherwise stated data are mean $\pm$ standard error of the mean (SEM). Subjects' characteristics between control and GDM groups were compared using two-sample independent t-tests for continuous outcomes and either chi-square or Fisher's exact tests for discrete outcomes. Repeated measures ANOVAs with appropriate transformation were used to analyze CPC and CEC data. The models compared outcomes at different time periods between the groups and within the groups. To adjust for pre-pregnancy body mass index (BMI) in comparing two groups at each time period, the model included pre-pregnancy BMI. Areas under curve were computed to analyze vascular reactivity, and Exact Wilcoxon two-sample rank sum tests were applied to compare groups. Spearman rank correlations were used for all correlation analyses.

RESULTS

Forty-six pregnant women were enrolled; 27 with GDM and 19 controls with a normal GDM screening test. Control subjects had no history of previous GDM and were not taking any prescription medications. One control subject had a diagnosis of hypertriglyceridemia and smoked tobacco. Of the GDM group, two women had GDM with a previous pregnancy, one woman smoked tobacco, and one woman was on sertraline for depression. Age, parity, blood pressure, and pre-pregnancy BMI, based on self-reported weight, were not different between control and GDM subjects (Table 1). The groups had similar ethnic and racial compositions. Women with GDM had higher fructosamine and HgA1C levels compared to controls, though these values were clinically insignificant.

Sixty-three percent of subjects completed the first post-partum visit (control n=12, 63% and GDM n=17, 63%). Major factors contributing to attrition included loss to follow up (control n=6 and GDM n=7), delivery <35 weeks gestation (GDM n=2), blood sample inadequate (GDM n=1), and delivery at another hospital (control n=1). All 12 women in the control group completed the 6–8 week post-partum visit while 3 GDM subjects were lost to follow-up. Though blood samples and VR testing were not conducted on these women during post-partum visits, pregnancy outcomes were available and were analyzed. No differences were detected in the clinical characteristics for women in each group that completed all study visits compared to those that did not.

Between the first study visit and delivery, diabetes drug therapy was initiated in 13 GDM subjects (Table 1) who failed traditional diet and exercise management (6 on glyburide and 7 on insulin). During labor and delivery, women in the control group had few complications (n=2) with one woman requiring an emergent cesarean delivery for fetal distress and one woman with spontaneous preterm delivery at 36 weeks gestation. In contrast, women with GDM experienced increased pregnancy complications (n=12, Table 1) including 9 preterm deliveries <37 weeks gestation (6 spontaneous vaginal deliveries and 3 cesarean deliveries for fetal distress), pre-eclampsia (n=3, requiring magnesium sulfate and hydralazine), cholestasis of pregnancy (n=1), emergent cesarean delivery for fetal distress (n=3), and abruption requiring transfusion (n=1). No subjects were diagnosed with gestational hypertension. Cesarean delivery rates were not different between control and GDM groups (42% vs. 33% respectively).

Infants born to women with GDM were more likely to be premature and have increased ponderal indices, an indicator of fetal growth (Table 2). Five neonates were admitted to the neonatal intensive care unit; all born to women with GDM. The discharge diagnoses were

prematurity (n=2), respiratory distress syndrome/suspected sepsis (n=1) and hypoglycemia (n=2). All were discharged to home after a short hospital stay.

Women with GDM have reduced CPCs, but not CFU-ECs or CECs

Since subpopulations of EPCs inversely correlate with vascular disease risk in non-pregnant adults,^{1–3} we hypothesized that women with GDM would have reduced CFU-ECs and CPCs compared to women with non-GDM pregnancies. Also, given recent data from our group demonstrating that a ratio of CPCs to non-angiogenic progenitors (nonCPCs, CD34+CD133–CD45+CD31+) correlate with vascular dysfunction, we hypothesized that women with GDM would have a decreased CPC:nonCPC ratio compared to controls. CFU-EC assays revealed no difference between GDM and control subjects during the third trimester of pregnancy (32.0 ± 8.2 versus 29.1 ± 11.0 colonies/ 10^6 MNCs, respectively). However, significant variability was observed using this technique.

Next, CPCs, nonCPCs, and CECs were quantitated using PFC to identify co-expression patterns of the antigens CD34, CD133, CD45, and CD31.^{12–14} Applying this novel technology, GDM subjects had one-third the number of CD34+CD133+CD45+CD31+ cells or CPCs during the third trimester, which remained low 1–2 days post-partum compared to controls (Figures 1A–B). When subjects with pre-eclampsia were excluded from the analyses, these differences remained, supporting an independent effect of GDM. CPCs tended to be lower 6–8 weeks post-partum in women with GDM compared to controls (Figure 1C). Interestingly, profound differences were detected at all timepoints between GDM and control CPC:nonCPC ratios, which is a sensitive measure of vascular dysfunction in adults with peripheral arterial disease (Figures 2A–C). Additionally, in controls, CPCs were higher in the third trimester compared to 1–2 days post-partum (0.24 ± 0.04 versus 0.14 ± 0.05 , $p < 0.01$) and 6–8 weeks post-partum (0.24 ± 0.04 versus 0.13 ± 0.04 , $p < 0.01$). In contrast, in GDM subjects, CPCs were not different at the three time points (third trimester: 0.08 ± 0.02 versus 1–2 days post-partum: 0.07 ± 0.01 and 6–8 weeks post-partum: 0.05 ± 0.01). CD34+CD133–CD45–CD31+ cells or CECs were no different between control and GDM subjects at any time point (data not shown).

Next, we questioned whether women with GDM treated with diabetes medications had lower CPCs compared to women with GDM managed conservatively. Interestingly, women on medications for hyperglycemia had fewer CPCs shortly after delivery compared to women managed with diet and exercise alone ($0.03 \pm 0.01\%$ versus $0.07 \pm 0.01\%$, $p < 0.02$). These differences were not apparent at the first study visit. Although drug therapy was instituted in women with impaired glycemic control, additional analyses did not demonstrate an inverse correlation between CPCs and measures of glycemia (i.e. fasting blood glucose, HgA1C, fructosamine).

Since obesity is associated with GDM and hypertensive disorders of pregnancy,¹⁹ we evaluated whether pre-pregnancy BMI inversely correlated with CPCs during the third trimester. No direct correlation was detected between pre-pregnancy BMI and CPCs in the third trimester. However, 6–8 weeks post-partum there was a negative correlation between pre-pregnancy BMI and CPCs in both control and GDM subjects ($r = -0.59$, $p < 0.002$). Using repeated measures ANOVA to examine for differences in CPCs between GDM and control subjects while controlling for pre-pregnancy BMI, we found that GDM subjects continued to have reduced CPCs compared to controls until 6–8 weeks post-partum. Thus, these data demonstrate that increased pre-pregnancy BMI and development of GDM have independent negative effects on CPCs.

A trend towards reduced Ach induced vasodilation was apparent in GDM subjects, though statistical significance was not achieved (Figure 2D). A trend towards a positive association

was observed between CPCs and vascular reactivity using area under the curve in women with GDM ($r = 0.5$, $p < 0.1$), while no association was detected in the control group. These data suggest that for women with GDM reduced CPCs may correlate with decreased endothelial-dependent vasodilation.

Infants born to women with GDM have reduced CPCs

Given our previous data demonstrating decreased numbers and function of ECFCs from cord blood of pregnancies complicated by pre-gestational diabetes,¹⁶ we examined whether ECFCs and CPCs were reduced in cord blood from GDM pregnancies. No differences were detected in ECFCs/ 10^8 MNCs between GDM and control samples (34.3 ± 7.9 vs. 20.1 ± 5.1 , $n = 10$ per group). However, GDM cord blood samples had a significant reduction in CPCs and the CPC:nonCPC ratio compared to controls (Figure 3A–B). CFU-ECs and CECs from cord blood and vascular reactivity were normal in neonates born to women with GDM compared to controls (data not shown). Collectively, these data suggest that fetal exposure to GDM alters CPC frequency, though endothelial function is intact at birth.

COMMENT

Here, we demonstrate that CPCs are reduced in women with GDM compared to pregnant controls during the third trimester and early post-partum period. Additionally, in uncomplicated pregnancies CPCs are higher in the third trimester compared to 1–2 days and 6–8 weeks post-partum. Interestingly, CPCs in control subjects 1–2 days post-partum approach levels detected in non-pregnant individuals,^{13–14} suggesting a quick return to baseline after delivery. Our data are consistent with the limited prior studies assessing circulating angiogenic cells during pregnancy. Previous reports show that in healthy pregnancies some EPC subsets increase during gestation, and in pre-eclamptic pregnancies fewer EPCs are detected in the third trimester.^{4–7, 9–10, 20} Though, conflicting reports do exist,^{5, 8, 21} primarily because diverse methods were used to examine EPCs resulting in the evaluation of different cell types. Taken together, these data suggest that the normal physiologic increase in CPCs during pregnancy does not occur in women with GDM or pre-eclampsia.

A limitation of our study was loss to follow-up leading to a reduction in sample size at post-partum visits. Additionally, due to the modest sample size, it was not possible to reliably evaluate the effect of adverse intrapartum events on maternal and neonatal data. While these are important considerations, our study advances current knowledge. Though published reviews speculate a role for EPC dysfunction in the genesis of gestational disorders associated with endothelial dysfunction including GDM,^{4–5} limited studies have been conducted during pregnancy. In fact, to our knowledge, only a single study included women with GDM.²² Buemi et al showed that GDM subjects ($n = 7$) had reduced CD34+ and CD34+VEGFR2+ cells compared to controls ($n = 7$) using conventional flow cytometry, not PFC. Strengths of the current study are the inclusion of additional GDM subjects ($n = 27$ during third trimester) followed through the post-partum period ($n = 17$ and $n = 13$ at 1–2 days and 6–8 weeks, respectively) as well as the evaluation of cord blood ($n = 13$) to examine impact on offspring. Additionally, conducting PFC to enumerate rare, circulating cells during pregnancy is a novel approach that has not been applied to the study of gestational disorders.

In the current study, similar results were not detected for CFU-ECs and CPCs, which is interesting since both assays measure hematopoietic progenitors that facilitate angiogenesis.¹¹ However, progenitors identified by colony formation and flow cytometry likely represent distinct subsets.²³ Additionally, this observation may be explained by inherent assay differences. Colony formation requires individual progenitors to proliferate *in vitro*, and

scoring is conducted manually, enhancing the risk for variability. In contrast, PFC is a highly sensitive and reproducible technique that requires minimal *ex vivo* manipulation,^{12–14} increasing the reliability to recapitulate endogenous conditions.

The divergent effect of GDM on CPCs and CECs is also intriguing and may provide insights into the pathogenesis of endothelial dysfunction in GDM. For example, when transplanted into a tumor xenograft model, CPCs enhance tumor angiogenesis and growth.¹⁴ Thus, CPCs may facilitate vascular remodeling during pregnancy via paracrine mechanisms. In contrast, CECs contain differentiated endothelial cells. Increases in CECs during acute coronary artery disease or in adolescents with type 1 DM suggest that CECs may serve as indicators of active endothelial damage.^{18, 24} Therefore, it is encouraging that women with GDM do not have increased CECs and suggest that interventions to enhance CPCs could ameliorate associated endothelial dysfunction.

Measures of glycemia did not correlate with CPCs or endothelial function in this study, which is disheartening since the mainstay of treatment is to achieve glycemic control. It is possible that these findings reflect the complexity of GDM with factors independent of glycemia being involved in the evolution of endothelial dysfunction. Previous studies using non-pregnant subjects with diabetes support this idea by not demonstrating correlations between HgA1C and vascular reactivity.^{18, 25} Our observation that pre-pregnancy BMI inversely correlates with CPCs independent of GDM also supports this notion.

Despite advances in obstetrical care, neonatal morbidities associated with GDM are significant. Moreover, fetal exposure to a diabetic environment has long-term health implications for offspring, including an enhanced risk for hypertension.^{26–30} We show that a GDM intrauterine environment results in reduced cord blood CPCs. Endothelial function is normal in these neonates, which is encouraging and suggests that irreversible endothelial injury has not occurred. Future studies of older children born to women with GDM will be required to examine whether CPCs remain low during childhood and to investigate whether endothelial dysfunction evolves after dramatic growth of the vascular system.

Additional studies that interrogate whether altered CPC function contributes to the genesis of endothelial dysfunction during pregnancy are warranted. Moreover, it will be interesting in future studies to examine whether CPC enumeration by PFC can be exploited to develop novel gestational treatments as is being evaluated for cardiovascular disease and cancer.^{12–14} Thus, studies that carefully define EPC subsets using well-established methods during normal and high-risk pregnancies will be needed to answer these important questions.

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Figure 1. Women with GDM exhibit reduced CPCs

Polychromatic flow cytometry demonstrates alterations in circulating EPC subpopulations. Maternal peripheral blood MNCs were evaluated for the proportion of CD34+CD133+CD45+CD31+ cells. The mean proportion of circulating CD34+CD133+CD45+CD31+ cells or CPCs are shown for samples evaluated at (A) 24–32 weeks gestation, control n=19, GDM n=27, *p<0.0001, (B) 1–2 days post-partum, control n=12, GDM n=18, *p<0.006, and (C) 6–8 weeks post-partum, control n=13, GDM n=14. Error bars represent SEM.

D

Figure 2. Women with GDM have a decreased CPC:nonCPC ratio with a trend towards impaired endothelial function

Ratios of CPCs to nonCPCs are shown for samples at (A) 24–32 weeks gestation, control n=19, GDM n=27, *p<0.0001, (B) 1–2 days post-partum, control n=12, GDM n=18, *p<0.05, and (C) 6–8 weeks post-partum, control n=13, GDM n=14, *p<0.0003. Each symbol represents data from an individual subject. The horizontal line on each scatter graph represents the mean of the group. (D) Vascular reactivity was evaluated using laser Doppler iontophoresis. Endothelial function was assessed by measuring vasodilation in response to 6 repeated stimulations with acetylcholine.

Figure 3. Cord blood from GDM pregnancies have reduced CPCs compared to samples from uncomplicated pregnancies
 Cord blood MNCs were evaluated for the proportion of CD34+CD133+CD45+CD31+ (CPCs) and CD34+CD133–CD45+CD31+ (nonCPCs) using PFC. (A) The mean proportion

of cord blood CD34+CD133+CD45+CD31+ cells are shown, control n=10, GDM n=13, *p<0.04. Error bars represent SEM. (B) Ratios of CPCs to nonCPCs are shown for cord blood samples, control n=10, GDM n=13, *p<0.0001. Each symbol represents data from an individual sample. The horizontal line on each scatter graph represents the mean of the group.

Table 1

Characteristics of Women Participants

Characteristic	Control (n=19)	GDM (n=27)
Age (years)	27.6 ± 1.6	29.8 ± 1.0
Parity	1.4 ± 0.2	1.6 ± 0.2
Pre-pregnancy Body Mass Index (Kg/m ²)	29.4 ± 1.2	30.5 ± 1.0
Systolic Blood Pressure (mmHg)	108 ± 2	112 ± 2
Diastolic Blood Pressure (mmHg)	64 ± 1	68 ± 2
Fructosamine	180 ± 4	202 ± 5 *
HgA1C (%)	5.1 ± 0.1	5.8 ± 0.1 *
% Black	21	33
% Hispanic	53	59
% Requiring Drug Therapy	Not Applicable	48
% Women with an Obstetric Complication	11	44 **

Mean ± SEM

*
p≤0.003**
p≤0.02

Table 2

Infants' Characteristics at Birth

Characteristic	Control (n=19)	GDM (n=27)
Gestational Age (weeks)	38.4 ± 0.4	37.4 ± 0.4 *
Birth Weight (g)	3,249 ± 113	3,404 ± 95
Length (cm)	50.5 ± 0.5	50.6 ± 0.4
Ponderal Index [†]	2.52 ± 0.06	2.68 ± 0.05 *
Preterm Newborns (% < 37 weeks gestation)	11	33
Large for Gestational Age (% > 90 th percentile)	21	26

Mean ± SEM

[†] Calculated as birth weight/length³×100*
p≤0.05