OBSTETRICS

Role of HIF-1α in maternal hyperglycemia-induced embryonic vasculopathy

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OBJECTIVE: Maternal diabetes adversely impacts embryonic vasculogenesis, which results in embryonic vasculopathy. The purpose of our study is to determine whether hypoxia inducible factor (HIF)-1α plays a role in diabetic embryonic vasculopathy.

STUDY DESIGN: Levels of HIF-1α were determined in mouse conceptuses. Conceptuses on day 7 of pregnancy were cultured under euglycemic (150 mg/dL glucose) and hyperglycemic (300 mg/dL) conditions with or without AdCA5, or in the presence or absence of 2.0 μg/mL human recombinant thioredoxin, an endogenous antioxidant protein. AdCA5 is an adenovirus encoding a constitutively active form of HIF-1α.

RESULTS: Maternal diabetes significantly reduced HIF-1α protein expression. The administration of 1 μL (1×10^7 infectious units/mL) per 1 mL culture medium AdCA5 completely reversed hyperglycemia-reduced vasculature morphological scores and vascular endothelial growth factor expression. Thioredoxin treatment reversed hyperglycemia-reduced HIF-1α levels.

CONCLUSION: We conclude that reduced HIF-1α plays a critical role in the induction of diabetic embryonic vasculopathy, and that oxidative stress is implicated in hyperglycemia-induced HIF-1α reduction.

Key words: constitutively active hypoxia inducible factor-1α, diabetic embryonic vasculopathy, hypoxia inducible factor-1α, maternal diabetes, vascular endothelial growth factor

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Congenital malformations occur in up to 10% of babies born to diabetic women, and the recent rise in diabetes[1] makes this pregnancy complication an extraordinarily important issue.[2] The embryonic vasculature is the first system to be developed and is the most vulnerable to the in utero environmental conditions. Hyperglycemia has been shown to be associated with embryonic vasculopathy, which leads to embryonic lethality or malformations.[3,4] The mechanism underlying maternal diabetes-induced embryonic vasculopathy is elusive. Most studies have focused on the mechanisms of maternal diabetes-induced malformations and have determined that congenital malformations during maternal hyperglycemia are the result of a disruption in the balance between intracellular reactive oxygen species and endogenous antioxidant capacities.[5-8] This oxidative-stress hypothesis is also applicable to maternal diabetes-induced embryonic vasculopathy because our recent study has found that a natural antioxidant purified from green tea, epigallocatechin-3-gallate, is effective in amelioration of hyperglycemia-induced embryonic vasculopathy in vitro.[9] Although oxidative stress mediates the negative impact on embryonic vasculogenesis, it is not clear how maternal diabetes and consequent oxidative stress adversely regulate vascular factors leading to embryonic vasculopathy.

Hypoxia inducible factor (HIF)-1 is a key transcriptional factor for hypoxic regulation of embryonic vascular development. HIF-1α is the oxygen-sensitive subunit of HIF-1.[10] Regulation of HIF-1 activity is critically dependent on the degradation of the HIF-1α subunit in normoxia.[11] HIF-1 acts as a master regulator of angiogenesis by controlling the expression of multiple angiogenic growth factors, including vascular endothelial growth factor (VEGF).[12] Mice lacking HIF-1 activity due to HIF-1α null mutation develop extensive vascular defects similar to those observed in diabetic embryonic vasculopathy, including inadequate vessel formation and aberrant vascular remodeling.[13,14] Increased[15] or reduced[16-18] HIF-1α protein levels contribute to the pathogenesis of several diabetic complications. Because the stability of HIF-1α is pivotal to its functions in embryonic vasculogenesis, we chose to assess both HIF-1α gene expression and protein levels in diabetic embryonic vasculopathy.

Embryonic vasculogenesis begins in the yolk sac (extraembryonic membrane) prior to vasculogenesis in the embryo. In addition, development of the embryonic cardiovascular system and yolk sac vasculature are regulated by the same group of angiogenic and survival

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factors via common mechanisms.[3,19,20] Thus, previous studies in hyperglycemia-induced embryonic vasculopathy have specifically focused on the yolk sac vasculature because it has provided a highly reliable experimental system.[4,19,21,22] The yolk sac is an extraembryonic membrane derived from the same progenitor cells that produce the embryo,[23] and it plays an important role in supporting development of embryos.[23,24] Adverse effects of hyperglycemia have been documented in the yolk sac of maternal diabetic animal models and in vitro cultured rodent embryos.[3,19,22,24] Under hyperglycemic conditions, vasculogenesis of the blood vessels in the yolk sac is disrupted, and the cellular structures in the vessels are altered.[4,19,21,22]

We have morphologically characterized the various adverse effects of hyperglycemia on yolk sac vasculature development by arbitrarily assigning morphological scores to individual vasculatures.[4] We have successfully used this established morphological score system in our studies to quantify the adverse effect of hyperglycemia on yolk sac vasculature development.[4,9]

In the present study, we used this yolk sac morphological system to test the hypothesis that hyperglycemia reduces HIF-1α expression, and blockade of HIF-1α reduction ameliorates diabetic embryonic vasculopathy. By using in vivo and in vitro models with a constitutively active form of HIF-1α, AdCA5, we have demonstrated that hyperglycemia reduces HIF-1α protein expression, but not messenger RNA (mRNA) expression, and reversal of HIF-1α reduction by AdCA5 reduces diabetic embryonic vasculopathy.

MATERIALS AND METHODS

Animals and reagents

C57BL/6J mice (median body weight 22 g) were purchased from Jackson Laboratory (Bar Harbor, ME). Streptozotocin from Sigma (St Louis, MO) was dissolved in sterile 0.1 mol/L citrate buffer (pH 4.5). Sustained-release insulin pellets were purchased from LinShen Canada Inc. (Toronto, Canada). Adenoviruses expressing the LacZ (AdLacZ) and the constitutive active form of HIF-1α (AdCA5) were provided by Dr Gregg L. Semenza at the Johns Hopkins University School of Medicine, Baltimore, MD. Human recombinant thioredoxin (Trx) was purchased from EMD Chemicals (San Diego, CA).

Mouse models of diabetic embryopathy

The procedures for animal use were approved by the Institutional Animal Care and Use Committee of University of Maryland School of Medicine. Eight-week-old C57BL/6J mice were intravenously injected daily with 75 mg/kg streptozotocin over 3 days to induce diabetes. Once a level of hyperglycemia indicative of diabetes ($\geq$250 mg/dL) was achieved, insulin pellets were subcutaneously implanted in these diabetic mice to restore euglycemia prior to mating. The mice were then mated with male mice of the same respective genotype. On day 5 of pregnancy (E5), insulin pellets were removed to permit frank hyperglycemia (>250 mg/dL glucose level), so the developing conceptuses would be exposed to a hyperglycemic environment during organogenesis (E7–11). Nondiabetic female mice with vehicle injections and sham operation of insulin pellet implants served as nondiabetic controls. On E7 and E8, mice were euthanized, and conceptuses were dissected out of the uteri for analysis.

Whole-conceptus culture

C57BL/6J mice were paired overnight. The next morning was designated E0 if a vaginal plug was present. Mouse conceptuses at E7 were dissected out of the uteri in phosphate-buffered saline (Invitrogen, La Jolla, CA). The parietal yolk sac was removed using a pair of fine forceps and the visceral yolk sac was left intact. Conceptuses (4/bottle) were cultured in 4 mL rat serum at 38°C in 30 rpm rotation in the roller bottle system. The culture bottles were gassed with 5% oxygen/5% carbon dioxide/90% nitrogen. Conceptuses were cultured under euglycemic (150 mg/dL glucose, a value close to the blood glucose level of nondiabetic mice) and hyperglycemic (300 mg/dL glucose) conditions in the presence or absence of 0.5 μL or 1 μL (1×10^7 infectious units/mL) adenoviral AdCA5 per 1 mL culture medium, or in the presence or absence of 2.0 μg/mL human recombinant Trx.

Morphologic assessment of the yolk sac vasculature

Conceptuses were examined under a stereomicroscope (MZ16F; Leica Microsystems Inc, Bannockburn, IL) to assess yolk sac vasculature defects. Images of conceptuses were captured by a DFC420 5 MPix digital camera with software (Leica, Wetzlar, Germany) and processed with Adobe Photoshop CS2 (Adobe Systems Incorporated, San Jose, CA).

Yolk sac vasculatures were morphologically scored based on visible maldevelopment as previously described.[4,9] Briefly, a morphological score of 4 indicated a full development of the E11-like yolk sac vasculature with an arborizing interconnecting vascular network composed of arteries, veins, and capillaries exhibiting blood flow. A score of 3 represented only a minor defect of the yolk sac vasculature with fewer blood vessels than that of the yolk sac vasculature with a score of 4. A score of 2 indicated an arrest of the yolk sac vasculature development at the primary capillary plexus stage resulting in few yolk sac vessels. A score of 1 indicated a major defect of the yolk sac vasculature displaying an ectastic vascular plexus with no signs of arborization and large, nonfused blood islands toward the ectoplacental cone. A score of 0 represented a yolk sac completely devoid of blood vessels with no visible or scattered blood islands.

Embryonic malformations were not examined because at early embryonic stages (E7-E9), structural malformations were not manifested. Our previously studies have extensively described the malformations in embryos of diabetic mice or cultured embryos exposed to hyperglycemia.[9,25] Especially, at E11, about 25% of embryos from diabetic mice exhibited neural tube defects.[25] Our ongoing studies are testing the hypothesis that the early molecular changes involving embryonic vasculopathy play