In Vitro Hyperglycemia or a Diabetic Intrauterine Environment Reduces Neonatal Endothelial Colony-Forming Cell Numbers and Function

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OBJECTIVE—Emerging data demonstrate that maternal diabetes has long-term health consequences for offspring, including the development of hypertension. In adults, circulating endothelial progenitor cells (EPCs) participate in vascular repair, and EPC numbers and function inversely correlate with the risk of developing vascular disease. Therefore, our objectives were to determine whether hyperglycemia or exposure to a diabetic intrauterine environment alters EPC function.

RESEARCH DESIGN AND METHODS—We used well-established clonogenic endothelial colony-forming cell (ECFC) assays and murine transplantation experiments to examine human vasculogenesis.

RESULTS—Both in vitro hyperglycemia and a diabetic intrauterine environment reduced ECFC colony formation, self-renewal capacity, and capillary-like tube formation in matrigel. This cellular phenotype was linked to premature senescence and reduced proliferation. Further, cord blood ECFCs from diabetic pregnancies formed fewer chimeric vessels de novo after transplantation into immunodeficient mice compared with neonatal ECFCs harvested from uncomplicated pregnancies.

CONCLUSIONS—Collectively, these data demonstrate that hyperglycemia or exposure to a diabetic intrauterine environment diminishes neonatal ECFC function both in vitro and in vivo, providing potential mechanistic insights into the long-term cardiovascular complications observed in newborns of diabetic pregnancies. ***Diabetes* 57:724–731, 2008**

The incidence of diabetes is increasing exponentially. According to the U.S. Department of Health and Human Services, ~10% of the population over 20 years of age have diabetes (type 1 + type 2), whereas another 26% have pre-diabetes (defined as impaired fasting glucose levels), many of whom are unaware of their diagnosis (1). Vascular diseases associated with diabetes contribute significantly to the morbidities and mortality of this chronic disease. In fact, 73% of adults with diabetes are hypertensive and >65% die from cardiovascular disease or stroke (1,2). Intensive treatment of type 1 diabetic patients decreases cardiovascular and microvascular complications (3), suggesting that the degree of hyperglycemia correlates directly with the development of vascular morbidities and is a key pathogenic factor in the development of endothelial dysfunction.

The burden of diabetes in pregnancy (type 1, type 2, and gestational) is also increasing, currently affecting ~6–10% of all pregnant women (1,4). It has long been recognized that maternal diabetes is associated with numerous fetal and neonatal morbidities including congenital anomalies, premature delivery, fetal macrosomia, neonatal respiratory distress, and neonatal hypoglycemia. However, emerging data suggest that maternal diabetes also has long-term health consequences for offspring (5–18). The most compelling data are studies in Pima Indians where offspring of women with either gestational diabetes or type 2 diabetes have an increased risk of developing the metabolic syndrome, hypertension, type 2 diabetes, and obesity, and in many cases, elevated blood pressure, insulin resistance, and increased BMI occur in childhood (10,11,14,17). The concept that alterations in the intrauterine environment results in fetal adaptations that increase the risk for subsequent disease is well described. However, the current challenge is to understand the underlying mechanisms responsible for these effects to design preventative strategies for individuals. Given that hypertension is prevalent in offspring of diabetic mothers, it is critical to address how the diabetic intrauterine environment affects the developing vascular system.

Multiple factors contribute to the maintenance of vascular integrity, thereby protecting an individual from vascular disease. A critical component of vascular health is efficient repair of damaged endothelium and the ability to form new blood vessels. Endothelial progenitor cells (EPCs) participate in both of these processes. Whereas significant debate remains over the optimal method for defining EPCs, numerous studies demonstrate an indirect correlation between circulating EPC numbers and vascular disease risk. Initial studies reported a reduction in circulating EPCs in individuals with coronary artery disease and in those adults with a high cardiovascular disease risk predicted by a Framingham risk factor score (19,20). Interestingly, this paradigm has held for multiple disease states in adults including diabetes, peripheral vascular disease, rheumatoid arthritis, and preeclampsia (21–24). However, no studies have been reported in infants or children.

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ECFC, endothelial colony-forming cell; EPC, endothelial progenitor cell; MNC, mononuclear cell; SA-β-gal, senescence-associated β-galactosidase.

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Previously, we characterized circulating EPCs as endothelial colony-forming cells (ECFCs) using functional parameters routinely used to define progenitor cells such as proliferative potential and self-renewal capacity (25–27). ECFCs circulate in the blood (cord blood and adult peripheral blood) and reside in the endothelium of vessel walls. In addition, ECFCs are highly proliferative, capable of 100 population doublings for cord blood, exhibit self-renewal capacity demonstrated by replating ability, and are capable of de novo vessel formation in vivo. Given that reduced circulating EPC numbers in adults are associated with increased vascular disease risk together with the observation that offspring of diabetic mothers have an increased incidence of hypertension, we questioned whether fetal exposure to a diabetic intrauterine environment would result in reduced cord blood ECFC numbers and function.

RESEARCH DESIGN AND METHODS

Umbilical cord blood samples and subject characteristics. Human umbilical cord blood samples (40–60 ml) from healthy newborns and infants of diabetic mothers (gestational age 38–42 weeks) were collected in heparinized solution. At Indiana University School of Medicine, screening oral glucose tolerance tests are performed as part of routine care on all nondiabetic pregnant women between 24 and 28 weeks' gestation. All control mothers had a normal screening 50-g oral glucose challenge, defined as a 1-h glucose <140, and normal blood pressure throughout pregnancy. All diabetic mothers had an established diagnosis of diabetes before pregnancy and were on insulin at delivery. Diabetic mothers had normal blood pressure and renal function throughout pregnancy documented by serum creatinine, serum blood urea nitrogen, and urinalysis. In addition, none of the women had evidence of retinopathy before pregnancy, and fundoscopic exams in three of the four women during pregnancy (16–32 weeks' gestation) were normal. The fourth diabetic woman did not have an ophthalmologic exam during pregnancy. Women with illnesses known to affect glucose metabolism (i.e., polycystic ovarian syndrome, Cushing syndrome) and taking medications known to affect glucose metabolism (i.e., glucocorticoids) were excluded from the study. The Institutional Review Board at the Indiana University School of Medicine approved all protocols, and informed consent was obtained from all pregnant women.

Culture of ECFCs. Immediately after collection of umbilical cord blood samples, human mononuclear cells (MNCs) were obtained by diluting blood with PBS 1:1, which was underlaid with an equivalent volume of Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ). Cells were centrifuged for 30 min at room temperature at 740*g*. MNCs were isolated and washed with EBM-2 medium (Cambrex, Walkersville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2% penicillin/streptomycin, and 0.25 μg/ml amphotericin (Invitrogen, Grand Island, NY) (complete EBM-2 medium).

MNCs were resuspended in complete EGM-2 medium (complete EBM-2 supplemented with EGM-2 bullet kit; Cambrex). Six-well tissue culture plates precoated with type 1 rat tail collagen (BD Biosciences, Bedford, MA) were seeded with 5×10^7 cells/well and cultured in a humidified incubator. After 24 h of culture, adherent cells were washed with complete EGM-2, and complete EGM-2 medium was added to each well. Medium was changed daily for 7 days and then every other day until the first passage.

Colonies of endothelial cells appeared between 5 and 8 days of culture and were identified as circumscribed monolayers of cobblestone-appearing cells. Upon confluency, endothelial cells were detached with 0.25% trypsin-EDTA (Invitrogen), resuspended in complete EGM-2 media, and plated on tissue culture flasks coated with type 1 rat tail collagen.

For this study, hyperglycemia treatment included a 7-day exposure of early passage (P3–P4) ECFCs of healthy newborns with D-glucose (Invitrogen) added to complete EGM-2 media. Complete EGM-2 media is considered euglycemic with a basal concentration of 5 mmol/l dextrose.

Colony-forming assays. For limiting dilution assays, ECFCs were seeded in six-well plates precoated with type 1 rat tail collagen (200 cells/well). Each condition was plated in triplicate wells. On day 7 after initial plating, wells were scored for colony formation by visual inspection with an inverted microscope (Olympus, Lake Success, NY) under 40× magnification.

ECFC growth kinetics. Cord blood ECFCs were passaged upon reaching 90% confluency. At each passage, ECFCs were counted by trypan blue exclusion for calculation of growth kinetic curve and cumulative population doubling levels. The number of population doublings occurring between passages was calculated according to the following equation: population doubling = $\log_2 (C_H/C_S)$, where C_H is the number of viable cells at harvest and C_s is the number of cells seeded. The sum of all previous population doublings determined the cumulative population doubling level at each passage.

Senescence-associated β-galactosidase (SA-β-gal) staining. ECFCs were cultured in six-well plates for 7 days before β-galactosidase staining to assess for senescence. Wells were washed, fixed with 2% formaldehyde/0.2% gluteraldehyde, and stained for 24 h with 150 mmol/l β-galactosidase solution (Acros, NJ), 2 mmol/l $MgCl_2$ (Fisher, Fair Lawn, NJ), 40 mmol/l trisodium citrate (Sigma-Aldrich, St Louis, MO), 5 mmol/l potassium ferricyanide (Sigma-Aldrich), and 5 mmol/l potassium ferrocyanide (Sigma-Aldrich), adjusted to 6 pH containing X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) (Invitrogen). Senescence was examined by visual inspection of deep blue stained cells with an inverted microscope under 40× magnification. All conditions were conducted in triplicate and at least 100 cells were scored per replicate.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay. ECFCs that were either untreated or treated with D-glucose for 1–7 days were examined for apoptosis using a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay per the manufacturer's recommendation (Roche, Indianapolis, IN). All conditions were conducted in triplicate, and at least 100 cells were scored per replicate.

H^3-thymidine proliferation assay. ECFCs were starved for 24 h without growth factors in EBM-2 media supplemented with 5% fetal bovine serum. Cells were plated at 50,000 cells/well in a six-well collagen-coated plate in triplicate and starved overnight in EBM-2 supplemented with 1% fetal bovine serum. ECFCs were then cultured in EBM-2 without serum for 8 h to achieve quiescence. Media were then changed to EBM-2 supplemented with 10% fetal bovine serum, and cells were untreated, stimulated with 25 ng/ml vascular endothelial growth factor, or stimulated with 25 ng/ml basic fibroblast growth factor (Peprotech, Rocky Hill, NJ) for 16 h. Cells were pulse-labeled with 1 μCi (0.037 MBq) tritiated thymidine (Perkin Elmer Life Sciences Products, Boston, MA) for 5 h and lysed in 0.1 M NaOH for 1 h before counting in a liquid scintillation counter (Beckman Coulter).

Matrigel assay. ECFCs were cultured at 8,000 cells/well in 96-well tissue culture plates coated with 50 μl Matrigel (BD Biosciences). At 24 h, images were collected using a Zeiss Axiovert one inverted microscope with a 5× CP-ACHROMAT/0.12 NA objective. Images were acquired using a SPOT RT color camera (Diagnostic Instruments, Sterling Heights, MI) with the manufacturer's software. Each experiment was done in triplicate.

Xenograft transplant. Cellularized gel implants were cast as previously described (28,29). Cord blood ECFCs were suspended (2×10^6 cells/ml) in complete EGM-2 supplemented with 1.5 mg/ml rat tail collagen I, 100 ng/ml human fibronectin (Chemicon), 1.5 mg/ml sodium bicarbonate (Sigma), 25 mmol/l HEPES (Cambrex), and 10% fetal bovine serum. Pericytes isolated from human adipose tissue were added to the gels to provide mural support to newly forming vessels as previously described (30). Cell suspensions were placed in a 12-well tissue culture dish for 30 min at 37°C for polymerization. The gels were then covered with complete EGM-2 for overnight incubation. Gels were implanted the following day into three anesthetized NOD/SCID mice. The right flank of each mouse was implanted with control ECFCs, and the left flank was implanted with ECFCs from diabetic pregnancies. Fourteen days after transplantation, grafts were excised and analyzed for vessel formation by immunohistochemistry. All experiments were approved by the Indiana University Laboratory Animal Research Center.

Immunohistochemistry. All reagents for immunohistochemistry studies were purchased from DAKO (Carpenteria, CA) unless otherwise specified. For anti-human CD31 staining, formalin-fixed paraffin-embedded tissue sections were deparaffinized and immersed in a retrieval solution for 20 min at 95–99°C. Slides were incubated at room temperature with anti-human CD31 (clone JC70A) for 30 min followed by 10-min incubations with LSAB2 link-biotin and streptavidin–horseradish peroxidase and then developed with DAB solution for 5 min. Slides were dehydrated and mounted using Cytoseal (Richard-Allen Scientific, Kalamazoo, MO). Enumeration of perfused vessel staining positively for anti-human CD31 was performed by visual inspection under 100 × magnification.

RESULTS

Hyperglycemia reduces cord blood ECFC clonogenic growth via premature senescence. To test whether neonatal ECFCs are sensitive to hyperglycemia stress, cord blood ECFCs from uncomplicated pregnancies were cultured for 7 days with increasing dextrose concentrations in clonogenic progenitor assays as described (29). A range of dextrose concentrations were tested including

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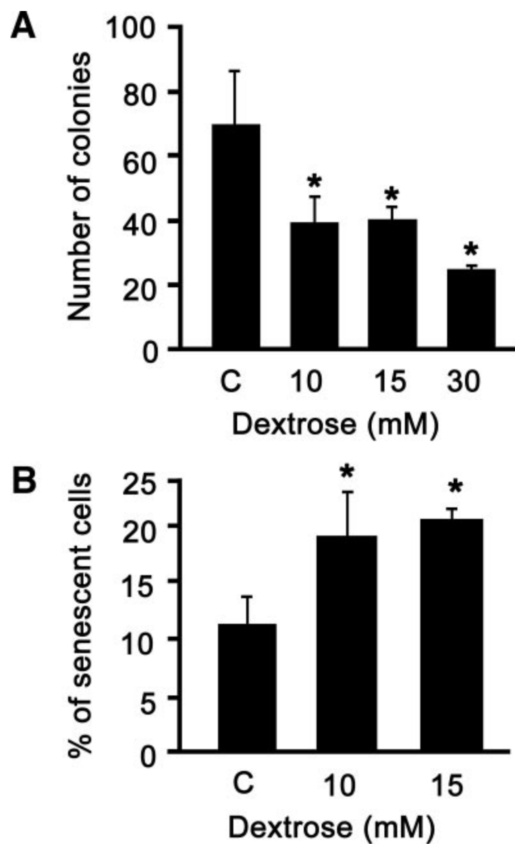

FIG. 1. ***A*** **and** ***B*****: Hyperglycemia reduces cord blood ECFC clonogenic capacity via senescence.** ***A*****: Number of ECFC-forming colonies 7 days after dextrose treatment. Results represent the mean ± SE, n = 4, *P < 0.03 for dextrose-treated versus the untreated control (C) by Student's t test.** ***B*****: Percentage of ECFCs that stained positively for SA-β-gal 7 days after dextrose treatment. Results represent the mean of three independent experiments ± SE; *P < 0.05 for dextrose-treated versus control by Student's t test.**

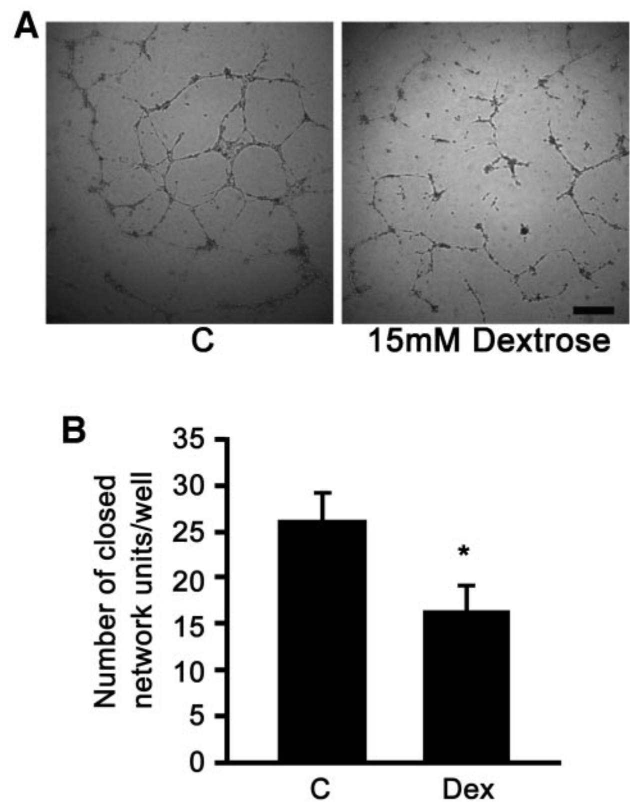

FIG. 2. ***A*** **and** ***B*****: Hyperglycemia reduces in vitro capillary formation.** ***A*****: Representative photomicrographs of ECFCs after plating on Matrigel. Results are representative of three independent experiments. Scale bar represents 200 μm.** ***B*****: Quantitation of capillary vessel density of control ECFCs (C) or ECFCs treated with 15 mmol/l dextrose (Dex). Data are means ± SE, n = 3. *P < 0.05 by Student's t test.**

levels of hyperglycemia observed in pregnant women with diabetes. ECFC colony formation was reduced at 10, 15, and 30 mmol/l dextrose concentrations compared with normoglycemic control subjects (5 mmol/l dextrose) (Fig. 1*A*). Interestingly, the most profound reduction was between the normoglycemic control and the lowest dextrose concentration tested (10 mmol/l or 180 mg/dl). To interrogate the mechanism responsible, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays and SA-β-gal staining were conducted to examine for increased apoptosis and/or senescence, respectively. No increase in apoptosis was observed in ECFCs treated with 10–15 mmol/l dextrose for 1, 3, 5, or 7 days (data not shown). However, cord blood ECFCs exhibited a twofold increase in SA-β-gal$^+$ cells after treatment with 10 and 15 mmol/l dextrose (Fig. 1*B*). Together these data suggest that enhanced senescence, but not apoptosis, contributes to the hyperglycemia-induced reduction of cord blood ECFC colony formation.

Given our observation that hyperglycemia induces enhanced senescence of cord blood ECFCs, we tested whether hyperglycemia diminishes tube formation of cord blood ECFCs using matrigel assays as described (29). Figure 2*A* shows representative photomicrographs of cord blood ECFCs cultured under normoglycemic and hyperglycemic conditions. Quantitative assessment of tube formation was conducted by scoring the number of closed capillary tube networks per well (Fig. 2*B*). A 30% reduction in tube formation was observed in cord blood ECFCs cultured under hyperglycemic conditions compared with control ECFCs. Collectively, these data demonstrate that exposure to levels of hyperglycemia seen routinely in pregnant diabetic women is sufficient to impair cord blood ECFC tube forming ability.

ECFCs exposed to a diabetic environment in utero exhibit premature senescence and reduced proliferation. Emerging evidence suggests that maternal diabetes increases the risk for offspring to develop cardiovascular disease; however, the underlying cellular mechanisms responsible are unknown. Given our data demonstrating that hyperglycemia induces significant functional deficits in cord blood ECFCs harvested from uncomplicated pregnancies, we hypothesized that maternal diabetes would alter fetal ECFCs in utero, resulting in reduced circulating cord blood ECFC numbers and function. To test this hypothesis, ECFC colonies were enumerated and cell lines established from nine pregnancies (four diabetic and five control subjects). Tables 1 and 2 include clinical data from the women and infants, respectively, in this study. Pregnant subjects with diabetes (two type 1 and two type 2) were considered well-controlled with A1C levels <6%, and none of the women had evidence of hypertension, nephropathy, retinopathy, or coronary artery disease. Two of the infants born to diabetic mothers had increased ponderal indexes with weight-to-height ratios above the 90th percentile. None of the infants born to the diabetic mothers developed hypoglycemia after birth. One infant born to a diabetic mother was diagnosed with respiratory distress

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TABLE 1
Clinical data for maternal subjects

	Maternal age (years)	Type of diabetes	Length of diabetes (years)	Medications	Maternal prepregnancy BMI (kg/m^2)	A1C (%)	Mode of delivery
Control 1	24	NA	NA	Zoloft	19	ND	Vaginal
Control 2	21	NA	NA	Topamax for seizures	30	ND	C-section
Control 3	40	NA	NA	None	30	ND	Vaginal
Control 4	25	NA	NA	None	20	ND	Vaginal
Control 5	21	NA	NA	None	27	ND	C-section
Diabetic 1	37	Type 1	20	Insulin	38	4.7–5.4	C-section
Diabetic 2	28	Type 2	14	Insulin	24	5.3–5.6	Vaginal
Diabetic 3	26	Type 1	14	Insulin pump	28	5.3–5.8	C-section
Diabetic 4	24	Type 2	5	Insulin, Synthroid	37	5.4	C-section

NA, not applicable; ND, not determined.

secondary to hyaline membrane disease versus pneumonia and was treated with oxygen and antibiotics in the neonatal intensive care unit.

To evaluate the effect of maternal diabetes on fetal ECFCs, the total number of ECFC colonies harvested from cord blood MNCs were enumerated. No differences were observed in the number of MNCs obtained from either experimental group (data not shown). However, a significant reduction in the number of cord blood ECFC colonies/10^8 MNCs was observed in samples from diabetic pregnancies compared with uncomplicated pregnancies (13.2 ± 2.6 vs. 40.1 ± 6.9, $P < 0.01$). Data from control ECFCs are similar to our previously published data (27,29). To examine whether in utero exposure to a diabetic environment alters the function of neonatal ECFCs, the primary cell progeny from all samples were expanded in culture, and functional assays were conducted. In addition to an overall decrease in the total number of ECFCs harvested from cord blood samples from diabetic pregnancies, the frequency of clonogenic precursors from these samples was significantly reduced compared with cord blood ECFCs from uncomplicated pregnancies (Fig. 3).

Because in vitro exposure of cord blood ECFCs to hyperglycemia increased senescence, we tested whether ECFCs from diabetic pregnancies also exhibited enhanced senescence, contributing to the reduction in clonogenic capacity. SA-β-gal assays demonstrated a twofold increase in senescent ECFCs from diabetic pregnancies compared with control subjects (Fig. 4*A* and *B*). Given that cord blood ECFCs are highly proliferative with self-renewal ability, we hypothesized that increased senescence would result in diminished proliferative capacity of ECFCs from diabetic pregnancies. To test this hypothesis, population doubling assays were conducted as described (29). These studies revealed that ECFCs from diabetic pregnancies exhibited a marked reduction in cumulative population doubling levels (Fig. 4*C*). To assess growth factor–induced proliferative responses, ECFCs from control and diabetic pregnancies were starved of serum and growth factors before stimulating with either basic fibroblast growth factor or vascular endothelial growth factor, two cytokines important for endothelial cell proliferation. ECFCs from diabetic pregnancies exhibited a significant reduction in baseline, basic fibroblast growth factor–induced, and vascular endothelial growth factor–induced proliferation compared with control subjects (Fig. 4*D*). Collectively, these data suggest that maternal diabetes results in an overall decline in proliferative capacity of cord blood ECFCs due to premature senescence.

ECFCs exposed to a diabetic environment in utero exhibit profound deficits in vascular regeneration. Impaired vascular regeneration at multiple anatomic sites leads to a variety of vasculopathies in diabetic patients. Given the effects of the diabetic environment on cord blood ECFCs outlined above, we next tested whether ECFCs from diabetic pregnancies exhibited decreased capillary-forming ability in vitro and diminished vasculogenesis in vivo. Figure 5*A* shows representative photomicrographs of capillary formation from ECFCs isolated from uncomplicated or diabetic pregnancies. Quantitative assessment of tube formation was conducted by scoring the number of closed capillary tube networks per well (Fig. 5*B*). A 66% reduction in tube formation was observed in cord blood ECFCs from diabetic pregnancies compared with control ECFCs (Fig. 5*B*).

TABLE 2
Clinical data for infant subjects

	Gestational age (weeks completed)	Infant weight (kg)	Infant weight/height percentile	Neonatal diagnoses
Control 1	40	3.45	75–90	Normal newborn
Control 2	39	3.26	10–25	Normal newborn
Control 3	39	3.79	50–75	Normal newborn
Control 4	39	3.2	5–10	Normal newborn
Control 5	41	3.96	50–75	Normal newborn
Diabetic 1	38	2.6	75–90	Normal newborn
Diabetic 2	38	3.85	95–97	Increased ponderal index
Diabetic 3	38	3.72	95–97	Increased ponderal index
Diabetic 4	38	4.01	75–90	Respiratory distress: hyaline membrane disease versus pneumonia

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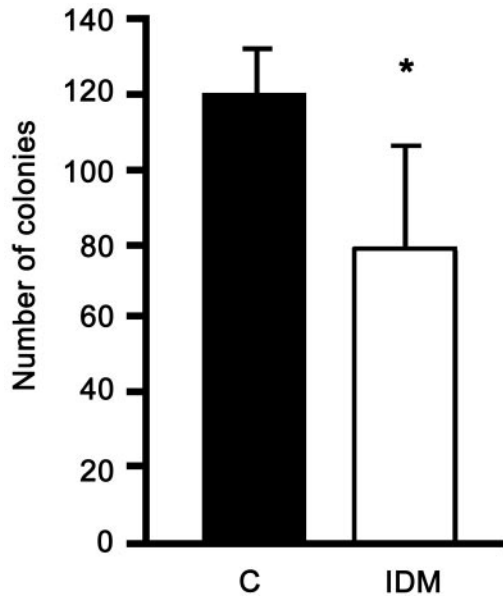

FIG. 3. In utero exposure to a diabetic environment reduces ECFC clonogenic capacity. Number of ECFCs derived from cord blood from uncomplicated (C) or diabetic (IDM) pregnancies, which formed colonies 7 days after plating at limiting dilution. Results are the mean ± SE, $n = 4$, $*P < 0.003$.

A characteristic of stem and progenitor cells, including ECFCs, is their ability to be adoptively transplanted into hosts and repopulate the mature cells of the intended lineage. Therefore, we compared the ability of cord blood ECFCs isolated from either diabetic or uncomplicated pregnancies to form vascular structures de novo using an established methodology for subcutaneous transplantation of cellularized gel implants into immunodeficient mice (28–30). Early passage cord blood ECFCs from diabetic or control pregnancies were generated from three different cord bloods, respectively, suspended in collagen/fibronectin gels and transplanted into immunodeficient mice. At 14 days, mice were killed and the grafts analyzed for chimeric vessel formation. Photomicrographs of cellularized gels and enumeration of vessels from diabetic or control ECFCs are shown (Fig. 5*C* and *D*). In three independent experiments, cord blood ECFCs from diabetic pregnancies had a twofold reduction in the number of chimeric vessels perfused with mouse erythrocytes in the transplanted graft compared with control ECFCs. Collectively, these data demonstrate that cord blood ECFCs from diabetic pregnancies exhibit diminished functional capacity both in vitro and in vivo.

DISCUSSION

Barker et al. (31) provided the initial epidemiologic evidence that infants born small for gestational age are at increased risk to develop a spectrum of adult diseases including the metabolic syndrome, cardiovascular disease, type 2 diabetes, and obesity. These seminal observations were the first to suggest that an individual's long-term risk for disease could be affected by prenatal events. Since these early reports, numerous studies in humans and

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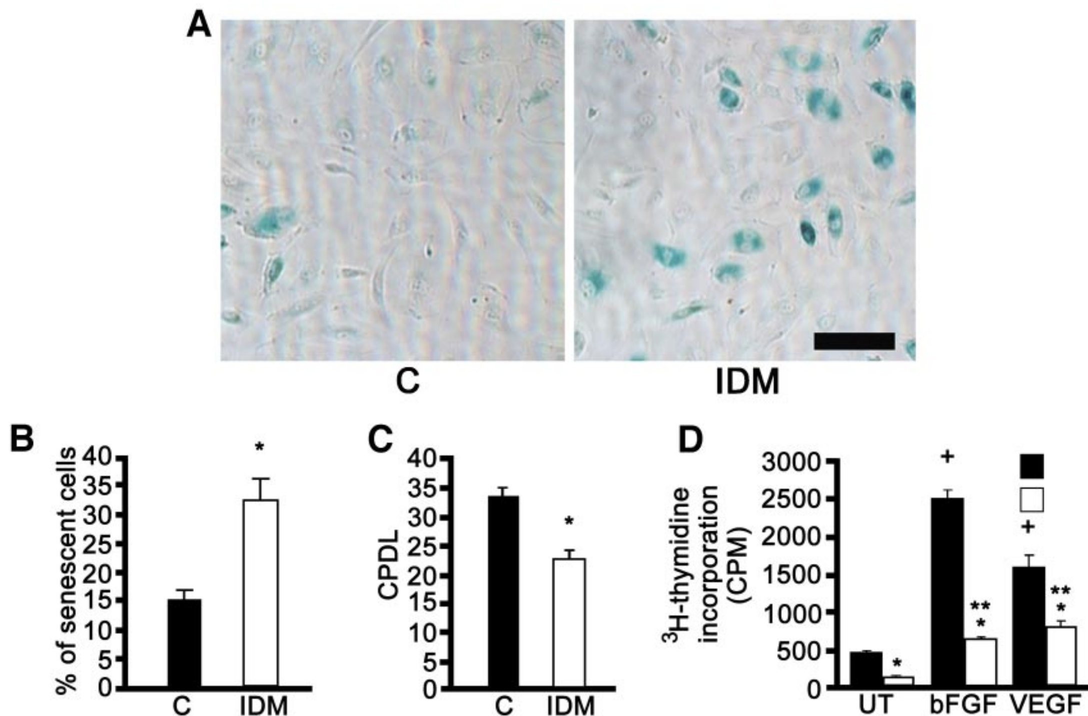

FIG. 4. *A–D*: Enhanced senescence of ECFCs exposed to a diabetic intrauterine environment correlates with reduced proliferative capacity. *A*: SA-β-gal staining of ECFCs derived from uncomplicated (C) or diabetic (IDM) pregnancies. Results are representative of four independent experiments. Scale bars represent 100 μm. *B*: Percentage of ECFCs derived from uncomplicated or diabetic pregnancies that stained positively for SA-β-gal. Results are means ± SM, $n = 4$, $*P < 0.002$ by Student's *t* test. *C*: Cumulative population doubling level (CPDL) of ECFCs derived from uncomplicated or diabetic pregnancies after 50 days of culture. Results are means ± SE, $n = 4$, $*P < 0.002$ by Student's *t* test. *D*: Proliferation of ECFCs derived from cord blood from uncomplicated (■) or diabetic (□) pregnancies in response to 25 ng/ml vascular endothelial growth factor or 50 ng/ml basic fibroblast growth factor stimulation. Results represent mean radiation counts per minute performed in triplicate from a representative experiment: $*P < 0.001$ compared with control, $**P < 0.002$ compared with untreated (UT) IDM condition, $+P < 0.002$ compared with UT control condition by Student's *t* test. Similar results were seen in two additional independent experiments. (Please see http://dx.doi.org/10.2337/db07-1507 for a high-quality digital representation of this figure.)

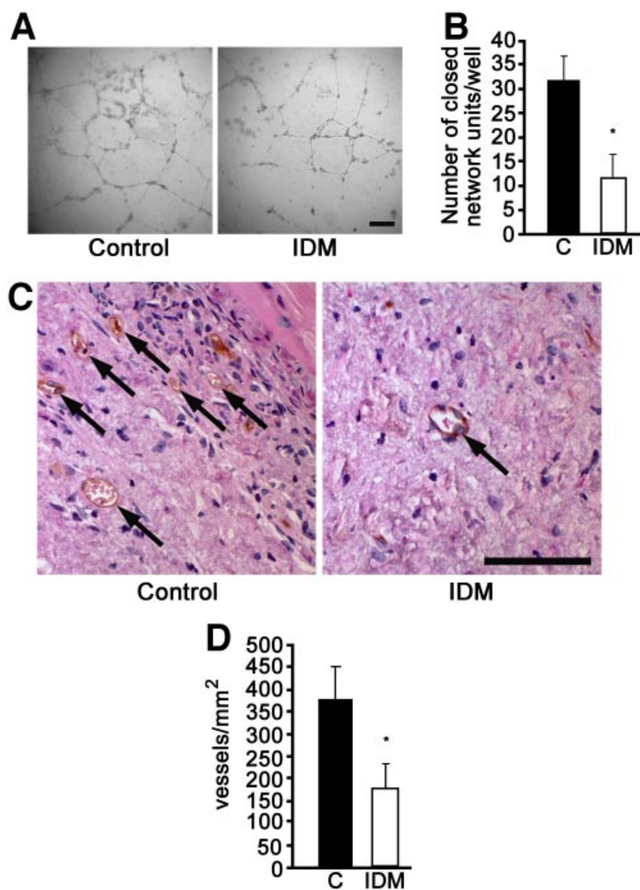

FIG. 5. *A–D*: In vitro and in vivo capillary formation of ECFCs exposed to a diabetic intrauterine environment is impaired. *A*: Representative photomicrographs of ECFCs derived from uncomplicated (C) and diabetic (IDM) pregnancies 24 h after plating on Matrigel. Results are representative of three independent experiments. Scale bar represents 30 μm. *B*: Quantitation of capillary vessel density 24 h after plating on Matrigel. Data are means ± SEM, $n = 4$. $*P < 0.002$ by Student's *t* test. *C*: Representative photomicrographs (100× magnification) of cellularized grafts and surrounding murine tissue 14 days after implantation into NOD/SCID mice stained with anti-human CD31 (brown) to identify human blood vessels. Grafts contained ECFCs from either an uncomplicated or diabetic pregnancy. Arrows indicate anti-human $CD31^+$ vessels and capillaries in the ECFC grafts, which were perfused with murine erythrocytes. Results shown are representative of three independent experiments using cells from different donors. *D*: Quantitation of capillary density within cellularized grafts containing ECFCs from uncomplicated or diabetic pregnancies 14 days after implantation. Results are the average number of capillaries containing murine erythrocytes/mm^2 of graft tissue ± SE, $n = 3$, $*P < 0.001$ by Student's *t* test. (Please see http://dx.doi.org/10.2337/db07-1507 for a high-quality digital representation of this figure.)

animals also demonstrate that intrauterine exposure to an energy-rich environment, such as a high-calorie diet, maternal diabetes, or maternal obesity, increase the risk for offspring to develop the metabolic syndrome, cardiovascular dysfunction, type 2 diabetes, and obesity (5,13,32, 33). Whereas clear evidence exists to show that alterations in the intrauterine environment increases disease risk in offspring, the underlying pathologic processes remain unknown.

Given the importance of EPCs in the maintenance and repair of the vascular system in adults together with the observed decrease in circulating EPCs in numerous adult disease states including diabetes, we speculated that in utero exposure to maternal diabetes during rapid growth of the fetal vasculature would diminish the numbers and function of circulating fetal EPCs. Previous studies by our group classified ECFCs as EPCs that circulate in cord blood and peripheral blood, are resident in blood vessel walls, and exhibit essential progenitor characteristics, having high proliferation potential, self-renewal capacity, and de novo blood vessel formation in vivo (26,27,29). Because maternal glucose freely diffuses across the placenta into the fetal circulation, initial studies examined whether hyperglycemia directly impairs fetal ECFC function. After hyperglycemia treatment, neonatal ECFCs from uncomplicated pregnancies exhibited significant functional deficits including a reduction in colony formation, enhanced senescence, and impaired tube-forming ability. The magnitude of the functional deficits observed at the lowest dextrose dose (10 mmol/l) was unexpected given that this concentration correlates with modest hyperglycemia in a diabetic pregnant woman (180 mg/dl) and can be seen routinely after a carbohydrate-rich meal or during an illness.

The majority of previous studies assessing the direct effect of hyperglycemia on EPCs were conducted on cell populations that express both hematopoietic and endothelial markers and have since been determined to be angiogenic macrophage precursors (34). Recently, however, Chen et al. (35) demonstrated that adult peripheral blood ECFCs treated with hyperglycemia exhibited enhanced senescence and reduced tube-forming ability in vitro, although the dextrose concentrations required to induce these phenotypes were much higher (20–30 mmol/l or 360–540 mg/dl) compared with our studies. The distinction between the degree of hyperglycemia required to induce functional changes in neonatal versus adult ECFCs may reflect important developmental differences that deserve further investigation.

To examine whether circulating ECFCs from diabetic pregnancies displayed similar functional deficits as hyperglycemia-treated ECFCs, cord blood ECFCs were harvested from uncomplicated and diabetic full-term pregnancies. Interestingly, cord blood from diabetic pregnancies had only one-third the number of circulating ECFCs compared with control cord blood samples. Our data support diminished growth factor–dependent proliferation and premature senescence of neonatal ECFCs from diabetic pregnancies as contributing factors involved in reduced ECFC colony-forming ability. To rigorously examine whether the functional capacity of neonatal ECFCs was impaired after exposure to a diabetic intrauterine environment, ECFCs were transplanted into NOD/SCID mice using a well-established in vivo vasculogenesis assay (28). Remarkably, ECFCs from diabetic pregnancies formed half as many human-murine chimeric vessels compared with control subjects. Collectively, these data suggest that in utero exposure to a diabetic environment severely diminishes the functional capacity of fetal circulating ECFCs.

Optimal glycemic control is the mainstay of therapy in diabetic pregnancies and is evaluated by serial blood glucose measurements and A1C levels. Given these criteria, the maternal subjects in our study were considered well controlled by their obstetricians, with A1C levels <6%. Therefore, the magnitude of cord blood ECFC functional deficits observed in the offspring of these women may initially appear surprising. However, intermittent episodes of mild-moderate hyperglycemia do not increase A1C levels appreciably. Our data showing that a short-term treatment with modest hyperglycemia (10 mmol/l dextrose or 180 mg/dl) significantly reduces neonatal ECFC function supports the idea that elevations in A1C

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levels may not be required for impairments in fetal ECFC function. Consistent with this notion, some investigators argue that targeting postprandial glucose levels, which are generally the daily peak hyperglycemia periods, should be routinely practiced in diabetic individuals to reduce their cardiovascular disease risk and improve neonatal outcomes (rev. in 36). Furthermore, whereas hyperglycemia is a major metabolic disturbance in diabetes, numerous metabolic, cellular, and biochemical derangements may be observed in diabetes, each of which could independently contribute to diminished fetal ECFC function. In support of this idea, evidence for fetal effects of an intrauterine diabetic environment was present in our study infants, even though the mothers had normal A1C values. For example, two of the infants had increased ponderal indexes (weight-to-height ratio >90th percentile), and a third infant (4.01 kg and weight-to-height ratio 75–90th percentile) experienced respiratory distress after delivery at full term. Collectively, these observations suggest that hyperglycemia directly impairs fetal ECFC function; however, additional factors likely contribute to fetal ECFC dysfunction in vivo given the complexity of the intrauterine diabetic environment. A limitation of this study was the number of cord blood ECFCs analyzed. However, the consistency of the data in all ECFC cell lines derived from diabetic pregnancies compared with five different control ECFC cell lines strongly supports the contention that fetal exposure to maternal diabetes has detrimental effects on neonatal circulating ECFCs. Future studies to explore additional factors involved in ECFC dysfunction are planned.

Our data demonstrating enhanced senescence in cord blood ECFCs from diabetic pregnancies are intriguing given previous studies supporting a role for enhanced endothelial cell senescence in the pathogenesis of vasculopathies (rev. in 37). Senescent cells are active participants in vascular disease progression via the induction of vascular structural changes, inflammatory cytokine production, and loss of vascular reparative capacity. Typically, endothelial cell senescence is observed during the process of aging or after damage from a chronic disease such as diabetes. Therefore, our data showing that neonatal ECFCs from diabetic pregnancies prematurely senesce are disconcerting given the tremendous vascular growth that occurs from infancy through adolescence. Collectively, our data together with previous studies correlating reduced circulating EPC numbers with cardiovascular disease risk in adults suggest that reduced circulating cord blood ECFC numbers and premature ECFC senescence may predispose infants born to diabetic mothers to develop endothelial dysfunction and ultimately cardiovascular disease.

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