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Short Communication

Hyperglycemia-Induced Vasculopathy in the Murine Conceptus Is Mediated via Reductions of VEGF-A Expression and VEGF Receptor Activation

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Major congenital malformations, including those affecting the cardiovascular system, remain the leading cause of mortality and morbidity in infants of diabetic mothers. Interestingly, targeted mutations of several genes (including VEGF and VEGF receptors) and many teratogenic agents (including excess D-glucose) that give rise to embryonic lethal phenotypes during organogenesis are associated with a failure in the formation and/or maintenance of a functional vitelline circulation. Given the similarities in the pathology of the abnormal vitelline circulation in many of these conditions, we hypothesized that the hyperglycemic insult present in diabetes could cause the resultant abnormalities in the vitelline circulation by affecting VEGF/VEGF receptor signaling pathway(s). In this study we report that hyperglycemic insult results in reduced levels of VEGF-A in the conceptus, which in turn, leads to abnormal VEGF receptor signaling, ultimately resulting in embryonic (vitelline) vasculopathy. These findings and our observation that addition of exogenous rVEGF-A_{165} within a defined concentration range blunts the hyperglycemia-induced vasculopathy in the conceptus support the concept that VEGF levels can be modulated by glucose levels. In addition, these findings may ultimately lead to novel therapeutic approaches for the treatment of selected congenital cardiovascular abnormalities associated with diabetes. ***(Am J Pathol 2001, 158:1199–1206)***

Offspring of diabetic mothers (both humans and experimental animals) experience a two- to fourfold increase in congenital anomalies.[1,2] Although no particular organ system or tissue seems to be specifically targeted, a variety of cardiovascular anomalies are frequently observed.[3] In addition to the increased incidence of congenital anomalies noted in the live births and stillbirths of diabetic mothers, these mothers experience difficulties in becoming pregnant and exhibit an increased incidence of fetal resorption.[1,4–8] After implantation and before placentation, embryonic growth is dependent on proper development of the yolk sac vasculature and the vitelline circulation. Arrested development or maldevelopment of this vasculature would lead to fetal demise early resulting in the termination of pregnancy; whereas arrest and/or maldevelopment of vasculature(s) at later times, associated with specific organ or tissue development, would contribute to congenital abnormalities in a wide variety of organs and tissues.

It was previously shown that *in vitro* culture of rat and mouse conceptuses at the primitive streak stage is possible and that conceptuses develop nearly normally for 48 hours during the initial stages of organogenesis. During this period they are nourished via the vitelline circulation, after which further growth and development would require a placental circulation.[9,10] The addition of 20 mmol/L D-glucose (a plasma concentration of glucose often observed in diabetic mothers of humans and of experimental animals) to these cultures results in significant yolk sac and embryonic vasculopathy and abnormal embryonic development.[10] In light of these findings and the data illustrating the crucial roles of VEGF and VEGF receptors (VEGFRs) during early vasculogenesis and angiogenesis,[11–18] we hypothesized that the vasculopathy observed in our cultured conceptuses and in embryos of streptozotocin-induced diabetic pregnant mice[10] was due to abnormalities in the VEGF signaling pathways.

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Table 1. Effects of VEGF Supplementation on Yolk Sac Vascular Arborization and on the Development of a Functional Vitelline Circulation

Condition/dose	Normal		Abnormal	
	Arborization	Functional circulation	Arborization	Functional circulation
Normoglycemic				
−VEGF	120/126 (96%)	120/126 (96%)	120/126 (4%)	120/126 (4%)
+$VEGF_{165}$:				
0.2 pg/ml	9/10 (90%)	9/10 (90%)	1/10 (10%)	1/10 (10%)
2.0 pg/ml	49/54 (91%)	49/54 (91%)	5/54 (9%)	5/54 (9%)
20.0 pg/ml	0/20 (0%)	0/20 (0%)	20/20 (100%)	20/20 (100%)
200.0 pg/ml	0/10 (0%)	0/10 (0%)	10/10 (100%)	10/10 (100%)
Hyperglycemic				
−VEGF	0/20 (0%)	0/20 (0%)	20/20 (100%)	20/20 (100%)
+$VEGF_{165}$:				
0.2 pg/ml	2/10 (20%)	2/10 (20%)	8/10 (80%)	8/10 (80%)
1.0 pg/ml	8/22 (36%)	8/22 (36%)	14/22 (64%)	14/22 (64%)
2.0 pg/ml*	72/80 (90%)	72/80 (90%)	8/80 (10%)	8/80 (10%)
10.0 pg/ml	48/62 (77%)	48/62 (77%)	12/62 (23%)	12/62 (23%)
+$VEGF_{165}$ @ 3 hours				
2.0 pg/ml	0/34 (0%)	0/34 (0%)	34/34 (100%)	34/34 (100%)
10.0 pg/ml				
+$VEGF_{120}$:				
0.2 pg/ml	8/15 (53%)	0/15 (0%)	7/15 (47%)	15/15 (100%)
10.0 pg/ml	12/20 (60%)	5/20 (25%)	8/20 (40%)	15/20 (75%)
+PGF:				
0.2 pg/ml	0/19 (0%)	0/19 (0%)	19/19 (100%)	19/19 (100%)
10.0 pg/ml	0/19 (0%)	0/19 (0%)	19/19 (100%)	19/19 (100%)

*Optimal VEGF-A_{165} concentration.

In this report we demonstrate that hyperglycemic insult results in reduced levels of VEGF-A, which in turn, leads to abnormal VEGFR signaling, resulting in embryonic vasculopathy. These findings and our observation that addition of exogenous VEGF-A blunts the hyperglycemia-induced vasculopathy may ultimately lead to novel therapeutic approaches for the prevention and treatment of congenital abnormalities associated with diabetes.

Materials and Methods

Murine Conceptuses

Murine conceptuses 7.5, 8.5, and 9.5 days post-coitus (dpc) were harvested from pregnant CD1 mice (Charles River, Wilmington, MA) as described[19,20] and used for morphological and biochemical studies directly or after defined periods of culture.[10,20,21] VEGF-A-LacZ knock-in mice were generated as described[22] by inserting an internal ribosome entry site (IRES)-LacZ cassette into the 3′UTR (exon 8) of VEGF-A and using a sequence encoding the β-galactose reporter (LacZ cassette) inserted into the noncoding region of exon 8 (3′ end) of the VEGF-A stop codon. An IRES preceded the LacZ coding sequence. This strategy permitted the production of two functional proteins, VEGF-A and LacZ, from a single bicistronic transcript.

In vitro murine conceptuses were cultured as described, in the presence of pooled, heat-inactivated, undiluted rat serum, and oxygenated using a series of gas mixtures with increasing oxygen concentrations as previously described.[9,10] Hyperglycemic culture conditions were achieved by addition of α-D-glucose (Sigma Chemical Co., St. Louis, MO) to a final concentration of 20 mmol/L as described.[10] VEGF/placental growth factor (PlGF) supplementation of control and hyperglycemic cultures was accomplished by addition of 0.2 to 20 pg/ml rVEGF-A_{165}, rVEGF-A_{120}, or rPlGF (Chemicon International, Inc., Temecula, CA; R&D Systems, Inc., Minneapolis, MN). Addition of growth factors or vehicle alone was made either at the start of culture or 3 hours after initiation of culture.

Conceptuses were assessed for the presence or absence of major structural and functional defects including heartbeat, yolk sac circulation, vitelline vessel branching, neural tube closure, and completion of axial rotation. Growth and development were scored by their organ primordia development as described.[23] Heartbeat and blood flow through the vitelline circulation was assessed visually by an Olympus dissecting microscope (Olympus Optical Co., Ltd., Japan) in each group of conceptuses at the termination of the culture period, as recorded and summarized in Table 1.

β-Galactosidase Staining of Conceptuses

Conceptuses harvested from VEGF-A-LacZ knock-in mice were fixed in 0.2% glutaraldehyde, 2% paraformaldehyde, 2 mmol/L EGTA, and 2 mmol/L $MgCl_2$ in Pipes buffer, pH 6.9. Staining was performed at 37°C in 0.02% X-Gal, 5 mmol/L $K_3Fe(CN)_6$, 2 mmol/L $MgCl_2$ in phosphate-buffered saline overnight. Stained conceptuses were washed, postfixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm. Sections were mounted on glass slides and counterstained with Nuclear Fast Red as described.[22]

Whole Mount Immunostaining of Conceptuses

Whole mount staining of conceptuses with anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) was performed as described.[10,20] Approximately 20 to 25 randomly selected *in vivo* grown and cultured conceptuses were evaluated for each experimental group.

Transmission Electron Microscopy (TEM) of Conceptuses

Light level semithin and TEM level thin sections of 8.5 and 9.5 dpc conceptuses were examined using a Zeiss Axiophot light microscope (Carl Zeiss, Oberkochen, Germany) and a Zeiss EM910 electron microscope (Carl Zeiss, Oberkochen, Germany) , respectively, as described elsewhere.[10]

Immunoprecipitation and Western Blotting

Preparation of yolk sac and embryo lysates and subsequent immunoprecipitation with anti-VEGFR-2/Flt-1 and Western blotting with anti-VEGF, anti-VEGFR-2/Flt-1 and anti-PY (PY 99) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were performed as described.[10,20,21,24] All blots were scanned into a Macintosh G3 computer (Apple Computer, Brea, CA) using an Arcus II scanner (Agfa-Gevaert, N.V.) and Photoshop 5.0 software (Adobe Systems, Inc. San Jose, CA). All experiments were repeated at least three times using independently prepared lysates.

Results

Hyperglycemia-Induced Embryonic Vasculopathy Is Associated with Decreased VEGF-A Levels

Although it is recognized that hyperglycemia elicits embryonic vasculopathy, the mechanism(s) responsible for this embryopathy remain unclear.[3,5,8,10,25,26] We have reported earlier that hyperglycemic insult results in arrest of vascular development at the primary capillary plexus stage in the yolk sacs of the conceptuses of streptozotocin-induced diabetic mothers and in cultured murine conceptuses.[10] From this observation we hypothesized that perturbations in VEGF-A expression, VEGFR expression, and/or VEGFR signaling may be crucial to the observed vasculopathy.

To assess VEGF-A levels in the murine conceptus, two approaches were taken. In an earlier project a β-galactosidase (LacZ) reporter gene with a preceding IRES was introduced by gene targeting into the 3′ UTR region of VEGF-A. This modification allows the production of both VEGF-A and LacZ from the same bicistronic mRNA created. Therefore the LacZ and VEGF-A expression are transcriptionally coupled.[22] In the first approach, these VEGF-A-LacZ knock-in heterozygous male mice were mated with CD1 mothers. Conceptuses recovered at 7.5 dpc were placed in normoglycemic and hyperglycemic culture as described.[10] Conceptuses were harvested after 24 (Figure 1, a and b) and 48 hours (Figure 1, d and e) in culture. Yolk sacs were isolated and 5-μm sections prepared and stained for activity. As illustrated in Figure 1, a, b, d, and e, we observed a reduced LacZ activity in the hyperglycemia-exposed yolk sacs compared with the normoglycemic yolk sacs, consistent with reduced levels of VEGF-A mRNA after hyperglycemic insult. Specifically, by 8.5 dpc the primitive capillary plexus formed from fused blood islands in the yolk sac. Abundant LacZ expression was observed in the visceral endodermal yolk sac cells, in extraembryonic mesoderm-derived cells and mesothelial cells forming the inner yolk sac layer. Hematopoietic and endothelial cells were unstained (Figure 1a). At 9.5 dpc an arborizing vascular network had developed and LacZ/VEGF-A was extensively expressed in the yolk sac endodermal cells and mesenchymal/mesothelial cells (Figure 1d). In hyperglycemia-exposed conceptuses, reductions in LacZ/VEGF-A expression

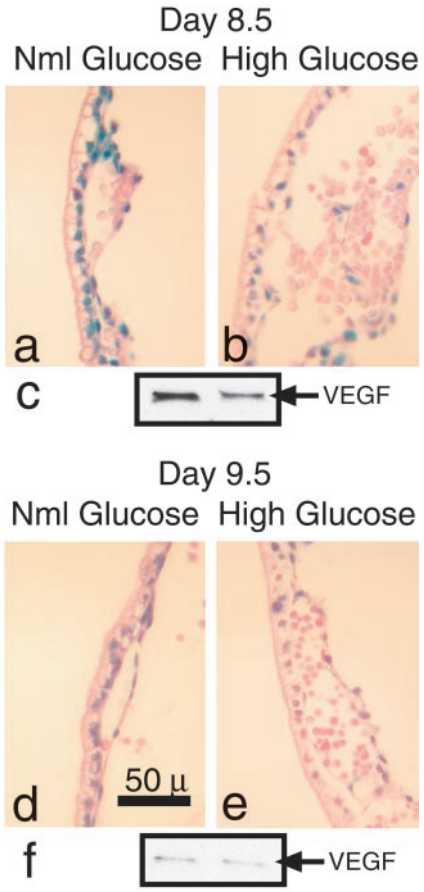

Figure 1. Hyperglycemia causes reduction of VEGF-A mRNA and protein in the murine conceptus. Histochemical analysis of 5-μm sections of yolk sacs from 8.5 (**a** and **b**) and 9.5 (**d** and **e**) dpc. VEGF-A/LacZ knock-in heterozygous conceptuses. The blue color present in the endodermal, mesodermal and mesothelial cells represents VEGF-A gene induction, whereas the endothelial cells and primitive blood cells are negative. Note that there is decreased intensity of staining in the hyperglycemia-exposed conceptuses (**b** and **e**). **c** and **f:** Representative Western blots of lysates of 8.5 (**c**) and 9.5 (**f**) dpc yolk sacs derived from normoglycemic (**left lanes**) and hyperglycemia-exposed (**right lanes**) conceptuses immunoblotted with anti-VEGF. Note the decreased band intensities in the lysates derived from the hyperglycemia-exposed conceptuses.

were noted (Figure 1, b and e), being more pronounced in the visceral endodermal cells compared to mesenchymal/mesothelial cells in the inner layer of the yolk sacs.

In the second approach, we performed Western blots on yolk sac lysates at 8.5 and 9.5 dpc. VEGF-A expression was noted throughout all stages of yolk sac vascularization, being barely detectable at 7.5 dpc (data not shown); increased at 8.5 dpc, when the primary capillary plexus has formed but arborization is not yet manifested; and decreased at 9.5 dpc, when there has been completion of branching morphogenesis and establishment of the vitelline circulation. Figure 1, c and f, illustrates the reduction in VEGF-A protein in the 8.5 and 9.5 dpc hyperglycemic cultures compared to the normoglycemic cultures.

Exogenous VEGF-A Prevents Hyperglycemia-Induced Embryonic Vasculopathy

In light of our findings of reduced levels of VEGF-A in the hyperglycemic cultures, we hypothesized that perhaps treatment of these cultured embryos with exogenous VEGF-A would abrogate or blunt the effects of the hyperglycemic insult.

Because VEGF-A levels are known to be tightly regulated during vasculogenesis,[11,12,27–30] we determined the effects of exogenous VEGF-A_{165} on yolk sac vascularization. Concentrations of 0.2 to 2.0 pg/ml of VEGF-A_{165} had no detectable adverse effects on vascular morphology. However, concentrations higher than 20.0 pg/ml elicited abnormalities in yolk sac vascular development, manifested as edematous conceptuses (data not shown). This abnormal yolk sac vasculature was characterized by the presence of an enlarged, tortuous, ectatic vasculature. Our findings are consistent with known VEGF concentration-dependent effects on vascular development and function.[11,12,31,32]

When 2 or 10 pg/ml of VEGF-A_{165} were added to hyperglycemia-exposed cultures at the start of the culture period a marked improvement in the branching morphology of yolk sac vessels was noted (see Table 1). Figure 2 illustrates the effects of exogenous VEGF-A_{165} on conceptuses exposed to hyperglycemic culture conditions at 8.5 and 9.5 dpc. At 8.5 dpc the effects of hyperglycemia and VEGF-A_{165} treatment are not apparent on low power examination of intact conceptuses (Figure 2, a–c). However, at 9.5 dpc the effects of hyperglycemic insult are readily observable (Figure 2, d, e, g, and h). An arborized yolk sac vasculature, an actively beating heart and blood flow in the vitelline vessel were noted in the normoglycemic conceptuses (Figure 2, d and g). In contrast, the yolk sacs of the hyperglycemia-exposed conceptuses displayed an ectatic vascular plexus with no signs of arborization (Figure 2, e and h) and large, non-fused blood islands toward the ectoplacental cone (Figure 2h). The embryos were malformed, with slowly beating hearts and no appreciable blood flow. However, when exogenous VEGF-A_{165} was added to the cultures (0.2 to 10 pg/ml), the hyperglycemia-exposed conceptuses displayed arborizing yolk sac vascular networks

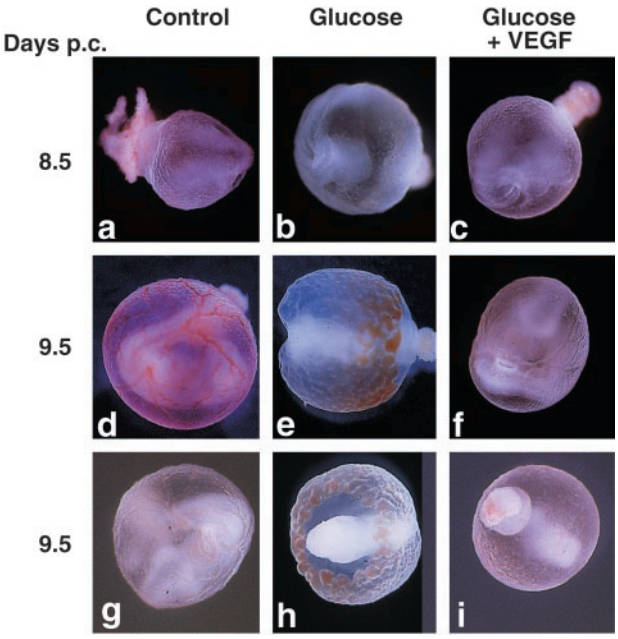

Figure 2. Hyperglycemia-induced vasculopathy is blunted by treatment with exogenous VEGF-A_{165}. Low power representative micrographs of whole conceptuses at 8.5 (**a–c**) and 9.5 dpc(**d–i**) under normoglycemic conditions (Control, **a**, **d**, and **g**), hyperglycemic conditions (Glucose, **b**, **e**, and **h**), and hyperglycemic conditions + exogenous VEGF-A_{165} (Glucose + VEGF-A, **c**, **f**, and **i**). Note the vasculopathy consisting of non-arborizing, ectatic vessels in the hyperglycemic conditions (**e** and **h**) and the rescue of a normal arborizing phenotype in the hyperglycemia-exposed conceptuses treated with exogenous VEGF-A_{165} (**f** and **i**).

(Figure 2, f and i). A striking difference between this group and the hyperglycemia-exposed group was the presence of a faster beating heart and a vigorous blood flow through the yolk sac vasculature.

It is important to note that this significant improvement in yolk sac vascularization and circulation was achieved when exogenous VEGF-A and D-glucose were added to the medium at the start of the culture period and were present for the duration of the culture. To ascertain whether VEGF-A treatment could be preventive, we exposed 7.5 dpc conceptuses to 20 mmol/L D-glucose for an initial 3-hour period (known to elicit yolk sac vasculopathy[10]) and then transferred the conceptuses into VEGF-A_{165}-supplemented normoglycemic media for the remaining 45 hours of culture. We observed that VEGF-A_{165} added at this time failed to correct the vasculopathy, indicating that VEGF-A is preventive rather than corrective and its effects are time-dependent.

PECAM-1 staining of whole mounts of yolk sacs harvested from 9.5 dpc normoglycemic, hyperglycemic, and hyperglycemic VEGF-A-treated conceptuses confirmed our results. Specifically, we noted an arrest of yolk sac vascular arborization after hyperglycemic insult (compare Figure 3a to Figure 3b) and a rescue of yolk sac vascular arborization in hyperglycemia-exposed conceptuses treated with 2 to 10 pg/ml VEGF-A_{165} (Figure 3c). Further, high power and TEM examination of the yolk sac vasculature revealed that addition of exogenous VEGF-A_{165} to hyperglycemic conceptus cultures prevented the morphological abnormalities of the yolk sac vasculature (Figure 3, d–m). Specifically, the loss of intimate endo-

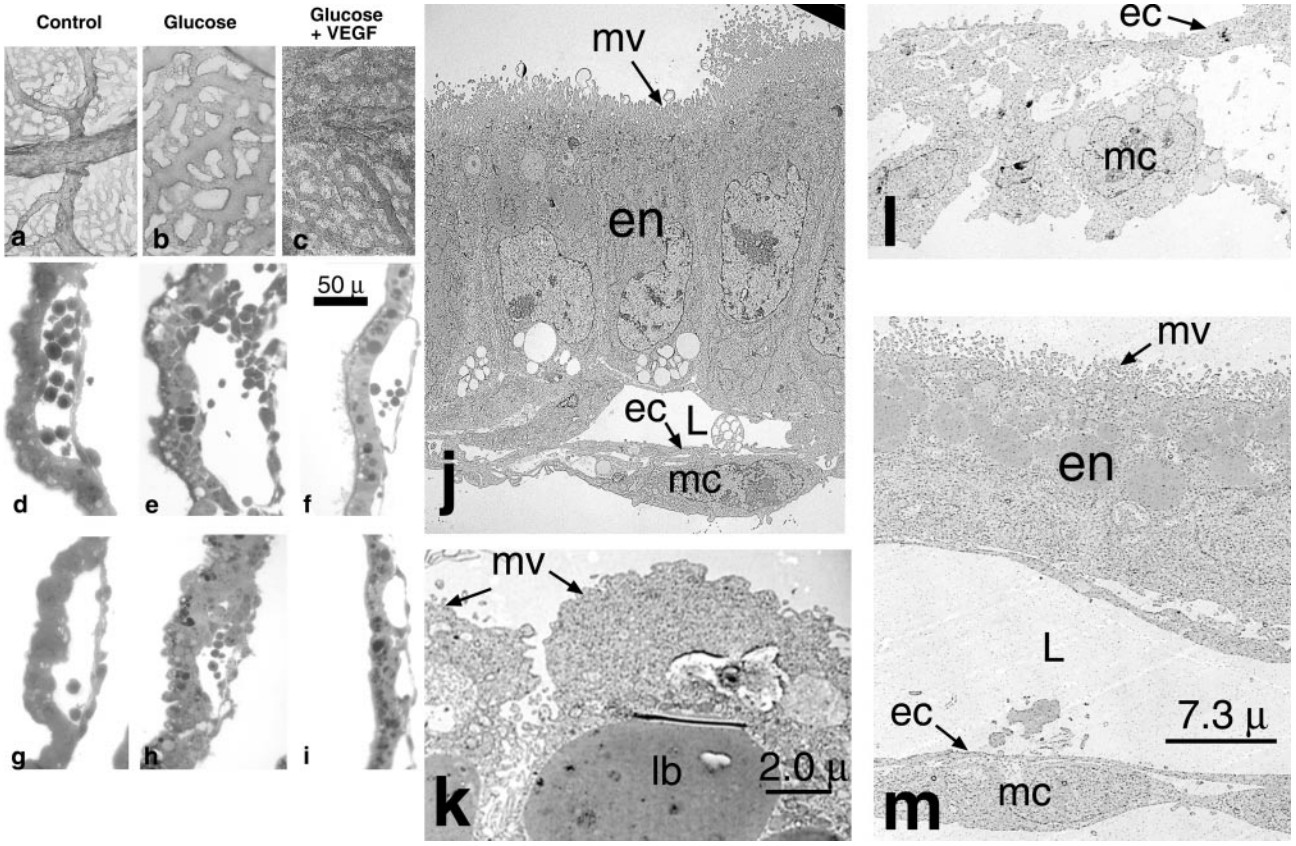

Figure 3. Hyperglycemia-induced disorganization of endothelial cells and adjacent mural cells is rescued by treatment with exogenous VEGF-A_{165}. Low power representative micrograph of PECAM-1 stained 9.5 dpc yolk sacs harvested from normoglycemic (**a**), hyperglycemic (**b**), and hyperglycemic + exogenous VEGF-A_{165} cultures (**c**). Note the lack of arborization and ectatic vessels in the hyperglycemia-exposed conceptuses (**b**) and the rescue of an arborizing phenotype in the in the hyperglycemia-exposed conceptuses treated with exogenous VEGF-A_{165} (**c**). **d–i:** High power micrographs illustrating the perturbation of the intimate endothelial cell-mural cell interactions following hyperglycemic insult at 8.5 (compare normoglycemic, **d**, with **e**) and 9.5 dpc (compare normoglycemic, **g**, with **h**). **f** and **i:** Illustrations of the rescue of intimate endothelial cell-mural cell interactions after addition of exogenous rVEGF-A_{165} to the cultures (compare **d** with **f** and **g** with **i**). Scale bar, 50 μm (**d–i**). **j–m:** Representative transmission electron micrographs of control (**j**), hyperglycemic (**k** and **l**), and hyperglycemic 9.5 dpc conceptuses treated with VEGF-A_{165} (**m**). **j:** Illustratation of a yolk sac comprised of polarized endodermal cells (en) with apical microvilli (mv) and a microvascular lumen (L), lined by flattened endothelial cells (ec) intimately invested by mesothelial cells (mc). In contrast, **k** illustrates a hyperglycemic yolk sac comprised of endodermal cells with blunted microvilli and large lysosomal bodies (lb). **l:** Illustratation of the yolk sac vasculature, which is comprised of plump endothelial cells (ec) and mesothelial cells (mc) that have lost their intimate associations with the endothelium. **m:** A hyperglycemic yolk sac treated with exogenous rVEGF-A_{165}. Its endodermal cells (en) are polarized with apical microvilli (mv). Its vasculature is again noted to be comprised of flattened endothelial cells (ec) intimately invested by mesothelial cells (mc). Scale bars, 7.3 μm (**j**, **l**, and **m**) and 2.0 μm (**k**).

thelial cell-mural cell (pericyte) interactions observed after hyperglycemic insult was prevented with VEGF-A supplementation (compare Figure 3, e, h, and l with Figure 3, f, i, and m). Interestingly, the endodermal cells of the yolk sac also exhibit a morphological appearance similar to control normoglycemic conceptuses when treated with exogenous VEGF-A (compare Figure 3j with Figure 3, k and m), raising the possibility that VEGF-A may exert effects on nonvascular cells during embryonic development.[33,34]

Hyperglycemia-Induced Vasculopathy Correlates with a Reduction in VEGFR-2/Flk-1 Activation and Complex Formation

In light of our findings of reduced VEGF-A expression after hyperglycemic insult and partial rescue of yolk sac vascular development by addition of exogenous VEGF-A, we reasoned that decreased VEGF-A_{165} levels could alter VEGFR-2 activation and subsequent interaction with a variety of signaling and adapter moieties which associate with the VEGFR-2 after VEGF engagement.[35–37]

We conducted experiments to investigate the patterns of VEGFR-2/Flk-1 expression under normoglycemic and hyperglycemic culture conditions. Western blot analysis of VEGFR-2 levels revealed that although expression levels changed over time (7.5 to 9.5 dpc) in culture, hyperglycemia did not appear to alter expression levels (Figure 4A). Immunoprecipitation of VEGFR-2 followed by Western blotting using anti-VEGFR-2/Flk-1 antibodies confirmed this (Figure 4B).

Investigation of the signaling complex formed following engagement of VEGFR-2/Flk-1 was performed by immunoprecipitation of VEGFR-2 followed by Western blot analysis using an anti-phosphotyrosine antibody (Figure 4C). This pull-down study revealed differential tyrosine phosphorylation of VEGFR-2/Flk-1 and several phosphoproteins associated with VEGFR-2/Flk-1[37] at 7.5, 8.5, and 9.5 dpc (see lanes 7.5 NG, 8.5 NG, and 9.5 NG). Hyperglycemic insult was noted to diminish dramatically the

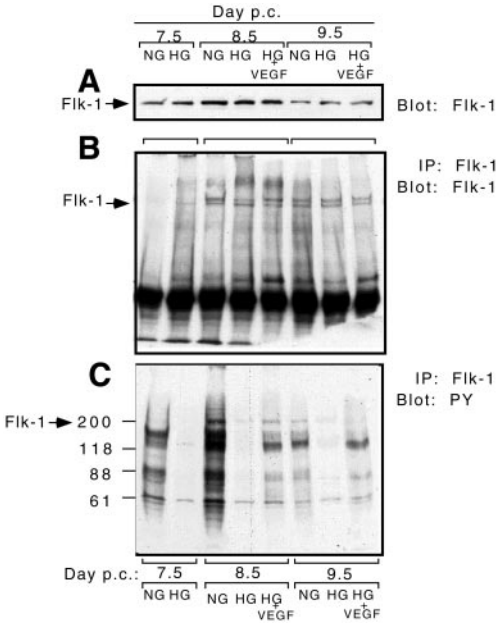

Figure 4. VEGFR-2/Flk-1 signaling is blunted in hyperglycemic conditions, but partially restored by treatment with exogenous VEGF-A_{165}. **A:** Representative Western blot of 7.5, 8.5, and 9.5 dpc yolk sac lysates derived from conceptuses cultured in normoglycemic (NG), hyperglycemic (HG), and hyperglycemic conditions plus exogenous VEGF-A_{165} (HG + VEGF) probed with antibodies directed against VEGFR-2. **B:** Representative immunoprecipitation of VEGFR-2 followed by a Western blot with anti-VEGFR-2/Flk-1 antibody of 7.5, 8.5, and 9.5 dpc yolk sac lysates derived from conceptuses cultured in normoglycemic (NG), hyperglycemic (HG), and hyperglycemic conditions plus exogenous VEGF-A_{165} (HG + VEGF). Note that although there appears to be differing expression levels of VEGFR-2 over the time period studied, no changes were observed after hyperglycemic insult or VEGF-A_{165} addition. **C:** Representative immunoprecipitation of VEGFR-2 followed by a Western blot with anti-phosphotyrosine (PY) antibodies of 7.5, 8.5, and 9.5 dpc yolk sac lysates derived from conceptuses cultured in normoglycemic (NG), hyperglycemic (HG) and hyperglycemic conditions plus exogenous VEGF-A_{165} (HG + VEGF). Note that hyperglycemic insult significantly reduces VEGFR-2/Flk-1 tyrosine phosphorylation and the complement of phosphoproteins coprecipitating with VEGFR-2 at all three time points and that addition of exogenous VEGF-A_{165} to the hyperglycemic cultures partially restores VEGFR-2/Flk-1 tyrosine phosphorylation and the complement of coprecipitating phosphoproteins.

tyrosine phosphorylation of VEGFR-2/Flk-1 itself and the association of these phosphoproteins with VEGFR-2 (see lanes 7.5 HG, 8.5 HG, and 9.5 HG and compare them to the corresponding NG lanes).

Exogenous VEGF-A Treatment Partially Prevents the Effects of Hyperglycemia on VEGFR-2 Tyrosine Phosphorylation and Complex Formation

After supplementation of hyperglycemic conceptus cultures with exogenous VEGF-A_{165}, partial restoration of the tyrosine phosphorylation of the VEGFR-2 and the associated phosphoproteins was noted at 8.5 and 9.5 dpc (see lanes 8.5 HG + VEGF-A and 9.5 HG + VEGF-A and compare them with lanes 8.5 NG and HG and 9.5 NG and HG).

Rescue of the Embryonic Vasculature by VEGF Is Specific for VEGF-A_{165} and Time-Dependent

In light of our findings of robust prevention of hyperglycemia-induced yolk sac vasculopathy with VEGF-A_{165} supplementation (86% restitution in arborization and in functional circulation) compared with a complete (100%) arrest of arborization and lack of a functional vitelline circulation caused by hyperglycemic insult,[10] we examined the protective capabilities of other VEGF-A isoforms and family members. The freely diffusible murine form of VEGF-A_{120} used in the identical concentration range elicited moderate, partial improvement of yolk sac vessel branching (60% restitution in arborization), but only a modest improvement in functional circulation (25%) in the vitelline vasculature. Parallel studies using PlGF at the identical concentration range resulted in no detectable rescue in arborization or functional circulation of the embryonic vasculature. In addition, when VEGF-A_{165} was added to the cultures 3 hours after initiation of hyperglycemia, it failed to improve either arborization (0%) or functional circulation (0%; Table 1).

Discussion

Congenital defects known to be associated with maternal diabetes mellitus are numerous and varied. Several theories have been postulated regarding potential mechanisms by which maternal diabetes might induce dysmorphogenesis; however, the pathogenesis remains unknown.[1–10,38]

Similarities of the vasculopathies observed after hyperglycemic insult at the primitive streak stage of development[10] and that noted in mice containing targeted disruption of either VEGF or its receptors[11–18,27–29] suggested the possibility that the arrest of yolk sac vascular development at the primary capillary plexus stage noted after hyperglycemic insult could be due to modulation of VEGF/VEGFR signaling pathways. Indeed, our findings of reduced VEGF-A expression in 8.5 and 9.5 dpc conceptuses cultured in 20 mmol/L D-glucose support this hypothesis. Furthermore, prevention of yolk sac vasculopathy by supplementation of the hyperglycemic cultures with exogenous rVEGF-A_{165} supports this concept, as do our findings that the observed VEGF-mediated prevention was mediated by a specific VEGF isoform, VEGF-A_{165}.

That the observed hyperglycemia-induced vasculopathy was related to altered VEGFR engagement and subsequent complex formation was supported by our findings of attenuations of VEGFR-2 (Flk-1) tyrosine phosphorylation and coprecipitation with several phosphoproteins known to be associated with VEGFR-2[37] after engagement. That reduced VEGF-A levels were responsible for this blunting of VEGFR tyrosine phosphorylation and signaling complex formation was confirmed by restitution of VEGFR-2 tyrosine phosphorylation levels and increased coprecipitation of tyrosine-phosphorylated signaling complex components in cultures supplemented with exogenous rVEGF-A_{165}. Our examination of VEGFR-1 (Flt-1) revealed low constitutive expression that made analysis of its tyrosine phosphoryla-

tion profiles unreliable (data not shown). Further, exogenous addition of its high affinity ligand, PlGF-1, did not improve the phenotype of the hyperglycemic cultured conceptuses and was noted to elicit yolk sac vasculopathy in normoglycemic cultured conceptuses (data not shown). Additionally, previous studies have shown that although VEGFR-1 (Flt-1) deficiency results in embryonic death, reconstitution with a truncated VEGFR-1 (Flt-1) devoid of its cytoplasmic domain rescues the phenotype.[39] These data, along with our current findings, are consistent with the notion that VEGFR-1 (Flt-1) may function as a cell surface reservoir or presenter of VEGF family ligands, rather than as a direct signaling moiety in certain circumstances. The roles played by the other VEGF-A receptors, VEGFR-3[15] and neuropilin-1,[16] in this model are unknown and beyond the scope of this report. Future studies focusing on these receptors may shed more light on the mechanisms involved in hyperglycemia-induced yolk sac and embryonic vasculopathy.

The exact mechanisms by which altered VEGF/VEGFR signaling cause yolk sac vasculopathy are unknown. Other investigators have demonstrated changes in PECAM-1 phosphorylation state after VEGF treatment of endothelial cells.[40] In prior studies using this conceptus culture model and conceptuses harvested from diabetic mice we have noted changes in PECAM-1 tyrosine phosphorylation, leading to persistent PECAM-1/Src homology 2 domain-containing protein tyrosine phosphatase (SHP-2) association; increased expression and activation of protein kinase C (PKCϵ), and increased serine/threonine phosphorylation leading to decreased PECAM-1/γ-catenin association.[10,21] Because these changes in PECAM-1 phosphorylation state have been associated with modulation of cell proliferation, cell migration, gene expression, and cytoskeletal organization,[21,24,41–43] it is possible that these alterations in PECAM-1 phosphorylation state are, in part, responsible for the resulting vasculopathy. Figure 5 is our working model, illustrating the effects of hyperglycemia-induced reduced VEGF-A expression on VEGFR tyrosine phosphorylation, adapter and signaling molecule association, and tyrosine phosphorylation and subsequent downstream signaling, involving changes in PECAM-1 phosphorylation state and binding of adapter, signaling, and cytoskeletal molecules. It remains to be determined whether these observed changes in PECAM-1 phosphorylation state and protein associations are causative and/or related to the arrest in vasculogenesis and angiogenesis observed after hyperglycemic insult during vasculogenesis and angiogenesis in the murine conceptus.

In addition to its well-documented effects on vascular endothelial development, maintenance, remodeling, proliferation, differentiation, and permeability, VEGF also has been shown to affect non-endothelial cell behavior. Selected epithelial and mesenchymal cell populations have been documented to respond to VEGF isoforms,[33,34,44] indicating that this growth factor/receptor family is likely to affect the development of a wide range of cells, tissues, and organs during development. In our model we noted striking changes in the endodermal cells of the yolk sac with supplementation of the hyperglycemic cultures

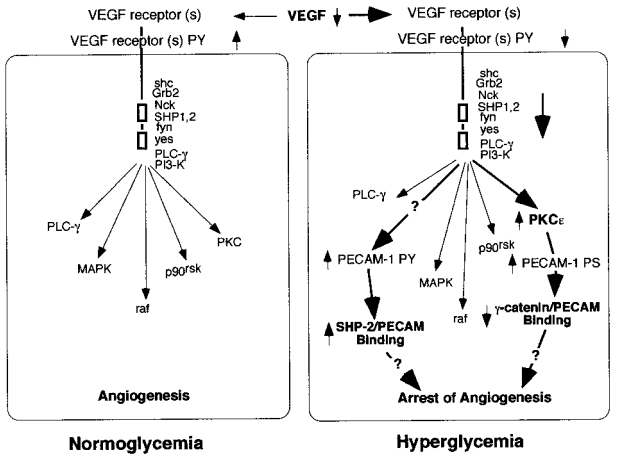

Figure 5. Current working model of the effects of hyperglycemia on vascular development of the murine yolk sac. Reduction of VEGF-A expression by endodermal, mesodermal and endothelial cells in the yolk sac leads to reduced VEGFR-2 activation and subsequent interactions with several adapter and signaling molecules. This, in turn, leads to altered downstream signaling cascades which result in arrest of vascular development. Our prior studies demonstrating increased PECAM-1 tyrosine phosphorylation and SHP-2 binding as well as increased PKCϵ activity, increased PECAM-1 serine/threonine phosphorylation, and decreased γ-catenin binding after hyperglycemic insult[10,21] are consistent with this working model.

with exogenous rVEGF-A_{165}. Specifically, the endodermal cells exhibited reformation of their apical microvilli and re-established their polarity and intimate interactions with the endothelium of the yolk sac vasculature on rVEGF-A_{165} supplementation. In addition, rVEGF-A_{165} supplementation also correlated with the re-establishment of intimate endothelial cell-mural cell interactions.[45,46] It remains to be determined whether these observed changes in non-endothelial cellular behaviors are directly caused by addition of rVEGF-A_{165}, or are the result of changes in the endothelial cells elicited by rVEGF-A_{165} supplementation.

Interestingly, diabetes in adults also results in vasculopathy; however, it has been associated with increased VEGF expression.[25,26] The elevated VEGF expression is thought to be a major mediator of retinopathy, nephropathy, and neuropathy, but also may play a beneficial role in collateralization in diabetic cardiovascular disease. This increased level of VEGF expression is in apparent contradiction with our findings, however; in postnatal diabetes hyperglycemia targets mature organs, tissues, and vasculature, whereas in our experiments developing organs, tissues, and vascular beds are exposed and therefore may respond differently to a similar insult.

The *in vitro* murine whole conceptus culture used in our studies is a useful model to study *in situ* vasculogenesis in the yolk sac. It permits evaluation of the effects of a single factor (eg, excess glucose, VEGF) on conceptuses, which cannot be achieved *in vivo*. Furthermore, the anatomically intact relationships among all three germ layers allow signaling (physiological or pathological) among different cell types necessary to form a vessel, which cannot be observed in other *in vitro* approaches.

Our observations also raise the possibility of future use of targeted delivery of selected VEGF isoforms to prevent specific congenital abnormalities. This approach, how-

ever, awaits the further development of specific delivery and exquisitely controlled expression systems.

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