Angiogenesis and Diabetes: Different Responses to Pro-Angiogenic Factors in the Chorioallantoic Membrane Assay

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Hyperglycemia induces defects in angiogenesis without alteration in the expression of major vascular growth factors in the chicken chorioallantoic membrane (CAM) model. A direct negative effect of hyperglycemia on angiogenesis may participate in failures of "therapeutic angiogenesis" trials. Here, we tested the hypothesis that the response to pro-angiogenic molecules such as angiotensin-converting enzyme (ACE), endothelin-1 (ET-1), and vascular endothelial growth factor-A (VEGF) is altered by hyperglycemia. Transfected (Chinese hamster ovary (CHO) or human embryonic kidney (HEK)) cells overexpressing ACE, ET-1, or VEGF were deposed onto the CAM of hyperglycemic or control embryos. The proangiogenic effect was evaluated 3 d later by angiography and histological analyses. Gene expression in response to these factors was assessed by *in situ* hybridization. Only VEGF overexpression evoked a proangiogenic response in the CAM from hyperglycemic embryos, upregulating the expression of endogenous VEGF, VEGF-R2, and Tie-2, all of them related to activation of endothelial cells. In conclusion, in a model where hyperglycemia does not alter the major vascular growth factor expression, the negative effect of diabetes on capillary density was overcome only by VEGF overexpression, whereas responses to other vasoactive peptides were practically abolished under hyperglycemic conditions.

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INTRODUCTION

The direct effects of hyperglycemia on angiogenesis, the growth of new capillaries from pre-existing ones, have been differently appreciated and differ according to experimental models. In most situations, angiogenesis is impaired in patients with diabetes, and this participates in impaired wound healing, and in an impaired outcome of transplantation of solid organs (1). Furthermore, arteriogenesis, a process of formation or remodeling of arteries, is decreased in patients with diabetes (2). In this context, there has been considerable interest in "therapeutic angiogenesis/arteriogenesis" in patients with peripheral arterial disease or coronary artery disease not amenable to surgical revascularization (3). However, most controlled therapeutic angiogenesis trials have been largely negative and the reasons for these failures have been discussed (4). A direct negative effect of hyperglycemia on neovascularization may explain in part failure of "therapeutic angiogenesis/arteriogenesis" trials. Although patients with overt diabetes often have been excluded from such trials, patients with modest impairment of glucose tolerance or undiagnosed diabetes were not, and they constitute a significant proportion of the patients with coronary artery disease (5).

Various mechanisms have been postulated to explain the impaired angiogenic response in diabetes: first, the presence of vascular dysfunction characterized by both endothelial and vascular smooth muscle impairments (2); second, the exposure to chronic hyperglycemia that leads to the nonenzymatic glycation of proteins and impaired formation of new blood vessels; and lastly, the presence of diabetes is associated with abnormalities in growth factor signaling (2,6) and/or expression (7), disturbing the local balance of vascular growth factors.

The chicken chorioallantoic membrane (CAM) model is used widely to study angiogenesis. In the CAM, quantification of angiogenesis is easy, and angiogenic responses can be altered by the direct administration of pro- or antiangiogenic factors directly onto the surface. Using this model, we have shown that a modest degree of hyperglycemia decreased

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angiogenesis by increasing apoptosis and decreasing proliferation of the cells of vessel walls, although we didn't detect alterations in the expression of vascular growth factors or their receptors in this model (8).

Here, we have explored further this model by investigating the capacity to build an angiogenic response to pro-angiogenic factors in hyperglycemic conditions.

MATERIAL AND METHODS

The investigation conforms to the *Guide for the Care and Use of Laboratory Animal* published by the United States National Institutes of Health (NIH Publication 85–23, revised 1996).

Chick Embryo Culture and Hyperglycemia Induction

Fertilized White Leghorn chicken eggs were incubated at 38° C in a humidified environment. By d 3 post-incubation, a window was opened in the shell to expose the CAM. By d 9 post-incubation, hyperglycemia was induced by a single intravitellus injection of 5 mg glucose/g whole egg (glucose stock solution: 30% [w/v] in water), as described previously (8). Except in the control experiment of grafting wild-type CHO cells, where control embryos were uninjected, water-injected (1 mL) embryos were used as control. By the day of cell-grafting (2 d after the glucose injection), blood glucose level was measured in samples taken from a CAM vessel using a One Touch Profile reflectancemeter (Lifescan, Milpitas, CA, USA). Three d after the grafting, by d 14, blood glucose was measured again, and nodule formation and CAM vascularization were analyzed as described below. Embryos were considered hyperglycemic when both blood glucose assays were > 180 mg/dL (10 mM).

Establishment and Characterization of HEK-VEGF Cells

HEK-293 cells (here called HEK) were maintained in mimimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and glutamine. The day before transfection, 10^6 cells were seeded on 10-cm tissue culture dishes and transfected with murine VEGF (pBLAST49-mVEGF; InvivoGen, California, USA) using Fugene-6 (Roche, Basel, Switzerland) and selected using the blasticidin agent (1mg/mL; InvivoGen). The pBLAST49 plasmid contains an EF-1α composite promoter that consists of the elongation factor 1 α core promoter fused to the five prime untranslated region (5′ UTR) of the human T-lymphotropic virus (HTLV). The level of secreted VEGF was assessed by Enzyme-Linked ImmunoSorbent Assay (ELISA) (R&D Systems, Lille, France).

The effect of VEGFA-transfected (HEK-VEGF) cells on angiogenesis was tested *in vivo* using a chamber model based on a method described by Yonekura *et al*. (9). Balb/c mice were anesthetized with sodium pentobarbital (60 mg/kg) by intraperitoneal (i.p.) route. Dorsal air pouches were induced by the subcutaneous injection of 5 mL of air into each mouse. Both sides of a Millipore ring (ref PR0001401) were covered with Millipore filters of 0.45 μm pore size (ref HAWPO1300). Both filters were stuck with a nail varnish (Gemey, Paris, France). The resulting Millipore chamber was filled with a suspension of HEK or HEK-VEGF cells (10^6 cells) in 0.15 mL of PBS. The HEK- or HEK-VEGF-containing chamber was implanted into the preformed air sac in the back of the mice. Five d after the Millipore chamber implantation, angiogenic response was assessed as follows: mice were anesthetized with pentobarbital, and peripheral vasodilatation was raised by putting mice on a heated plate at 40° C for 10 min. A cast was formed by the intravenous (i.v.) injection of 1 mL 5% carmine red in 10% gelatin into the warmed mice. The carcasses were chilled and the air pouch linings dissected. The tissues were oven dried at 56° C for 48 h and weighed. The dried tissues were digested for 24 h at 56° C in 1.8 mL digestive buffer (dithiothreitol 2 mM, disodium hydrogen orthophosphate 20 mM, EDTA 1 mM, papain 12 U/mL). The dye was dissolved by the addition of 0.2 mL 5 M sodium hydroxide, and the digest were centrifuged at 2000*g* for 10 min and filtered through a 0.2 μm filter. The dye content of 200 μL samples was assayed spectrophotometrically using a 96-well plate reader at 492 nm against a standard curve. The results were then expressed as mg dye content.

Grafting of Cells onto the CAM

Transfected CHO cells producing both ET-1, by stable cotransfection of pre-pro-ET-1, and endothelin-1-converting enzyme (ECE) (CHO-ET-1) have been described previously (10,11). Cells producing chicken angiotensin-converting enzyme (cACE) (CHO-ACE) were established and characterized enzymatically. These cells express cACE at the cell membrane and are able to hydrolyze the substrate Hippuryl-His-Leu into hippuric acid and His-Leu dipeptide as described for CHO expressing human ACE (12). The activity of cACE in these cells is inhibited by ACE-inhibitors including lisinopril, captopril, and fosinoprilate (A Michaud and K Savary, unpublished data).

CHO and HEK cells were grown in F-12 (Ham) Nutrient Mixture (Glutamax; GIBCO, Paisley, Scotland) and DMEM (4,500 mg/L glucose, glutamax, and pyruvate; GIBCO), respectively. Both media were supplemented with 10% FCS and 100 units/mL penicillin-streptomycin (GIBCO).

Grafting of cells was performed as described previously (10). The cells were cultured until confluence, detached from the tissue culture dish (100 × 20 mm) by scraping in presence of culture medium, and poured into centrifuge tubes (one tube for each dish). The cells were pelleted by centrifugation at 1000*g* for 5 min, and the supernatant was discarded. Cells were submitted to a second centrifugation at 1000*g* for 3 min, the supernatant was carefully aspirated and, when necessary, the tubes were inverted and the cells were allowed to dry under laminar flow. The cells were not

resuspended between the centrifugations, and the second centrifugation was added to remove the medium completely. Then, 10 to 15 μL of cells (approximately 3×10^6 cells) were drawn up into a sterile pipette and layered onto the CAM of 11-d-old embryos. Grafted cells were allowed to grow on the CAM for 3 d where they formed nodules, which were analyzed by angiography.

Angiography

Embryos were injected through a CAM vessel with 20 mg/mL 2-MDa fluoresceinated dextran (Sigma, St Louis, MO, USA) in PBS as described previously (8). The nodules and vessels were observed with a MZ FLIII Leica stereomicroscope and digitalized pictures were taken with a Coolsnap digital camera (Roper Scientific, Trenton, NJ, USA). All analyses of the pictures of the treatment group and type of nodules were made blindly. For each type of cells, comparisons were made between normoglycemic (control, CT) and hyperglycemic (glucose, GLU) conditions.

Inhibition of VEGF-Mediated CAM Angiogenesis

The VEGF receptor tyrosine kinase inhibitor, PTK787/ZK222584 (succinate salt; gift from Dr P Traxler, Novartis Pharma, Basel, Switzerland) was applied twice per d (20 μL at 0.1 μg/μL in PBS-0.1% DMSO) onto the CAM in the vicinity of the cells, starting 48 h after grafting. After 2 d of treatment, the nodules were analyzed by angiography as described previously (10).

Immunohistochemistry

After angiography, 4% paraformaldehyde (PFA) was poured directly onto the nodule-surrounding area for 10 min; CAMs were then excised, immersed in PFA for 2 h, dehydrated in a graded series of increasing alcohol concentrations, cleared in xylene, and routinely embedded in paraffin (13). Seven μm thick sections were cut. Sections were then deparaffinized, rehydrated, and submitted to immunohistochemistry analysis.

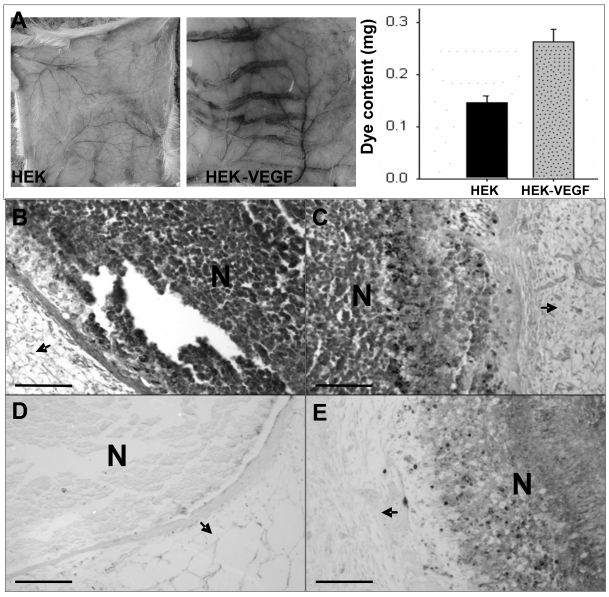

Figure 1. Validation of bioactivity of VEGF expressed by HEK cells. In (A), the pro-angiogenic action of HEK-VEGF cells was checked using a chamber model. After 5 d of subcutaneous implantation of a chamber containing HEK or HEK-VEGF cells on the back of mice, a vascular cast was made by i.v. injection of a solution of carmine red and gelatin, and angiogenesis was quantified by carmine content of the dorsal skin. Representative photographs taken from the dorsal skin before tissue digestion evidence an increased number of blood vessels in HEK-VEGF treated mice, and these newly formed vessels were tortuous and morphologically distinct from preexisting vessels. After digestion, the dye content of the samples was assayed spectrophotometrically and the results were then expressed quantitatively as mg dye content as shown in the graphic. Further, HEK nodules formed upon the CAM after grafting of HEK or HEK-VEGF cells were reacted with specific antibodies against VEGF and EPO as positive control. EPO expression was detected in both HEK (B) and HEK-VEGF (C) nodules. VEGF was detected only in HEK-VEGF (E) nodules, not in HEK nodules (D). *N* = nodule, and arrows indicate the CAM tissue subjacent to the cell nodule. Bars = 50 μm.

Expression of VEGF and erythropoietin (EPO) was assessed in HEK and HEK-VEGF nodules. The sections were incubated with a rabbit polyclonal antibody against VEGF that does not recognize chicken (endogenous) VEGF (1/500 in PBS; Santa Cruz Biotechnology, CA, USA), or EPO (1/1000 in PBS).

To identify capillary formation into the nodules, endothelial cells were stained using biotin-labeled *Sambucus nigra* lectin (1/3000; Vector Laboratories, Burlingame, CA, USA) as described previously (8) and pericytes were characterized using a monoclonal anti-desmin antibody (Desmin II, EURODIAGNOSTICA, Netherlands). Briefly, deparaffinized sections were incubated sequentially in 0.3 mg/mL albumin for 30 min, *Sambucus nigra* lectin diluted in 0.1 mg/mL albumin for 1–2 h, and an avidin-biotin-peroxidase complex (Vectastin ABC Elite Kit; Vector Laboratories). For anti-desmin staining, sections were incubated with antimouse antibody (1/200; Vector Laboratories) conjugated to biotin before subsequent steps. Peroxidase activity was detected with diaminobenzidine and H_2O_2 (Polysciences, Warrington, PA, USA).

Assessment of Nodule Vascularization

Nodule vascularization was investigated both on angiography pictures and on histological slides stained with the *Sambucus nigra* lectin and monoclonal an-

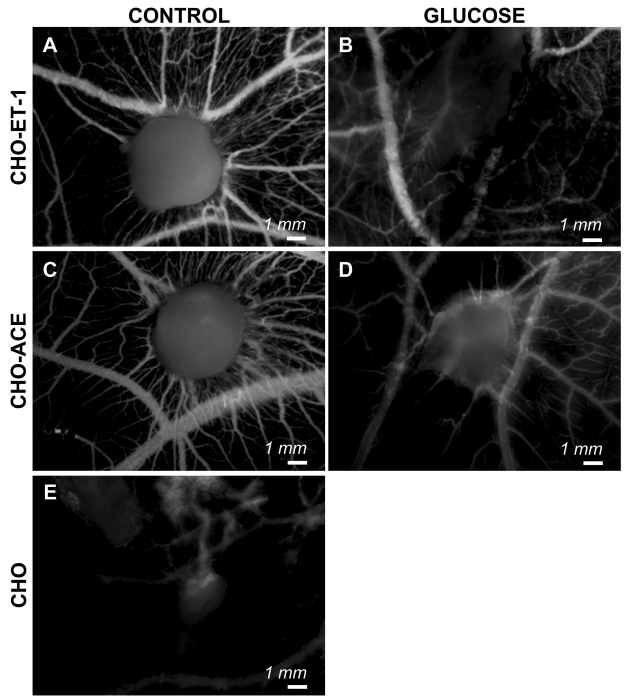

Figure 2. Angiogenic response in the CAM after implantation of CHO cell aggregates in control (water-injected) (left) or glucose-treated embryos (right). Representative nodules after 3 d of grafting of CHO-ET-1 (A, B) and -ACE (C, D). Cell aggregates from both ET-1 and ACE- transfected CHO cells did not induce the formation of nodules in glucose-treated embryos (B and D, respectively). Wild-type CHO cells were unable to form nodules in control embryos (E).

tibody anti-desmin. In all cases, all images and slides were analyzed by two different investigators who were blinded of both the cell type and the experimental group. In the case of comparison of HEK and HEK-VEGF nodules, both types of cells and treatment were analyzed in the same series, including four different groups: both HEK and HEK-VEGF in both hyperglycemic and control embryos. On angiography pictures we scored 1) nodule size, 2) nodule vascularization, and 3) peri-nodular vascularization. The nodule size was the mean of two orthogonal measures of the nodule. Nodule vascularization was scored 2 when a typical spoke-wheel pattern of vascularization was observed, 1 when undubious penetration of vessels into the nodule was observed, 0.5 when equivoquial attraction of vessels was observed, and 0 when no evidence for attraction of vessel was observed. Peri-nodular vascularization was scored 1 when no alteration was observed, and 0.75, 0.5, and 0 when it had disappeared in about a 1/4, 1/2, and 3/4 of perinodular zone, respectively. On histological slides stained with *Sambucus*, we evaluated the positive staining for *Sambucus nigra* lectin, that is, capillary density inside the nodule. Vessels immunostained with a monoclonal antibody to desmin were scanned with a digital camera and the following parameters were directly measured on the computer monitor using the IPLab software (IPLab; Scanalytic) (14): 1) vessel density, i.e, number of vessels per surface area, 2) perimeter, 3) length of major axis, and 4) length of minor axis.

In situ Hybridization

In situ hybridization was performed as described previously on paraffin sections (8,13). Single-strand antisense and sense riboprobes, labeled with ^{35}S-UTP were generated by *in vitro* transcription from the following cDNA fragments: Quek1 (chicken VEGF-R2), chicken VEGF, chicken angiopoietin-2, chicken Tie-2 (angiopoietin receptor), chicken ET-1, and chicken ET_B (endothelin receptor) (15).

The hybridization signals were scored according to the intensity and density, and evaluated semiquantitatively as described previously (16). The observations were performed by two independent investigators who were blinded to the treatment group. In case of comparison of HEK and HEK-VEGF nodules, both types of cells and treatment were analyzed in the same series as described above.

Statistical Analysis

Statistical analysis was assessed by a nonparametric test Kruskal-Wallis, and by Mann-Whitney test. Results were expressed as means ± SEM. A $P < 0.05$ was considered significant. Analyses were performed using the StatView 4.51.1 statistical package.

For the characterization of HEK-VEGF cells, analyses were performed using SAS V8.2 via Everstat v5.0 interface. Significant differences between groups were assessed by Student *t* test along the objectives on carmine dye content parameter.

RESULTS

Characterization of Growth Factor Production by HEK and HEK-VEGF Cells

Since transfected CHO cells producing both ET-1 (CHO-ET-1) and ACE (CHO-ACE) were characterized previously as described in Material and Methods (10,11), we first determined the production of VEGF by HEK and HEK-VEGF cells. Using an ELISA assay, the VEGF concentration in a medium of HEK-VEGF cells was 13.9 ± 1.3 ng/mL compared with 0.1 ng/mL in a medium of control HEK cells ($P < 0.05$). The effect of HEK-VEGF cells on angiogenesis was tested first in a mammalian model after subcutaneous implantation of a chamber

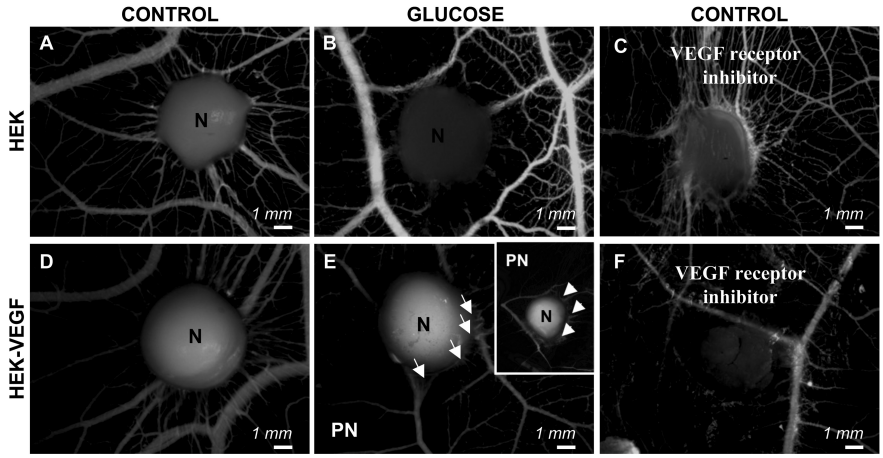

Figure 3. Angiogenic response in the CAM after implantation of HEK cell aggregates in control (water-injected) or glucose-treated embryos. HEK (A,B,C) and HEK-VEGF (D,E,F) cells induce nodule formation in both control and hyperglycemic embryos. However, note that in HEK-VEGF-nodules, although nodules were of the same size in both conditions (D = control; E = glucose) and vessels penetrated the nodule in both of them, VEGF secreted by the nodule did not correct hyperglycemia-induced vascular defects in the adjacent CAM. Upper and lower quarters of the image in (E) show obvious vascular defects in the vicinity of the nodule compared with (D). *N* = nodule, *PN* = perinodular area, and arrows show vessels penetrating in the nodule. In addition, in HEK-VEGF-nodules, the newly formed vessels are tortuous and morphologically distinct from vessels found in HEK-nodules vascularization. PTK787/ZK222584 (0.1 μg/μL), a VEGF receptor inhibitor, applied close to the HEK-VEGF cell aggregates, 2 d after grafting, inhibited vascularization of the nodule (F), but did not inhibit vascularization of HEK nodule (C). In detail (E), representation of an extreme case showing a strong local proangiogenic response around the HEK-VEGF nodule and decreased angiogenesis in the perinodular area in the hyperglycemic CAM.

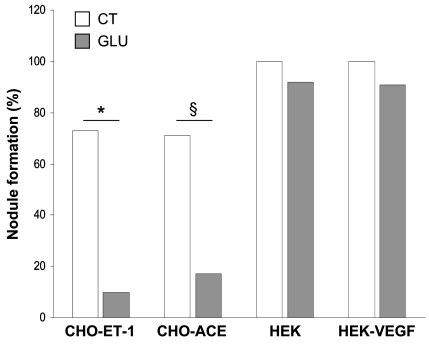

Figure 4. Effect of hyperglycemia on ET-1-, ACE-, and VEGF-mediated nodule formation in the CAM assay. Control and glucose-injected animals received a single intravitellus injection of water or glucose 48 h before the grafting of CHO or HEK cells transfected or not with angiogenic factors. Data are expressed as percentage of cell depots that grew and formed a nodule. The number of embryos that received a cell depot was: 37 and 40 for CHO-ET-1; 14 and 12 for CHO-ACE; 11 and 12 for HEK; and 16 and 11 for HEK-VEGF, control (CT, water-injected) and glucose (GLU), respectively. $^{*}P < 0.001$ and $^{\S}P = 0.008$ for the comparison of CT versus GLU.

containing HEK or HEK-VEGF cells on the back of mice (Figure 1A).

As the HEK cells express and secrete EPO (17), we verified that only HEK-VEGF cells express VEGF. EPO staining was then used as a positive control to show that transfected cells gained expression of VEGF, but did not lose expression of EPO. After grafting onto the CAM, EPO protein was detected by immunohistochemistry both in HEK- and HEK-VEGF cells (Figure 1B, Figure 1C), while VEGF was detected only in HEK-VEGF cells (Figure 1E). HEK cells did not present immunoreactivity against VEGF (Figure 1D).

Hyperglycemia

In the setting of the model, we had determined that the blood glucose level of control embryos was 103 ± 11 mg/dL (max. 145 mg/dL; *n* = 103). All hyperglycemic embryos presented blood glucose above 180 mg/dL, both at time of grafting and at the end of the experiment: 296 ± 55 mg/dL (*n* = 75) (Mean ± SD).

Nodule Formation in the CAM

Further, we compared separately the angiogenic effects of ET-1, ACE, or VEGF (Figure 2, Figure 3) in control and hyperglycemic embryos. The Figures show the angiogenic response observed 3 d after grafting upon the CAM in control and hyperglycemic embryos. Wild-type CHO cells (*n* = 5) (Figure 2E) and CHO cells transfected with a control plasmid were unable to form a nodule in control embryos, as previously described (10).

In control embryos, CHO cells expressing ET-1 (Figure 2A) or ACE (Figure 2C), as well as HEK-VEGF (Figure 3D), were able to form a nodule (a compact structure of cells growing upon the CAM) displaying a spoke-wheel pattern of vascularization in the CAM. HEK cells (Figure 3A), used as control for the experiments using the HEK-VEGF cells, also were able to form a nodule upon the control CAM. In glucose-treated embryos, both ET-1- and ACE-transfected CHO cells (Figure 2B, Figure 2D, respectively) failed to present the same response, showing the same pattern as that observed after grafting wild-type CHO-cells. In contrast, HEK and HEK-VEGF transfected cells conserved their property of forming nodules even upon the hyperglycemic CAM (Figure 3B, Figure 3E, respectively).

By using PTK787/ZK222584, we showed that the pro-angiogenic effect observed after grafting HEK-VEGF cells onto the CAM is due mainly to VEGF. The HEK-VEGF nodules degenerated after a 2-d PTK787/ZK222584 treatment (Figure 3F) (*n* = 4). However, we did not observe the same effect when the inhibitor was applied to HEK nodules (Figure 3C) (*n* = 3).

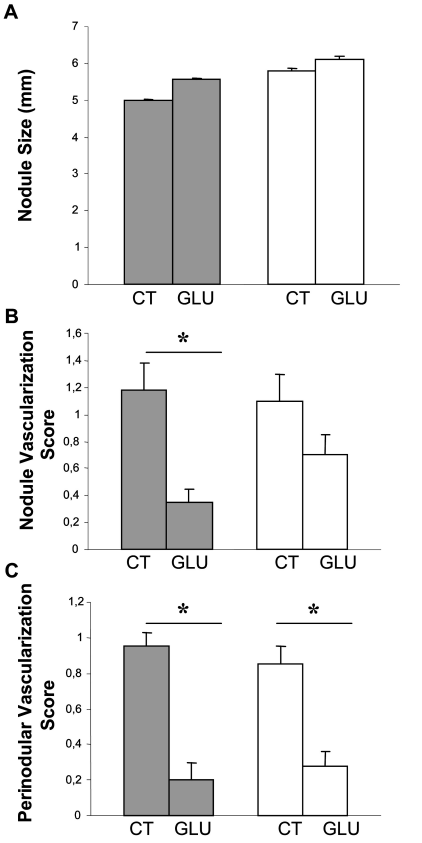

Figure 5. Angiographic analysis of HEK and HEK-VEGF nodules in the CAM of control and glucose-treated embryos. The pictures were scored according to (A) nodule size, (B) vascularization, and (C) perinodular vascularization. Grey bars: HEK; white bars: HEK-VEGF. CT = control (water-injected) CAM; GLU = glucose-treated CAM. Values are mean ± SEM; $P < 0.05$.

Figure 4 summarizes the results of grafting experiments. High glucose impaired ET-1- and ACE-mediated nodule formation and, consequently, vascularization, but did not interfere with the ability of HEK and HEK-VEGF cells to form nodules.

As only HEK and HEK-VEGF cells were able to form nodules upon the hyperglycemic CAM, we focused our study on these cells and we investigated 1) the potential differences between control and glucose-treated CAM in the degree of vascularization of the nodules, and 2) the mechanisms involved in the pro-angiogenic responses, such as the regulation of gene expression.

Nodule Vascularization

Both HEK and HEK-VEGF then were able to form nodules at similar sizes as assessed on angiography pictures (Figure 5A); however, they differed concerning the degree of vascularization. After grafting with HEK cells, the number of blood vessels that penetrated the nodules was much lower in hyperglycemic embryos than in control embryos. After grafting with HEK-VEGF cells, the vascularization score of the nodules formed onto the CAM was similar in both conditions (Figure 5B). The pro-angiogenic activity observed after grafting either HEK- or HEK-VEGF-cells was restricted to the nodular region, while the peri-nodular vascular density stayed altered in hyperglycemic embryos (Figure 5C), suggesting that secreted proangiogenic factors did not diffuse enough to correct the hyperglycemia-induced vascular defects in the surrounding CAM.

Vascular density also was assessed on histological sections taken 3 d after grafting. Immunohistochemistry using either the *Sambucus nigra* lectin, a marker of endothelial cells, (Figure not shown, Figure 6, respectively) or a monoclonal antibody to desmin, a marker of pericytes (Figure 7, Figure 6), confirmed that both HEK- and HEK-VEGF cells formed vascularized nodules in control embryos (Figure 7A, Figure 7C). In contrast, in hyperglycemic embryos, only HEK-VEGF (Figure 7D), not HEK (Figure 7B) nodules, were vascularized. In vascularized nodules, adjacent to the graft, there is a strongly disorganized area of the CAM mesoderm, rich in newly formed blood vessels as evidenced by lectin and desmin staining. These newly formed vessels, in response to HEK-VEGF cells, were tortuous and morphologically distinct from the preexisting ones. Staining with the monoclonal antibody to desmin allowed the automated assessment of the number of vessels in the CAM underlying the grafted nodules. The density of vessels in the CAM underlying the nodules formed by HEK cells was lower in hyperglycemic embryos ($n = 4$) compared with control ($n = 5$) ($P = 0.03$), whereas no statistically significant difference was observed after grafting with HEK-VEGF cells (hyperglycemic embryos $n = 6$, and control embryos $n = 5$) (Figure 7E).

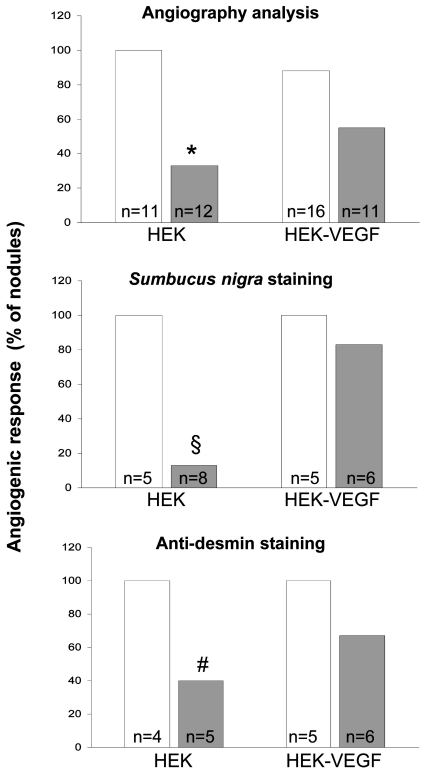

Figure 6. Effect of hyperglycemia on HEK and HEK-VEGF-mediated vascularization in the CAM assay. Angiogenic response was assessed both *in vivo* by angiography using fluoresceinated dextran and histologically after staining the slides with either the *Sambucus nigra* lectin, a marker of endothelial cells, or using an anti-desmin monoclonal antibody, a marker of pericytes and vascular smooth muscle cells. Results are expressed as percentage of nodules that triggered an angiogenic response in control (water-injected) or hyperglycemic conditions. White bars: control; gray bars: glucose. $^{*}P = 0.001$. $^{\S}P = 0.03$, and $^{\#}P = 0.16$ for the comparison between control versus glucose.

As shown in Figure 6, we observed that even after grafting of HEK cells, all

analyzed parameters (angiography and the histological analyses) were altered in the glucose-treated group as compared with the control group. On the other hand, after grafting of HEK-VEGF cells, there were no differences between the control and treated groups. However, even though there is no statistically significant difference concerning the angiography analysis, we have to emphasize here that this parameter was more affected by the hyperglycemic milieu than the other two.

Gene Expression

Gene expression in response to HEK- and HEK-VEGF cells was analyzed by *in situ* hybridization. A semiquantitative scoring was used to characterize the expression of mRNA as already validated in our laboratory (16). In control CAM, the expression of all probes that were analyzed was low (basal level) after grafting of HEK-cells. After grafting of HEK-VEGF cells, the expression of endogenous (chicken) VEGF, VEGF-R2, and Tie-2 was increased as compared with control CAM grafted with HEK-cells. In hyperglycemic embryos, both after grafting HEK- and HEK-VEGF-cells, the pattern of expression of the same markers of angiogenesis was similar to that observed in control CAM. However, not only the expression of endogenous (chicken) VEGF, VEGF-R2, and Tie-2 was increased after grafting of HEK-VEGF cells compared with HEK cells, but also that of ANG-2, ET-1, and ET_B (Figure 8, Figure 9).

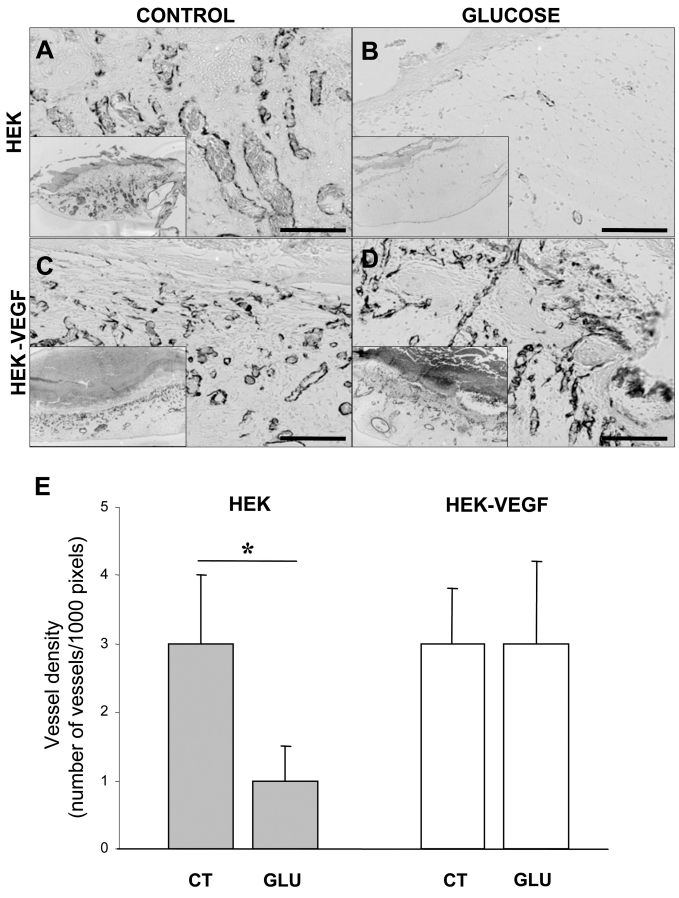

Figure 7. Vascular density in HEK and HEK-VEGF nodules. Blood vessels in control (A,C) and glucose-treated CAM (B,D) were labeled with monoclonal antibody to desmin 3 d after grafting. A dense network of new blood vessels is observed in the control CAM after (A) HEK- and (C) HEK-VEGF-grafting. In contrast, in glucose-treated CAM, only (D) HEK-VEGF, not (B) HEK, nodules are highly vascularized. Immunostained vessels were scanned, automatically measured and the vascular density was scored (E). Grey bars: HEK; white bars: HEK-VEGF. CT = control (water-injected) CAM; GLU = glucose-treated CAM. $n = 4$ for CT groups and $n = 5$ for GLU groups. The inserts represent the correspondent pictures at lower magnification. Values are mean ± SEM; $P < 0.05$. Bars = 100 μm.

DISCUSSION

Diabetes is associated with abnormal angiogenesis (1). In most models of diabetes, decreased angiogenesis is observed, and this participates in impaired wound healing, impaired outcome of grafted organs, and impaired opening of collateral vessels in patients with coronary heart disease and peripheral artery disease. Vascular growth factors are under investigation as potential therapies to stimulate angiogenesis/arteriogenesis in macroangiopathies, that is, coronary and lower limb atherosclerotic disease, and anti-angiogenic factors have reached clinical applications in patients with cancer or proliferative retinopathies (3,18). Although growth factor therapies are an attractive therapeutic option for these patients, a better understanding of their angiogenic influences, as well as their limitations, in diabetic patients is critical to maximize the effects of such therapies (6). Herein, we have tested the hypothesis that hyperglycemia, *per se*, could alter the pro-angiogenic effect of enzymes and growth factors.

Thus, we have investigated three different conditions to increase angiogenesis by using the CAM model, a model of defective angiogenesis upon hyperglycemic conditions (8). This model has been used to test the pro- or anti-angiogenic properties of molecules through direct gene transfer, cell grafting, tissue and sponge implantation (10,19,20).

Our first interest was on endothelin-1, because endothelins have been described to be involved in diabetic proliferative retinopathy (21), and impaired endothelin-1 mediated angiogenesis has been shown in diabetic animals (22).

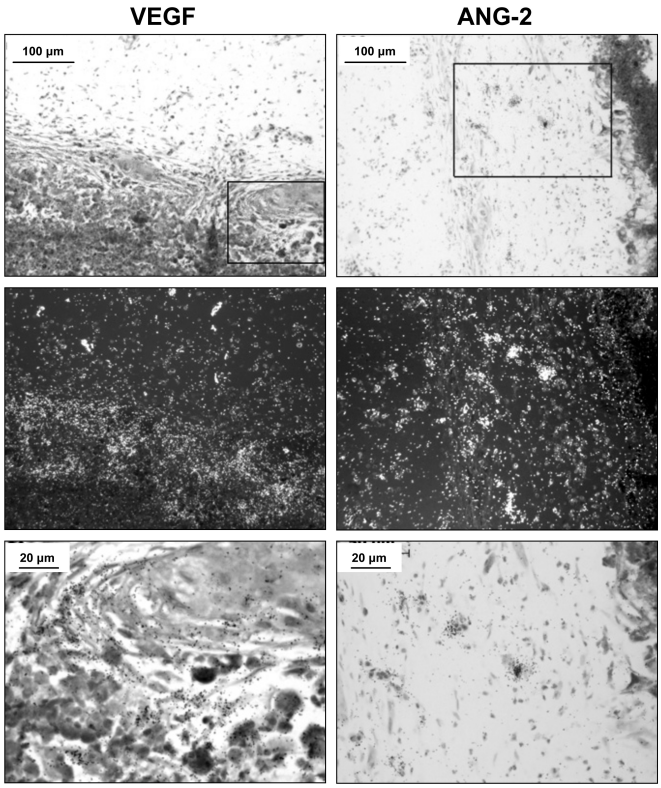

Figure 8. *In situ* hybridization. The gene expression pattern was evaluated using *in situ* hybridization. Panels represent *in situ* hybridization using cVEGF and cANG-2 antisense probes in glucose-treated embryos 3 d after HEK-VEGF-cell grafting. A very strong gene expression is observed in the area underlying the nodules, especially in the interface formed between the nodule and the CAM mesoderm, and in the proximity and in the wall of some blood vessels.

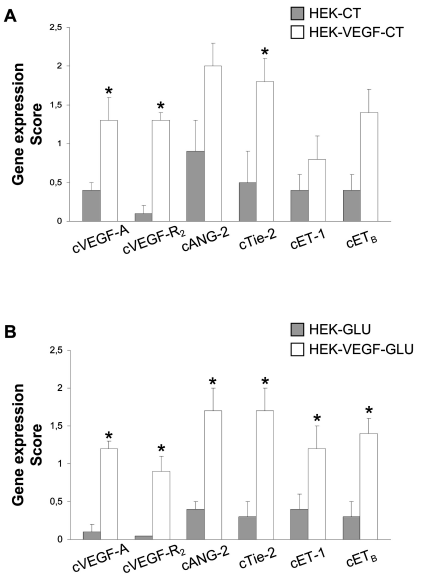

Figure 9. Semiquantitative evaluation of vascular growth factors and their receptors after *in situ* hybridization in control (A) and hyperglycemic embryos (B) after grafting of either HEK or HEK-VEGF cells. *In situ* hybridization slides were blindly assessed for both density and intensity of cells expressing the vascular growth factors or receptors. Results are expressed as score and values are mean ± SEM. The number of embryos was four in HEK-CT, five to seven in HEK-GLU, five in HEK-VEGF-CT, and seven in HEK-VEGF-GLU. *$P < 0.05$. CT: control (water-injected), GLU: glucose-injected animals. There are no significant differences on gene expression between CT and GLU after grafting with the same cell type (HEK or HEK-VEGF).

The second angiogenic condition we tested was the angiotensin converting enzyme. ACE blockade is a major therapeutic target in diabetes for the prevention of macroangiopathies, and the possibility that it may prevent diabetic retinopathy is tested in the DIRECT trial (21). In diabetic mice, ACE-inhibitors were shown to have dual favorable actions, both by inhibiting angiogenesis in the retina and by increasing it in the ischemic limb after ligation of the femoral artery (23). The feasibility of this approach in the CAM had been tested by the demonstration that angiotensin II was able to increase angiogenesis *in vivo* in the CAM model (24). Unpublished results obtained in the laboratory had likewise shown that CHO cells transfected with the chicken ACE gene were able to form vascularized nodules upon the CAM (K Savary, unpublished results). Finally, the VEGF was an obvious factor to test on the CAM, owing to its major role in angiogenesis.

To deliver vasoactive peptides at constant rates, we chose to use transfected cells grafted upon the CAM. In the present study, we have used cells that were transfected with ET-1 or ACE. However, hyperglycemia completely abolished ET-1-mediated angiogenesis and ACE-mediated angiogenesis. Only cells transfected with VEGF kept their ability to form well-vascularized nodules under hyperglycemic conditions.

These results are in line with those of Bek *et al.*, who, using a retina model, showed that diabetes impairs collateralization in response to ET-1-, but not to VEGF-mediated angiogenesis (22). Likewise, a plasmid coding for VEGF was able to correct the defective angiogenesis that was observed after ligation of the femoral artery in the NOD-mouse (25). In this context, a VEGF signaling defect may be one of the reasons for the reduced collateral blood vessel growth seen in patients and in experimental models of diabetes. It has been shown previously that patients with diabetes had reduced VEGF signaling despite normal or increased levels of VEGF ligand, and that a further increase in VEGF ligand expression is able to correct this VEGF signaling defect substantially (26). Our findings also are corroborated by the fact that

increased serum ACE activity is correlated with decreased angiogenesis in diabetic patients (27).

Focusing on the ability of VEGF overexpression to stimulate vascularization in hyperglycemic CAM, our data suggest that the effect of VEGF is more on angiogenesis than on the outgrowth of arterioles/venules (arteriogenesis). This finding is in agreement with other studies that have shown that the negative effect of diabetes on capillary density could be overcome by the application of VEGF, while the same strategy was less effective in the case of diabetes-associated impairment of collateral vessel formation (2,25).

In response to VEGF, we observed that a series of genes related to activation and proliferation of endothelial cells were expressed in the CAM of both control and hyperglycemic animals. Among these genes, we found endogenous VEGF-R2 and Tie-2, ET_B, ANG-2, and VEGF, all of them representing a common angiogenic response of endothelial cells (28). As discussed previously, both processes, angiogenesis and arteriogenesis, depend on the maintenance of the intact endothelial function/endothelial proliferation (2).

However, even though VEGF was able to exert a proangiogenic activity in the CAM of hyperglycemic embryos, this proangiogenic effect was restricted to the nodular area since the vascularization of the CAM areas in the close vicinity around the nodules was not altered. This still presents decreased vascularization, probably as a direct effect of hyperglycemia, an effect also observed in the perinodular area of HEK nodules. It is, however, reassuring in the context of diabetes, with regards to the dangers of growth factors therapies on the retinopathy that growth factors can be delivered locally.

One limit of the data is the analysis of transformed cell-induced angiogenesis. Experimental angiogenesis induced by grafting cells, as well as by inducing wounding, may not be the same of the natural angiogenesis on the CAM (29,30). However, as we are not interested in correcting the hyperglycemia-induced decreased spontaneous angiogenesis, but showing that hyperglycemia alters the angiogenic response to different proangiogenic factors, we believe that our model is appropriate.

In conclusion, our results have focused on potential pitfalls in the interpretation of proangiogenic therapies in patients with vascular disease, by showing that hyperglycemia *per se* can interfere with the response to some, but not all, growth factors. This is an advocacy to optimal glucose control in patients receiving such treatments.

Our results suggest that, in the context of a modest hyperglycemia during developmental angiogenesis, the negative effect of diabetes on capillary density was overcome only by VEGF overexpression, whereas responses to other vasoactive peptides were altered in the same conditions. In the evaluation of future trials testing pro-angiogenic factors in patients with diabetes, such interferences of hyperglycemia on the angiogenic response will have to be considered.

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