Potential Role of Hyperglycemia in Fetoplacental Endothelial Dysfunction in Gestational Diabetes Mellitus

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Key Words
Hyperglycemia • Endothelial cell • Angiogenesis • Gestational Diabetes Mellitus

Abstract
Background: Gestational diabetes mellitus (GDM) is associated with structural and functional alterations in various tissues including endothelial dysfunction. The aim of this study was to explore the effects of hyperglycemia on fibroblast growth factor 2 (FGF2)- and vascular endothelial growth factor (VEGF)-stimulated placental angiogenesis and the underlying molecular signaling mechanisms. Methods: The density of fetal placental capillaries was examined using immunohistochemistry. Human umbilical vein endothelial cells (HUVECs) derived from GDM (dHUVECs) and normal healthy patients (nHUVECs) were used as cell models in this study. Cell proliferation, migration and tube formation were measured with an MTS assay, a transwell system and a matrigel assay, respectively. The activation of ERK1/2 was analyzed with Western blot. The specific inhibitor of extracellular signal-regulated kinases 1/2 (ERK1/2) PD98059 was used to elucidate the involved signaling pathway. Results: GDM did not alter the capillary density of the fetus-placenta. Both the GDM and hyperglycemic conditions inhibited the proliferation of the FGF2- but not the VEGF-stimulated HUVECs and the basal migratory capacity. Hyperglycemia significantly inhibited tube formation and ex vivo angiogenesis. Moreover, hyperglycemia inhibited the FGF2- but not the VEGF-induced activation of ERK1/2. PD98059 significantly inhibited the FGF2-activated ERK1/2 phosphorylation and the FGF2-stimulated cell proliferation in HUVECs. Conclusion: Both GDM and hyperglycemia may impair placental angiogenesis by reducing HUVEC proliferation, migration and tube formation. Hyperglycemia-inhibited cell proliferation stimulated by FGF2 probably contributed to the suppression of the MEK1/2/ERK1/2 pathways in the HUVECs.

Introduction
Gestational diabetes mellitus (GDM) is defined as glucose intolerance with an onset or first recognition during pregnancy and affects up to 8% of all pregnancies [1–3]. These
metabolic disturbances are associated with increased risks of adverse maternal and neonatal outcomes [4, 5]. GDM is well known to be involved in structural and functional alterations in various tissues including endothelial dysfunction [6]. Although the effects of hyperglycemic conditions (25 mM) on endothelial cells in normal pregnancies have been extensively studied [7, 8], the proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs) from GDM patients who are cultured under physiological glucose conditions (5 mM) have not previously been studied. Moreover, the signaling mechanisms underlying the effects of hyperglycemia on endothelial cell proliferation, migration and tube formation are still unknown.

Normal fetoplacental vascular remodeling and function are required for normal fetal growth and development. Thus, alterations in placental structure and function have deleterious consequences on fetal development and growth and the health of the mother [9, 10]. The establishment and remodeling of the placental blood vessels are regulated by a broad spectrum of angiogenesis-associated factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2) [11, 12]. The expressions of VEGF and FGF2 in the placenta positively contribute to placental vascular growth and blood flow during pregnancy [13]. We and other researchers have provided solid evidence indicating that VEGF and FGF2 play major roles in placental angiogenesis and vasodilatation [13, 14]. Additionally, we have revealed that the mitogen-activated protein kinase kinase 1/2 (MEK1/2)/extracellular signal-regulated kinases 1/2 (ERK1/2) pathway is partially involved in FGF2- and VEGF-induced placenta endothelial cellular proliferation and migration [15, 16].

The functions of fetoplacental endothelial cells have been well studied and found to be abnormal in GDM [17, 18]. However, little is known about the effects of hyperglycemia on FGF2- and VEGF-stimulated placental angiogenesis and the underlying signaling mechanisms. Therefore, we tested the hypothesis that hyperglycemia reduces placenta endothelial functions by inhibiting the activation of the MEK1/2/ERK1/2 pathway in HUVE cells.

Materials and Methods

Study Subjects and Sample Collection

The study participants were recruited at the Department of Obstetrics, Shanghai First Maternity and Infant Hospital (Shanghai, China) from February 2013 to July 2014. The recruitment of women with normal pregnancies and pregnancies complicated by GDM and large for gestational age occurred at the time of admission for elective cesarean delivery at term (38-40 weeks). Women with any adverse underlying medical condition (including asthma, hypertension, preeclampsia and pregestational diabetes) were excluded. Women with GDM were diagnosed according to the IADPSGC criteria, i.e., women who had 1 or more fasting 1- or 2-h OGTT values equal or greater than the threshold values of 5.1, 10.0, and 8.5 mmol/L. All GDM subjects received dietary and exercise instruction treatment. Four women with GDM were prescribed insulin in the third trimester of pregnancy according to hospital guidelines for insulin therapy in GDM. Human umbilical cords from normal term pregnancies with no complications and pregnancies complicated by GDM were collected immediately after selective cesarean delivery. The women in the healthy control group had no histories of spontaneous miscarriage, ectopic pregnancy, preterm delivery, or stillbirth. The demographic and clinical characteristics of the study subjects, including maternal age, gestational age, gravidity and parity (Table 1), were recorded. No significant differences existed between the groups in terms of maternal age or gestational age at the time of pregnancy termination. Fresh chorionic villus samples were collected and immediately stored in liquid nitrogen until analysis. This study was approved by the ethics review board of the Shanghai First Maternity and Infant Hospital. Written consent for participation in this study was obtained from each individual after detailed explanations regarding the purpose and procedures were provided by the hospital staff.
Histology and Immunohistochemistry

Placental tissue sections (5 μm) were deparaffinized and dehydrated. The endogenous peroxidase activity was quenched by immersing the tissue sections in 3% H₂O₂ in methanol for 10 min. After blocking the non-specific binding with 1% horse serum albumin for 20 min, the tissue sections were immunostained using CD31 antibody ((Minneapolis, MN) and DAPI (Beyotime, Beijing, China). CD31 was used as an endothelial marker to quantify the fetoplacental capillaries.

Isolation and Identification of the HUVECs

The HUVECs were isolated using a standard collagenase enzyme digestion method [19]. Immediately after isolation, the cells were cultured and expanded steadily in Endothelial Cell Medium (ECM, ScienCell, San Diego, CA) containing 5% fetal bovine serum, 1% penicillin/streptomycin and ECGS (ScienCell). After 2 to 3 days of culture, the cells were sorted by flow cytometry based on their expression of platelet/endothelial cell adhesion molecule 1 (PECAM 1). The purity of the cells was also examined based on the uptake of 1,10-dioctadecyl-3,3,30,30-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL). HUVECs at passages 3 to 5 from the normal and GDM pregnancies were used for the studies of cell functional and the signaling pathway experiments. For high glucose treatment, we used ECM containing 25mM glucose. ECM containing 5mM glucose (5mM D-glucose+20 mM L-glucose) was used as normal osmotic control.

Cell Proliferation and Migration Assays

The cell proliferation assays were performed using MTS (Promega, Madison, WI) assays. The cells were seeded in 96-well plates (3000 cells/well) and cultured overnight. The HUVECs were starved in ECM containing 1% BSA and 1% penicillin/streptomycin for 8 h, and the cells were then treated with FGF-basic (FGF2; PeproTech Inc., Rocky Hill, NJ) or VEGF165 (VEGF; PeproTech) in ECM containing 1% heat-inactivated FBS (56°C, 30 min) for 48 h. VEGF and FGF2 were added at different concentrations (0-100 ng/ml). To examine the effects of the MEK1/2/ERK1/2 pathway on cell proliferation, additional cells were pretreated with PD98059 (an ERK1/2 inhibitor; Selleck Chemicals LLC, Houston, TX) for 1 h prior to the proliferation assay. Ultimately, we chose FGF2 and VEGF at concentrations of 100 ng/ml for the further cell experiments.

Cell migration was evaluated using a 24-multiwell FluoroBlok transwell insert system (BD Biosciences, San Jose, CA). After 8 h of starvation with ECM containing 1% BSA and 1% penicillin/streptomycin, the cells were seeded into the insert (10000-150000 cells/well), and FGF2 or VEGF was subsequently added to the bottom wells. After 16 h of incubation the fluorescent dye calceinacetoxymethyl ester (final concentration 0.5 µg/ml; Invitrogen) was added to the bottom wells and incubated for 30 min. The migrated cells were recorded (in four randomly chosen fields per well) under a Nikon inverted microscope connected to CCD camera. The cell numbers in the pictures were counted.

Matrigel plug assay

AMatrigel plug assay was performed in BALB/c mice, as described previously with some modifications. Briefly, matrigel (500 µl, growth factor reduced, BD Bioscience) mixed with FGF2 (100 ng/ml), heparin...
(20 U) and high glucose solution (25 mM D-glucose) or normal glucose solution (5 mM D-glucose + 20 mM L-glucose) was injected subcutaneously into the flanks of mice. After 10 days, the matrigel plugs were removed and hemoglobin content in the plugs was determined with Drabkin’s reagent kit (Sigma-Aldrich, St. Louis, MO).

**Tube formation assay**

The formation of capillary-like structures was assessed in a 48-well plate using Growth Factor Reduced Matrigel (BD Biosciences, San Jose, CA). HUVECs (20,000 cells/well) resuspended in serum-free ECM were plated on top of solidified Matrigel (200 μl/well). After 8 h of incubation at 37°C, 5% CO₂, endothelial cell tubes in randomly chosen microscopic fields were photographed with a Nikon inverted microscope (Nikon Eclipse Ti, Tokyo, Japan). Tubular structures were quantified by manual counting the number of branch points in 4 randomly chosen low-power fields (×40) from each well.

**Western Blotting Analysis**

The Western blotting analysis was performed as described previously [20] After starvation for 16 h in ECM containing 1% BSA and 1% penicillin/streptomycin with 5.5 mmol/L D-glucose or 25 mmol/L D-glucose, the cells were treated with FGF2 or VEGF (100 ng/ml) for 0–60 min. To examine the effects of PD98059 on ERK1/2 phosphorylation, additional cells were treated in a similar fashion with the exception that during the serum starvation and growth factor treatment, the cells were treated with PD98059 at 10 μM. The cells were harvested at different times (0, 5, 10, 30, 60 min, respectively) following FGF2 or VEGF treatment. The cells were lysed by sonication and centrifuged. The protein concentrations of the supernatants were determined. The proteins were separated on 12% SDS-PAGE gels, electroblotted onto Immobilon-P membranes (Millipore, Bedford, MA), and immunoblotted with either rabbit phospho-specific (1:2000, CST, Danvers, MA) or total ERK1/2 (1:2000, CST) antibody. The ERK1/2 on the membranes was visualized with a chemiluminescence system (Amersham Life Science Inc., Arlington Heights, IL) and quantified with scanning densitometry (model GS 670; Bio-Rad, Hercules, CA).

**Statistical Analyses**

The values are presented as the means ± the S.E.M. unless otherwise indicated. All statistics were performed with SigmaStat version 3.5 (Jandel Co., San Rafael, CA). One-way ANOVAs followed by Fisher’s LSD tests were applied for the comparisons of the clinical, cell proliferation and migration characteristics. Two-way ANOVAs followed by Bonferroni t-tests were applied for the Western blot analyses. The differences were considered significant when p < 0.05.

**Results**

**GDM did not change the capillary density of the fetal-placenta**

It has been reported that diabetic placentas primarily present with villous immaturity and fibrinoid necrosis, chorangiosis, and increased angiogenesis [21]. In the current study, we investigated the effects of GDM on fetoplacental capillaries using CD31 as an endothelial marker. Fig. 1 illustrates the expression of CD31 in the placental villi vascular system. In the digitized color images, the bright red color indicates positive CD31-labeled fetal capillaries, and the blue DAPI color identifies the nuclei. However, no significant difference in capillary density was detected between the GDM and healthy normal placentas.

Both the GDM and hyperglycemic conditions inhibited FGF2- but not VEGF-stimulated HUVEC proliferation.

To determine the role of GDM on placenta-fetal endothelial function, a cell proliferation assay was performed using the MTS method. We observed that both FGF2 and VEGF significantly increased (p<0.05) the proliferation of the HUVECs derived from the GDM group (dHUVECs) and the normal group (nHUVECs) (Fig. 2A). Importantly, GDM suppressed (p<0.05) the FGF2- but not the VEGF-stimulated HUVE cell proliferation compared with the normal control (Fig. 2A). These observations indicated that the dHUVECs exhibited impaired endothelial function after they were isolated and cultured in vitro. This impaired
endothelial function might have been caused by the high glucose environment in GDM or other disordered metabolic factors. To further test the effects of hyperglycemic conditions on
cell proliferation, nHUVECs from uncomplicated pregnancies were treated with increasing glucose concentrations (25 mM). Similarly, hyperglycemia significantly inhibited FGF2- but not VEGF-stimulated cell proliferation (Fig. 3A).

Both GDM and hyperglycemia inhibited the migratory capacity of the HUVECs but not FGF2- or VEGF-stimulated cell migration.

Endothelial cell migration is essential for angiogenesis and plays central roles in many physiological and pathological conditions. Here, we compared the migratory capacities of nHUVECs and dHUVECs utilizing the well-established and commonly used transwell assay for cell migration. As illustrated in Fig. 2B, the dHUVECs were characterized by a significant reduction in migratory capacity compared with nHUVECs. Moreover, the hyperglycemic condition also significantly impaired the migratory capacity compared with the normal culture condition (Fig. 3B). Furthermore, neither GDM nor hyperglycemia altered FGF2- or VEGF-stimulated nHUVEC migration (Fig. 2B and 3B). These results clearly demonstrated that the high glucose environment impaired cell migration activity.

**Fig. 3.** Effects of hyperglycemia (HG) on FGF2- and VEGF-stimulated HUVE cell proliferation and migration. Cell proliferation (A) and migration (B) were examined with MTS and transwell assays. The data are expressed as the mean ± the SEM percentages of the control (n = 4). *Different from the control (p < 0.05). *Different from normal (p < 0.05).
The hyperglycemic condition inhibited the tube formation and ex vivo angiogenesis in Matrigel Plug Assay

The formation of capillary-like structures is one of the critical steps to the formation of new blood vessels. Here, we compared the tube formation of nHUVECs and dHUVECs using an matrigel assay. As illustrated in Fig. 4, FGF2, but not VEGF significantly induced tube formation in both nHUVECs and dHUVECs. Neither GDM nor hyperglycemia altered FGF2-stimulated tube formation (Fig. 4 and 5A). Importantly, the hyperglycemic condition significantly impaired the basal level of tube formation compared with the normal culture condition (Fig. 5A). To further confirm the effects of hyperglycemia on placenta angiogenesis, matrigel plug assay was performed. As shown in Fig. 5B, FGF2-induced angiogenesis in the matrigel plug was significantly inhibited under high glucose conditions. These data indicated that hyperglycemia significantly impaired the tube formation of endothelial cells and angiogenesis in the matrigel plug.

The hyperglycemic condition inhibited the FGF2-induced activation of ERK1/2 in the HUVECs

It is well demonstrated that the MEK1/2/ERK1/2 pathway is partially involved in FGF2- and VEGF-induced fetoplacental endothelial cellular proliferation and migration [15, 16]. Therefore, we examined the effects of hyperglycemic conditions on the FGF2- and VEGF-induced activations of ERK1/2, which is one of the critical signaling molecules in HUVECs, using Western blot. We found that the basal levels of phospho-ERK1/2 (pERK1/2) at time 0 and the total ERK1/2 (tERK1/2) levels at all of the studied time points were similar between the hyperglycemic and normal conditions (Fig. 6). Both FGF2 and VEGF rapidly (> 10 min) induced ERK1/2 phosphorylation in the treated cells in both of these conditions (Fig. 6). Compared with the normal condition, the hyperglycemic condition significantly inhibited the FGF2- but not the VEGF-induced ERK1/2 phosphorylation (Fig. 6).
Effects of PD98059 on FGF2-induced ERK1/2 HUVEC activation and cell proliferation

To further investigate the role of the MEK1/2/ERK1/2 pathway in HUVE cell proliferation, the pharmacological kinase inhibitor PD98059 was used in a cell proliferation assay. Based on our previous study, the optimal concentration of PD98059 at 10 μM was used [15]. Importantly, PD98059 at 10 μM significantly decreased FGF2-induced ERK1/2 activation (Fig. 7A) and only partially FGF2-stimulated HUVE cell proliferation (Fig. 7B). These data clearly suggested that MEK1/2 may mediate the hyperglycemia-induced inhibition of the proliferation of HUVECs that is stimulated by FGF2. It is noteworthy that PD98059 only partially blocked FGF2-stimulated cell proliferation, suggested that other signaling pathways may involved in this cellular action. Indeed both FGF2- and VEGF-induced cellular responses (cell proliferation, migration) are mediated via activation of multiple protein kinases, including ERK1/2, p38 MAPK and AKT1[14], but the mechanism awaits further investigation.

Discussion

There are many proposed mechanisms by which maternal hyperglycemia could induce fetoplacental endothelial dysfunction [17, 18]. In the current study, we found that both
Fig. 6. Effects of hyperglycemia on FGF2- and VEGF-induced ERK1/2 phosphorylation in HUVE cells. Cells plated in 6-cm culture dishes were cultured under normal glucose conditions until they reached 70–80% confluence and were then cultured for 24 h in normal or hyperglycemic conditions followed by serum starvation for an additional 16 h. The cells were then treated with 100 ng/ml FGF2 or VEGF for 0–60 min. The proteins were subjected to Western blot analyses for total ERK1/2 (tERK1/2) and phospho-ERK1/2 (pERK1/2). The data were normalized to tERK1/2 and are expressed as the mean ± the SEMs fold-change relative to the controls at time 0 (n = 3). *Different (p < 0.05) from the corresponding time 0 control. #Different from normal (p < 0.05).

Fig. 7. Effects of PD98059 on FGF2-induced ERK1/2 phosphorylation (A) and FGF2-stimulated cell proliferation (B) in HUVE cells. The cells were cultured in normoxic conditions for 24 h. After serum starvation, the cells were treated with FGF2 (A) for 5 min in the absence or presence of PD98059 (10 mM, 1 h pretreatment). The proteins were subjected to Western blot analyses for total ERK1/2 (tERK1/2) and phospho-ERK1/2 (pERK1/2). Cell proliferation was examined with a MTS assay. The data are expressed as the mean ± the SEM fold-change relative to the control (no growth factor or kinase inhibitor; n = 3) *Different from the control (p < 0.05). #Different from the FGF2-treated group (p < 0.05).

GDM and the hyperglycemic condition significantly inhibited the FGF2- but not the VEGF-stimulated HUVEC proliferation in addition to basal levels of migration, tube formation and ex vivo angiogenesis. Importantly, we revealed that the MEK1/2/ERK1/2 signaling...
pathway may mediate the hyperglycemia-induced inhibition of HUVE cell proliferation that is stimulated by FGF2.

Placenta angiogenesis is considered to be a crucial process that is responsible for the correct function of the placenta [21]. It has been reported that the majority of placentas from GDM pregnancies exhibit increased [22] or abnormal angiogenesis [23, 24]. Our present data revealed that the placental weights from the GDM pregnancies were significantly increased compared with those of the normal healthy controls (Table 1). However, we did not find any substantial difference in villous capillary density between the diabetic and healthy placentas.

To further investigate the effects of hyperglycemia on placental angiogenesis, we performed in vitro proliferation, migration and tube formation assays involving constant control of the angiogenic factors, including FGF2 and VEGF, using both diabetic and normal HUVE cells. Reduced proliferation of diabetic endothelial cells has recently been reported [25, 26]. In keeping with this report, we found that the HUVECs from the GDM pregnancies exhibited an inhibition of cell proliferation that was stimulated by FGF2 compared with the control group. Further study indicated that the in vitro hyperglycemic condition (25 mM) also significantly inhibited FGF2-stimulated HUVEC proliferation. However, neither GDM nor hyperglycemia altered VEGF-stimulated cell proliferation. A possible explanation for this distinction is that hyperglycemia differentially alters these angiogenic factors and the expressions of their receptors in the fetoplacental endothelium [27, 28]. Indeed, a recent study indicated significant increases in total VEGF mRNA expression and protein release in diabetic HUVECs [27], and these increases probably counteract the inhibitory effect of hyperglycemia on VEGF-stimulated cell proliferation.

Another important finding is that both GDM and hyperglycemia impaired the basal migratory activity. Increasing evidence indicates that hyperglycemic conditions directly cause the deregulation of the transcription of multiple genes involved in cell movement, adhesion and cell migration [29-31], which in turn caused the dHUVECs to exhibit reduced migratory capacity compared with the nHUVECs. However, we did not observe significant inhibitory effects of hyperglycemia on FGF2- or VEGF-stimulated cell migration. We hypothesize that these results were due to the reduced basal migratory capacity. Finally we observed that hyperglycemia significantly inhibited the basal level of tube formation and ex vivo angiogenesis in Matrigel Plug Assay, further indicated that high glucose condition impaired the endothelial cells functions and placenta angiogenesis. Additional studies will be designed to clarify the molecular mechanisms that are involved in hyperglycemia-induced endothelial cells dysfunction.

Our previous work demonstrated that the MEK1/2/ERK1/2 signaling pathway plays major roles in placenta endothelial cell proliferation and migration. Consistent with these founding, our present data also indicated that both hyperglycemia and GDM activated the ERK1/2 signaling pathway. Importantly, hyperglycemia significantly suppressed FGF2-induced cell proliferation and ERK1/2 phosphorylation, which suggests that ERK1/2 is probably involved in the hyperglycemia-induced endothelial dysfunction in GDM. These data are supported by a recent study involving a rat model that found that hyperglycemia-induced placental and embryonic developmental abnormalities might be associated with a reduction in ERK1/2 phosphorylation [32].

Overall, our study demonstrated that maternal diabetes induces significant alterations in fetal endothelial function, including cell proliferation and migration. The inhibition of the MEK1/2/ERK1/2 signaling pathway by hyperglycemia may cause reduced cell proliferation in response to FGF2 stimulation. However, the molecular mechanisms underlying the hyperglycemia-induced reduction in cell migration warrants further investigation.

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Disclosure Statement

The authors declare that they have no competing interests.

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