Hypoxia Induces Vascular Endothelial Growth Factor Gene and Protein Expression in Cultured Rat Islet Cells

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The formation of new microvasculature by capillary sprouting at the site of islet transplantation is crucial for the long-term survival and function of the graft. Vascular endothelial growth factor (VEGF), an endothelial cell–specific mitogen with potent angiogenic and vascular permeability-inducing properties, may be a key factor in modulating the revascularization of islets after transplantation. In this study, we examined the gene expression of VEGF mRNA in three tumor cell lines and in isolated whole and dispersed rat islets in vitro by Northern blot hybridization in normoxic (5% CO_2, 95% humidified air) and hypoxic (1% O_2, 5% CO_2, 94% N_2) culture conditions. Increased expression of VEGF mRNA was observed in β-TC3, RAW 264.7, and IC-21 tumor cell lines when subjected to hypoxia. With isolated whole islets in normoxic culture, a threefold increase in VEGF mRNA ($P < 0.001$) was seen at 48 h as compared with freshly isolated islets. This response was similar to the 3.8-fold increase observed with islets subjected to hypoxia. Dispersed rat islet cell clusters cultured on Matrigel for 24 h under hypoxic conditions showed a 3.4-fold increase ($P < 0.01$) in VEGF mRNA compared with those cultured in normoxia. This correlated with increased VEGF secretion as determined by enzyme-linked immunosorbent assay. Immunohistochemical studies revealed the presence of increased expression of VEGF protein near the center of islets after 24 h of normoxic culture. Islet cell clusters on Matrigel showed intense cellular localization of VEGF in both β-cells and non–β-cells. These findings suggest that rat islet cells, when subjected to hypoxia during the first few days after transplantation, may act as a major source of VEGF, thereby initiating revascularization and maintaining the vascular permeability of the grafted islets. ***Diabetes*** **47:1894–1903, 1998**

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DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; VEGF, vascular endothelial growth factor.

A significant obstacle to successful pancreatic islet transplantation, besides immune rejection, is the inadequate vascularization of the graft during the first few days after implantation (1), which may result in the dysfunction and death of the islet tissue (2–4). Pancreatic islets are richly vascularized (5); they receive their blood supply from an afferent arteriole that branches into a glomerular-like network of microvessels, which forms a local intra-islet portal system (6,7) through which blood flows from the central core of β-cells to the non–β-cell mantle. Islet capillaries are fenestrated, which may be important for the efficient release of secreted products into the bloodstream (6). The process of isolating islets disrupts this specialized vasculature, thus threatening the survival of cells in the center of the islet (8). Several studies have demonstrated that decreased blood supply may lead to death of the β-cells in the core of cultured or transplanted islets (9,10).

The molecular mechanisms that initiate and regulate the process of revascularization are incompletely understood. Most of the revascularization takes place over a period of 7–10 days with almost all of the vessels arising from the recipient (10,11). Angiogenesis is a complex multistep process that includes remodeling of the extracellular matrix, migration and proliferation of endothelial cells, capillary tube formation, and maturation of blood vessels (12–14). This process is thought to be initiated by local activation of genes encoding angiogenic factors. Studies from several laboratories have demonstrated that multiple factors may contribute to an angiogenic response, including the peptides epidermal growth factor, transforming growth factor-α and -β, acidic or basic fibroblast growth factor, platelet-derived endothelial cell growth factor, β-cellulin (member of the EGF/TGF-α family), and vascular endothelial growth factor (VEGF) (15–17).

VEGF (also known as vascular permeability factor) is mitogenic for endothelial cells in various in vitro and in vivo systems (18–22) and has been shown to increase permeability of microvessels (20,23–25). VEGF has been shown to be expressed in many tumor cell lines and various normal tissues and organs (23). Gene expression of VEGF is markedly upregulated in several cell lines grown in hypoxic conditions (26–30). VEGF expression as determined with in situ hybridization is increased in ischemic areas surrounding regions of tumor necrosis (31,32). In humans, there are four VEGF isoforms—consisting of 121, 165, 189, or 206 amino acids—which are generated by alternative splicing of a single gene (23,33,34). The most abundant and widely distributed

form is $VEGF_{165}$ (14,33). VEGF binds with high affinity to two highly homologous tyrosine kinase receptors expressed mainly by endothelial cells: the tyrosine kinase receptor KDR (or the murine homolog fetal liver kinase *Flk-1*, also known as VEGF-R2) (35,36) and the *fms*-like tyrosine kinase receptor *Flt*, also called VEGF-R1 (37). Immunohistochemical studies have demonstrated the presence of the *Flk-1* receptor in fetal pancreatic duct cells, suggesting the importance of this receptor/ligand for the maturation of β-cells from duct cells (38).

In the current study, the expression and modulation of VEGF by hypoxia were evaluated in tumor cell lines established from rodents. In addition, the influence of regional hypoxia on the expression of VEGF mRNA in isolated islets was investigated in whole islets cultured in hypoxic and normoxic culture conditions, and compared with cultured dispersed islet cells. The relationship between VEGF mRNA expression and subsequent VEGF secretion was investigated using enzyme-linked immunosorbent assay (ELISA). Finally, localization of VEGF protein in whole islets and dispersed islet cells was determined using immunohistochemistry.

RESEARCH DESIGN AND METHODS

Cell lines and culture. β-TC3 (mouse β-cell tumor cell line) (39), RAW 264.7 (a mouse monocyte-macrophage line from the ascities of a tumor induced by intraperitoneal injection of Abelson leukemia virus) (40), and IC-21 (a mouse macrophage line derived by transformation of normal C57BL/6 mouse peritoneal macrophages with simian virus 40) (41) cells were grown to 80% confluence in sterilized culture dishes (tissue culture treated, 100 × 15 mm; Becton Dickinson, Bedford, MA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing a glucose concentration of 25 mmol/l (Cellgro; Mediatech, Herndon, VA), supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 100 U/ml penicillin, and 100 µg/ml streptomycin (Cellgro; Mediatech). The β-TC3 cell line was a gift from Dr. Shimon Efrat (Albert Einstein College of Medicine, Bronx, NY). RAW 264.7 and IC-21 cell lines were gifts from Dr. Michael Barret (Dana-Farber Cancer Institute, Boston, MA).

Isolation of islets and culture of islet cells on Matrigel matrix. Islets were isolated from SD rats (Taconic, Germantown, NY) weighing 200–250 g by distending the pancreatic duct with a collagenase-containing solution (Collagenase P, 1.5 mg/ml; Boehringer Mannheim, Indianapolis, IN), as previously described (42). After digestion, the islets were separated on a Histopaque density gradient (Histopaque-1077; Sigma, St. Louis, MO) and further purified by handpicking. Islets were cultured in RPMI containing a glucose concentration of 11.1 mmol/l (Cellgro; Mediatech) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in standard humidified culture conditions of 5% CO_2 and 95% air at 37°C. Dispersed islet cells were prepared by placing 24-h cultured rat islets pooled from four rats (~3,000 islets) in 1 ml of trypsin-EDTA solution (× 1) (Cellgro; Mediatech) for 10 min at 37°C. The islets were then washed in culture media and dissociated by trituration in a narrow bore sterilized and siliconized Pasteur pipette. The dispersed islet cells were kept in supplemented RPMI culture media for 3–4 h before being cultured on Matrigel matrix (Collaborative Biomedical Products, Bedford, MA). Sterilized culture dishes (tissue culture treated, 60 × 15 mm; Fisher Scientific, Pittsburgh, PA) were coated with Matrigel basement membrane matrix (1:3 diluted solution of Matrigel in cold DMEM; Collaborative Biomedical Products, cat. # 40234, Bedford, MA). After coating (150–200 µl of the diluted Matrigel/cm^2 of growth surface of culture dish), the dishes were placed in an incubator at 37°C for a minimum of 3 h to allow the Matrigel to dry. Thereafter, 7 ml of prewarmed culture media were gently placed on the Matrigel. Dispersed islet cells were aliquoted onto the Matrigel and cultured for 24 h followed by 100% media change. The dispersed islet cells were cultured for a further 48 h, after which the cells were allowed to continue for a further 24 h either in standard normal culture conditions or were exposed to hypoxia before extraction of RNA.

Recovery of islet cells from Matrigel matrix. MatriSperse cell recovery solution (Collaborative Biomedical) was used at full strength to recover cells from the Matrigel matrix. After removal of medium from the dishes, the layer of islet cells on the Matrigel was washed three times with cold phosphate-buffered saline (PBS). MatriSperse (3 ml) was then added and the cell/gel layer was scraped and placed into an ice-cold 50-ml Falcon conical tube at 4°C. The dish was rinsed with 2–4 ml of MatriSperse solution and transferred to the tube. The tube was kept on ice for at least 90–120 min to allow the Matrigel to dissolve completely. The islet cells were then centrifuged (800–1,000 rpm for 5 min) to a pellet and washed twice in cold PBS; total RNA was extracted as described below.

Hypoxia studies. Confluent cell lines monolayers were exposed to 1% O_2 using an advanced, computer-controlled, water-jacketed triple-gas incubator (model 480; Lab-Line, Melrose Park, IL) that controls the O_2 and CO_2 to within ±0.1% of the set point value. Cells were maintained at 37°C in a constant 5% CO_2 atmosphere in which nitrogen was substituted for oxygen for a minimum of 24 h. This hypoxic environment (1% O_2, 5% CO_2, 94% N_2) was used throughout the studies. Previously published studies (43) and our current observations indicated that cells cultured under these hypoxic conditions for up to 48 h would show no morphological changes by light microscopy, would exclude trypan blue dye (>97%), and subsequently could be passaged normally. In addition, viability was also determined by using viability stains, Calcein-AM and propidium iodide (Molecular Probes, Eugene, OR), as described below. We observed >95% of the cells to be viable either in normoxic culture conditions (5% CO_2 with 95% humidified air) or after 24 h in hypoxic culture conditions. Whole isolated islets or dispersed islet cells cultured on Matrigel were studied in similar hypoxic conditions. After hypoxic or normoxic culture periods, the medium of islet cells plated on Matrigel matrix was collected, passed through a 0.45-µm filter (Becton Dickinson), and stored at –80°C until measurement of VEGF protein.

RNA isolation and Northern blot analysis. Total cellular RNA was extracted by a single-step method (44) using the Trizol reagent kit (Life Technologies, Gaithersburg, MD). Briefly, cells or whole islets were lysed in 1 ml of phenol/guanidine-isothiocynate containing Trizol solution, followed by isopropanol precipitation. Total RNA was quantitated using a spectrophotometer (LKB, Pharmacia, Little Chalfont, U.K.). Samples of RNA (15–20 µg) were resolved by electrophoresis through 1.5% agarose gel containing 2.2 mol/l formaldehyde, with subsequent capillary transfer to Biotrans nylon membranes (ICN Biochemicals, Irvine, CA). This was followed by ultraviolet cross-linking using a Stratagene UV Stratalinker 2400 (Stratagene, La Jolla, CA). A full-length fragment of human $VEGF_{165}$ cDNA (provided by Dr. Napoleone Ferrara, Genentech, San Francisco, CA) probe was generated with [α-^{32}P]deoxy-cytosine triphosphate (Du Pont-NEN, Boston, MA) using the random-primed DNA labeling kit (Boehringer Mannheim). Prehybridization followed by hybridization was performed at 65°C for 18 h using 1×10^6 cpm of the labeled probe per ml of hybridization solution in a rotating oven (model 400; Robbins Scientific, Sunnyvale, CA). Thereafter, the blot was washed three times under medium stringency conditions at 65°C with 0.5 × sodium chloride–sodium citrate and 5% SDS for 20 min each and then exposed to Du Pont reflection NEF-496 film with an intensifying screen at –80°C for 48–72 h. Each Northern blot was repeated at least three times, with similar results being obtained; a representative blot from each of these experiments was chosen for the figures. Lane loading differences were normalized by stripping and subsequent rehybridization of the blots to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1.3 kb human GAPDH cDNA; Clontech, Palo Alto, CA) or to 36B4 (45,46). VEGF and internal control values were obtained by subtracting background signal in each lane from the corresponding band signal in that lane. Gene expression of insulin mRNA was also determined by stripping and rehybridizing the same membrane with mouse insulin cDNA generated by polymerase chain reaction using primers as previously described (47) to generate a labeled probe. Hybridization signals were analyzed within the linear range of the signal quantitation using the Molecular Dynamics Computing Densitometer and PhosphorImager (Molecular Dynamics, Sunnyvale, CA) (software from ImageQuant, version 3.3, Sunnyvale, CA).

Measurement of VEGF using ELISA. VEGF in supernatants of rat islet cells cultured on Matrigel matrix was measured by an ELISA chemiluminescent detection system. Mouse monoclonal anti-human VEGF (R & D Systems, Minneapolis, MN) diluted in PBS was used as capture antibody (0.5 µg/ml) in triplicate wells of Dynatech Microlite (#1) 96-well plates (Dynatech, Chantilly, VA) and incubated overnight at 4°C. Plates were then washed with blocking buffer (Tropix, Bedford, MA) (0.5% I-Block, 0.1% Tween-20 in PBS) and incubated for 2 h at room temperature, followed by three washes with wash buffer (0.2% I-Block, 0.1% Tween-20 in PBS). Recombinant human VEGF-165 (R & D Systems) diluted in supplemented RPMI medium was used as the standard with concentrations of 1–500 pg/well. Undiluted supernatant samples and standards were added in triplicate to wells and incubated at room temperature for 2 h. After three washes, each well was incubated with chicken anti-human VEGF antibody (gift from Dr. Don Senger) for 1.5 h at room temperature. After three washes with wash buffer, alkaline phosphatase conjugated rabbit anti-chicken IgG (1:5,000, Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 1 h at room temperature. The plate was then washed three times with wash buffer followed twice with assay buffer (0.1 mol/l diethanolamine [Tropix, Bedford, MA], 1 mmol/l $MgCl_2$, 0.02% sodium azide). Substrate/enhancer (10% Saphire II enhancer, 0.4 mmol/l 1,2-dioxetane chemiluminescent enzyme substrate [Tropix]) was added to each well and incubated for 30 min at room temperature, after which the plate was read in a computer-controlled Microtiter Plateluminometer (model ML 3000; Dynatech). The program Statview 512 was used to plot luminosity averages. The

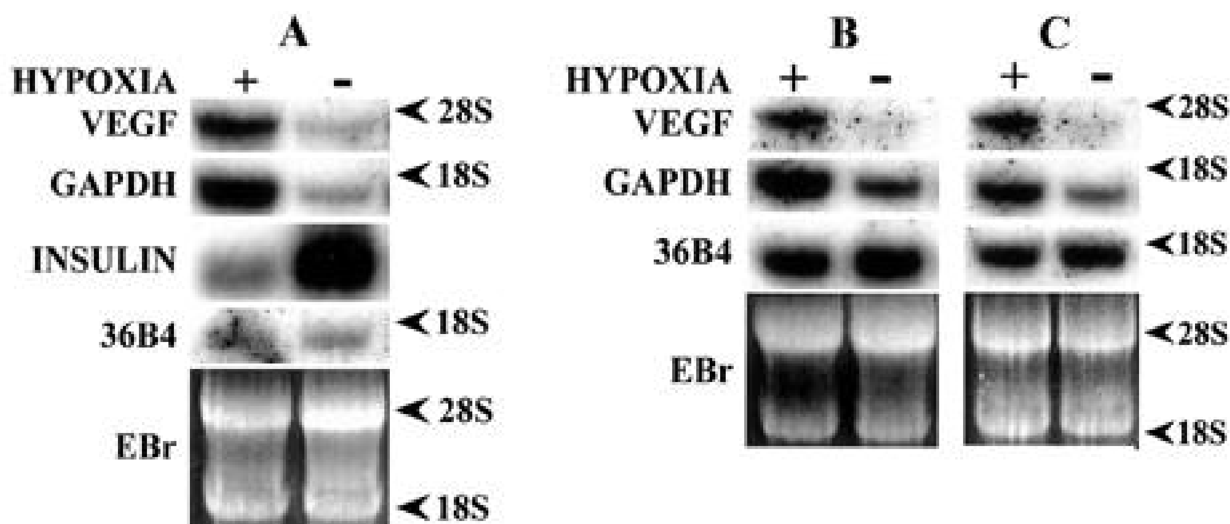

FIG. 1. VEGF mRNA expression in β-TC3 (*A*), RAW 264.7 (*B*), and 1C-21 (*C*) cells exposed to hypoxic (1% O_2, 5% CO_2, 94% N_2) or normoxic (5% CO_2, 95% air) culture conditions for 24 h. Total RNA (20 µg/lane) was analyzed by Northern blot hybridization using a ^{32}P-labeled cDNA probe for human $VEGF_{165}$. The same membrane was rehybridized with cDNA probes for GAPDH, insulin, and 36B4. Ethidium bromide (EBr) stains are shown in the bottom panel; locations of 28S and 18S rRNA are indicated by arrowheads. Results are representative of three independent experiments.

concentration of VEGF in the supernatant samples was calculated with a formula derived by linear regression analysis of luminosity values obtained with standard concentrations of VEGF. The correlation coefficient of the luminosity curve for VEGF was 0.984. The detection limit of this assay (2 SD above background or zero standard) was 4.5 pg/ml and the linear measurable range was 4.5–5,000 pg/ml. Because recombinant rat or mouse VEGF was not available at the time, we chose to use purified recombinant human VEGF to calibrate this assay. Therefore results are expressed as human VEGF equivalents in pg/ml.

Viability assay. Calcein-AM and propidium iodide (Molecular Probes) were used as viability stains (48). Solutions were freshly prepared by diluting the stock in PBS to a final concentration of 1 µg/ml Calcein-AM and 10 µg/ml propidium iodide. To assess viability of whole islets or dispersed rat islet cells on Matrigel, 500 µl of the staining solution was placed directly over whole islets or dispersed cells and incubated for 20 min at 37°C. The cells were then washed and assessed using fluorescence microscopy (on Zeiss LSM microscope; Carl Zeiss, Thornwood, NY). Live and dead cells were simultaneously visualized using a double-pass filter for fluorescein and Texas Red. Viable cells converted Calcein-AM into a green fluorescent product. Dead cells were permeable to propidium iodide, which bound to chromatin and was seen as red fluorescence.

Immunohistochemistry and confocal microscopy. Initial studies to determine the presence of VEGF in islets were conducted in whole pancreas. Pancreatic tissue was excised from SD rats (150 g), fixed in 4% freshly made formaldehyde in 0.1 mol/l phosphate buffer for 2 h, and then washed and stored in 0.1 mol/l phosphate buffer (pH 7.4) until embedded in paraffin. Freshly isolated rat islets and cultured islets were fixed in 4% formaldehyde for 30 min, enrobed in agar, and kept in 0.1 mol/l phosphate buffer until embedded in paraffin. Dispersed rat islet cells cultured on Matrigel-coated Lab-Tek Chamber slides (Nunc, Naperville, IL) in normoxic or hypoxic culture conditions were fixed with 4% formaldehyde for 30 min and then washed and stored in the same buffer at 4°C until staining. Immunofluorescent staining for VEGF was performed using the Tyramide Signal Amplification–Green kit (Du Pont-NEN). Briefly, dewaxed and rehydrated sections (5–7 µm) or islet cells on Matrigel in chamber slides were incubated for 30 min with 0.3% H_2O_2 in PBS (with extra 0.9% NaCl added) to block endogenous peroxidase activity. Sections and chamber slides were then incubated with PBS containing 1.5% goat serum for 30 min at room temperature (Vector Laboratories, Burlingame, CA) to block nonspecific binding. This was followed by an overnight incubation at 4°C with affinity-purified rabbit polyclonal anti-human VEGF antibody (1:100, A-20; Santa Cruz Biotechnology, Santa Cruz, CA) that was previously treated overnight at 4°C with 2 mg/ml poly-L-lysine (Sigma), spun down, and then used (49). After three washes in PBS (5 min per wash), the sections were incubated with biotinylated goat anti-rabbit antiserum (Vector) for 1 h at room temperature. After an additional three washes with PBS, the sections were incubated for 30 min with TNB-blocking buffer (0.1 mol/l Tris-HCl[pH 7.5], 0.15 mol/l NaCl, 0.5% blocking agent supplied with the kit) followed by a further 30-min incubation with streptavidin-horseradish peroxidase (1:500). After three washes with TNT buffer (0.1 mol/l Tris-HCl [pH 7.5], 0.15 mol/l NaCl, 0.05% Tween 20), the sections and islet cells were incubated with fluorescein tyramide (1:50) for 5 min. Specificity of VEGF staining was confirmed by the preabsorption of VEGF antibody (diluted 1:100) in 25-fold excess (by weight) of VEGF peptide (Santa Cruz Biotechnology) overnight at 4°C. For double-staining of insulin, sections and chamber slides stained for VEGF were then washed with PBS and incubated overnight at 4°C with antisera of guinea-pig anti-human insulin (1:200, Linco Research, St. Charles, MO), followed by 1-h incubation at room temperature with Texas Red conjugated affinipure donkey anti-guinea pig IgG (1:100, Jackson ImmunoResearch), washed, and then mounted in anti-fade mounting media.

Sections and chamber slides were viewed using an oil immersion 40 or 63× objective lens with a Zeiss LSM 410 invert laser scanning confocal microscopy unit (Zeiss). An argon/krypton mixed-gas laser giving excitation wavelengths of 488 and 568 nm was used to obtain fluorescent images relative to each marker. Images were handled in Adobe Photoshop.

Statistical analysis. Experiments were repeated at least three times. Results are expressed as means ± SE. Statistical analysis was performed using the unpaired Students' *t* test for pairwise comparisons and one way analysis of variance (ANOVA) for multiple comparisons. Post-hoc analysis for multiple comparisons was done using Tukey's test. Statistical significance was considered at $P < 0.05$.

RESULTS

Hypoxia-induced VEGF mRNA expression in mouse tumor cell lines. Hypoxic induction of VEGF mRNA gene expression was examined in β-TC3, RAW 264.7, and IC-21 cell lines. As shown in Fig. 1*A*, VEGF mRNA was increased 3.7-fold by hypoxia (when normalized to the expression of 36B4) in β-TC3 cells compared with normoxic conditions. In the same samples, expression of GAPDH mRNA in hypoxic culture conditions was upregulated 6.6-fold compared with normoxic culture conditions. Levels of transcripts of this housekeeping gene changed in hypoxic and normoxic culture conditions, so GAPDH could not be used for normalization purposes. This change in the gene expression of GAPDH mRNA in hypoxic conditions has also been observed by other investigators (50,51). Quantification of mRNA was therefore normalized to 36B4, as previously described (30,43). Stripping and reprobing the same blots with mouse insulin cDNA fragment revealed that in the presence of hypoxia, insulin mRNA levels were reduced to ~30% of nor-

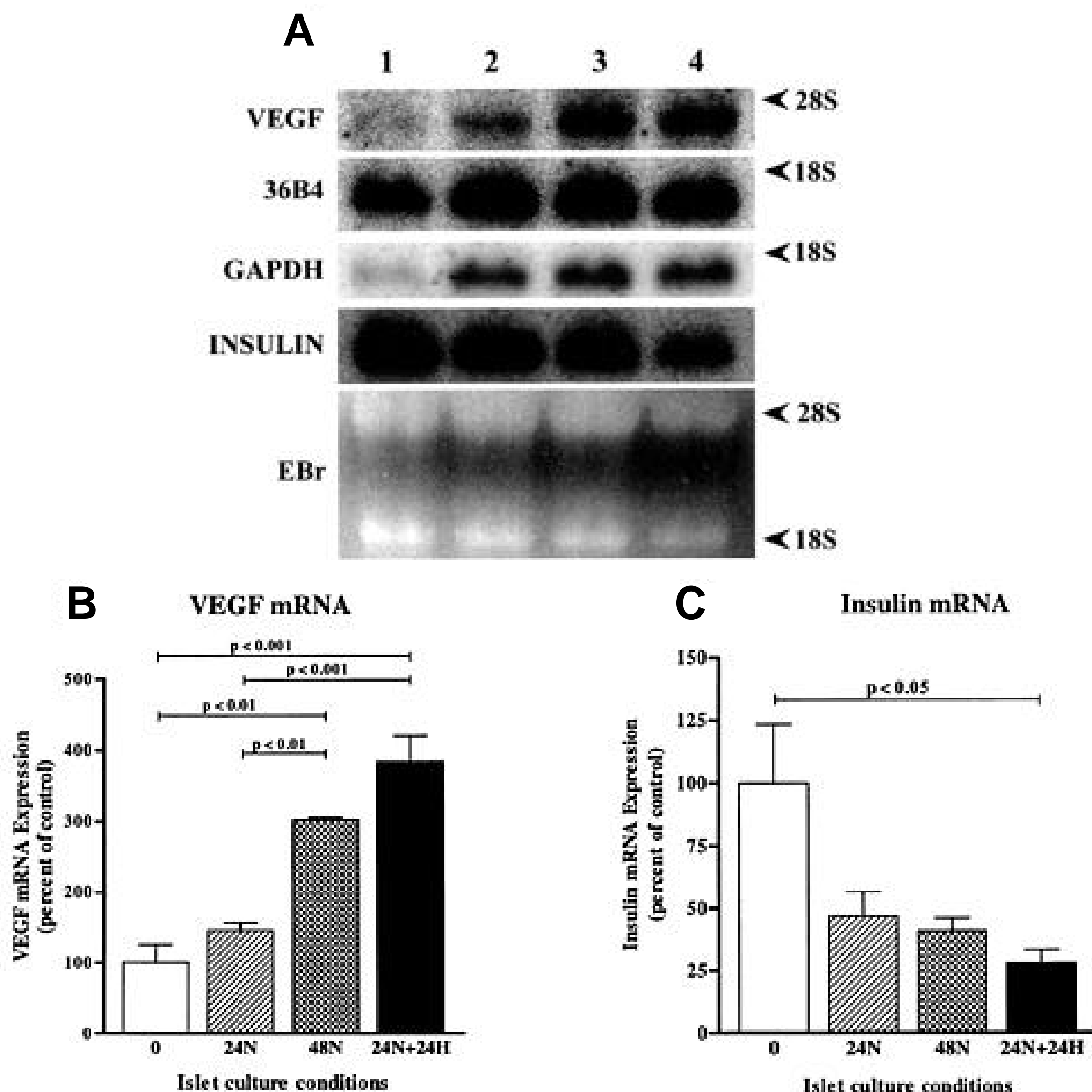

FIG. 2. ***A***: **Northern blot analysis for VEGF mRNA gene expression in isolated rat islets in culture.** ***Lane 1***: **Freshly isolated islets (0);** ***lane 2***: **24-h cultured islets in normoxic culture conditions (24N);** ***lane 3***: **48-h cultured islets in normoxic culture conditions (48N);** ***lane 4***: **24-h normoxic culture followed by 24-h hypoxic culture (1% O_2) conditions (24N + 24H). Total RNA was extracted from islets and 15 µg/lane was subjected to Northern blot hybridization using labeled probe for human $VEGF_{165}$. The same membrane was rehybridized with cDNA probes for 36B4, GAPDH, and insulin, as shown. Ethidium bromide (EBr) stains are shown in the bottom panel; locations for 28S and 18S are indicated by arrowheads. This experiment was performed three times; a representative Northern blot is shown. Bar graphs show quantitation of VEGF (*B*) and insulin (*C*) mRNA expression levels after normalization to the control signal 36B4; results are expressed as relative normalized mRNA expression (percent of control) ± SE. Data in *B* were analyzed by ANOVA.**

mal. This observation of increased GAPDH and decreased insulin mRNA in β-TC3 cells was consistent with the shunting of glucose metabolism to anaerobic respiration in the presence of hypoxia (51). We next sought to determine whether VEGF expression would be similarly upregulated in non–insulin-secreting cell lines in response to hypoxic culture conditions. As demonstrated in Fig. 1*B* and *C*, VEGF mRNA was similarly upregulated by hypoxia in RAW 264.7 (3.7-fold increase) and 1C.21 (4.2-fold increase) cells when normalized to 36B4. In addition, GAPDH mRNA was upregulated in both these cell lines by hypoxia. Increases of 2.7- and 3.6-fold of normoxic levels were observed in RAW 264.7 and 1C-21 cells, respectively, increases not as pronounced as in the β-TC3 cells. A similar pattern of expression of VEGF, GAPDH, 36B4, and insulin (β-TC3) mRNAs was observed in all three cell lines in hypoxic and normoxic culture conditions when 10 or 15 µg of total RNA were loaded per lane (data not shown).

VEGF mRNA expression in isolated cultured rat islets in vitro. The effect of normoxic and hypoxic culture periods on VEGF mRNA levels was determined by Northern blot analysis for VEGF. A progressive increase in VEGF mRNA expression was observed in islets cultured in normoxic culture conditions (Fig. 2*A* and *B*). No significant differences in the levels of VEGF mRNA expression in freshly isolated islets and those cultured for 24 h in normoxic culture conditions were found. However, after 48 h of normoxic culture,

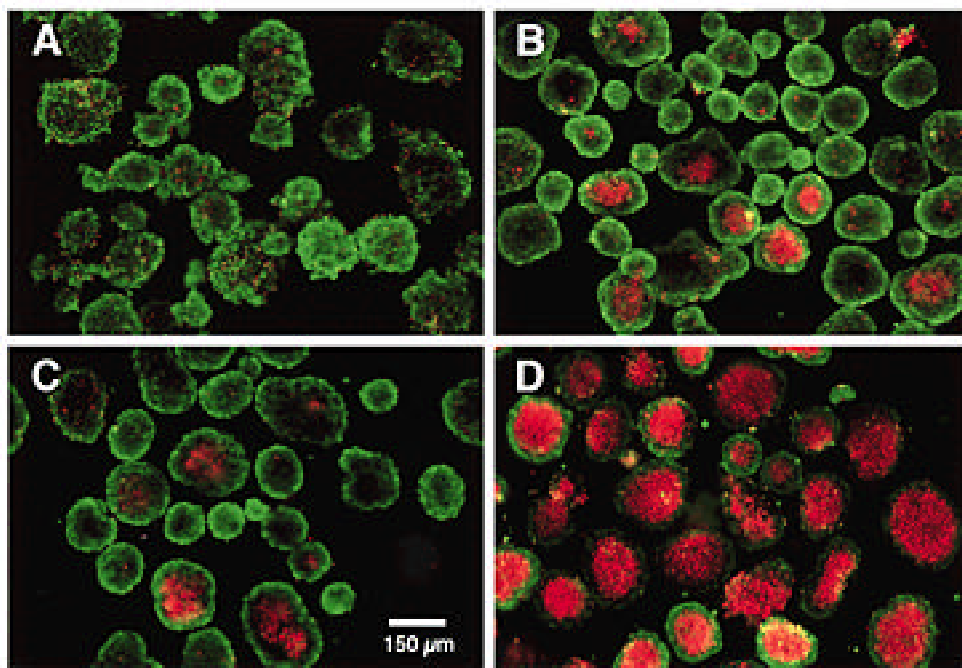

FIG. 3. Determination of viability of isolated rat islets by Calcein-AM and propidium iodide viability stains. Live (green fluorescence) and moribund or dead (red fluorescence) cells were visualized at the same time using specific filters for fluorescein and Texas red. *A*: Freshly isolated islets. *B*: 24-h cultured islets in normoxic conditions. *C*: 48-h cultured islets in normoxic conditions. *D*: Islets cultured for 24 h in normoxic culture conditions followed by 24 h in hypoxic (1% O_2) culture conditions.

VEGF mRNA levels were increased three- and twofold compared with those of freshly isolated islets ($P < 0.01$) and those of 24-h normoxic culture ($P < 0.01$), respectively (Fig. 2*B*). Islets cultured for 24 h in normoxic conditions followed by 24 h of hypoxic culture did not significantly increase the VEGF mRNA expression compared with in 48-h normoxic-cultured islets (Fig. 2*B*). Levels of VEGF mRNA expression increased 3.8- and 2.6-fold after 24 h of hypoxic culture compared with freshly isolated islets ($P < 0.001$) and those cultured for 24 h in normoxic culture ($P < 0.001$), respectively (Fig. 2*B*). The level of expression of insulin mRNA was significantly different only between freshly isolated islets and those exposed to 24 h of hypoxia, with there being a decrease to 28% after hypoxia ($P < 0.05$) (Fig. 2*C*). Increased expression of GAPDH mRNA levels was seen in all cultured islets (Fig. 2*A*). This upregulation of GAPDH mRNA (even in islets cultured in normoxic culture conditions as shown in Fig. 2*A*) may have been due to necrosis of cells and the decreased oxygen available in the central portion of large islets or those trapped in an islet clump.

The increase of VEGF mRNA in islets in normoxic culture conditions may have resulted from hypoxia of the central portion of the islets. Freshly isolated islets have little or no central necrosis, as demonstrated by Calcein-AM and propidium iodide viability stains (Fig. 3*A*). The majority of cells were viable and converted Calcein-AM to a green fluorescent product. After 24 h or 48 h of normoxic culture period, larger islets (diameter >150 μm) show increased cell death in the center of islets, surrounded by a mixture of moribund and viable cells (Fig. 3*B* and *C*). Almost all islets cultured for 24 h in normoxia followed by 24 h in hypoxic culture (1% O_2) had a rim of live cells surrounding a core of dead/dying cells (Fig. 3*D*). These combined observations suggest that VEGF mRNA expression in isolated islets in culture is progressively increased, since islets are separated from their vascular supply, and that the presence of hypoxia/anoxia is probably the major factor that modulates the expression of VEGF in isolated islets in culture.

VEGF gene expression in dispersed islet cells cultured on Matrigel matrix. Because the findings with whole islets showed increased VEGF expression even under normoxic conditions, additional experiments were performed to see if hypoxia per se directly mediated the expression of VEGF mRNA. Whole islets were dispersed into single cells or small fragments cultured on Matrigel matrix, which became adherent over the next 24 h. A medium change was performed after an overnight culture period to remove dead and nonadherent cells. After 48 h in normoxic culture, islet cells were allowed to continue for a further 24 h in normoxia or were subjected to hypoxic (1% O_2) culture. Viability studies using Calcein-AM and propidium iodide demonstrated that >98% of the cells under either conditions were viable after this 72-h culture period (Fig. 4*A* and *B*). In addition, >95% of the islet cells on the Matrigel matrix stained positive for insulin. Quantitative analysis of the islet cell pellet after recovery from the Matrigel matrix revealed >91% of the cells stained positive for insulin and 9% stained positive with non–β-cell hormones. Minimal, if any, fibroblast contamination was seen in our islet cell cultures after 72 h, and when present on rare occasions, the fibroblast cells accounted for <1%. Northern blot analysis (15 μg/lane) for expression of VEGF mRNA is shown in Fig. 5. Barely detectable signals of VEGF mRNA were observed in lanes bearing total RNA from islet cells cultured in normoxic culture conditions (Fig. 5*A*). Levels of VEGF mRNA expression in hypoxic culture conditions increased by 3.4-fold of normoxic levels ($P < 0.01$) (Fig. 5*B*). Similarly, GAPDH mRNA

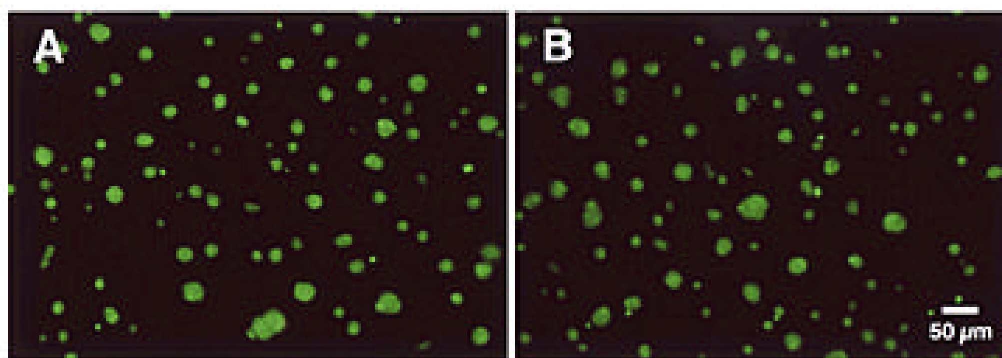

FIG. 4. Assessment of viability of islet cell clusters cultured on Matrigel matrix in normoxic (*A*) or hypoxic (*B*) culture conditions using Calcein-AM and propidium iodide as viability stains. In both culture conditions, all islet cells were observed to be viable, as indicated by the presence of green fluorescence and the absence of red staining with propidium iodide.

expression levels increased by 2.6-fold of normoxic levels ($P < 0.005$) (Fig. 5*C*), whereas insulin mRNA expression decreased to 69% of normal levels ($P < 0.05$) (Fig. 5*D*).

Presence of immunoreactive VEGF in culture medium of hypoxic islet cells on Matrigel matrix. A chemiluminescence ELISA was used to quantify VEGF in the culture media of rat islet cells plated on Matrigel matrix. Supernatants from experiments of Fig. 5 were analyzed for the presence of VEGF protein. As shown in Table 1, VEGF levels in supernatants of islet cells exposed to 24 h of hypoxic conditions were 10–19 pg/ml (average 14.8 pg/ml) compared with that of normoxic islet cells. There were no detectable lev-

FIG. 5. *A*: Expression of VEGF, GAPDH, and insulin mRNA in dispersed islet cells cultured on Matrigel matrix in normoxic or hypoxic culture conditions. Total RNA (15 μg/lane) from four sets of experiments was resolved simultaneously through agarose gel for Northern blot analysis with cDNA for $VEGF_{165}$, GAPDH, and insulin and for 36B4 as internal control. Staining for ethidium bromide (EBr) is shown in the bottom panel of *A*; locations for 28S and 18S are indicated by arrowheads. Bar graphs show quantitation of multiple experiments ($n = 4$) after normalization to the control signal 36B4 for VEGF (*B*), GAPDH (*C*), and insulin (*D*) mRNAs. Results are expressed as relative normalized mRNA expression (percent of control) ± SE, and differences were evaluated by unpaired *t* test.

TABLE 1
Measurement of VEGF by ELISA in supernatants of rat islet cells cultured on Matrigel matrix after exposure to normoxic or hypoxic culture conditions

Experiment	Normoxic supernatants (pg/ml)	Hypoxic supernatants (pg/ml)
1	Below detection	10
2	Below detection	14
3	Below detection	19
4	Below detection	16

Detection limit of the VEGF ELISA assay was 4.5 pg/ml. No VEGF activity was detected in culture dishes coated with Matrigel with culture medium and without cells kept in normoxic or hypoxic culture conditions. $P < 0.001$ assuming below detection = 4.5 pg/ml.

els of VEGF protein in supernatants of islet cells cultured in normoxic culture conditions. In addition, VEGF protein was not detected in control culture dishes coated with Matrigel that contained culture medium but were without cells.

Localization of VEGF protein in whole islets and dispersed islet cells by immunofluorescence. In normal adult rat pancreases, VEGF immunofluorescence was found in most islets and was diffusely distributed in whole islets (Fig. 6*A*). By double-immunostaining for insulin and VEGF, most of the VEGF staining was localized in β-cells (Fig. 6*B*), although some β also stained positive with the VEGF antibody. VEGF immunoreactivity was usually localized in the cytoplasm, especially in the perinuclear area. There was no detectable VEGF immunoreactivity using antibody preabsorbed with its immunogenic peptide (Fig. 6*C*). On sections of freshly isolated rat islets, VEGF immunostaining was observed in only a few β-cells located in the center of large islets (Fig. 7*A*). This unexpected difference in staining pattern observed in islets fixed in whole normal pancreas and in freshly isolated islets may have been a result of the isolation procedure. In contrast, strong VEGF immunostaining was detected in most islets cultured overnight in normoxic culture conditions for 24 h (Fig. 7*B*). In large islets, most islet cells demonstrated intense VEGF immunoreactivity. It is interesting that the cells in the very center of the cross-section were often less stained than those halfway from the center to the periphery. Dispersed islet cells cultured on Matrigel-coated chamber slides in hypoxic culture conditions also had VEGF immunoreactivity (Fig. 8). As demonstrated in Fig. 8*A*, an islet cell cluster cultured on Matrigel in hypoxic conditions showed strong VEGF immunoreactivity distributed throughout the cytoplasm in β-cell (stained positive for insulin) (Fig. 8*B*) and in two adjacent non–β-cells.

DISCUSSION

Revascularization of islet grafts after transplantation is an important process that influences the long-term viability and functional outcome of grafts. The formation of new blood vessels optimizes the provision of nutrients and oxygen to grafts and provides access for systemic delivery of insulin. The development of new capillaries from preexisting vessels is a highly organized process that is in direct response to tissue demand (52) and depends on changes in the balance of angiogenic stimulators and inhibitors (53). Although the underlying mechanisms of vascularization in situ are poorly understood, the sequence of events involves stimulation of preexisting quiescent vessels with subsequent cell-cycle progression, remodeling of cell adhesion and junctions, induction of proteolytic activities, and neutralization of inhibitors (52). Following transplantation of islets as an avascular graft, it is likely that a similar multistep process occurs, with remodeling of extracellular matrix, migration, and proliferation of host endothelial cells leading to capillary sprouting, lumen formation, and functional maturation of blood vessels (12,16,23).

Several groups have reported that exposure of tumor cell lines and primary cells to hypoxic culture conditions dramatically upregulates the expression of VEGF mRNA (26,30,54,55). Our studies confirmed and extended these observations to several tumor cell lines (β-TC3, RAW 264.7, and 1C-21) and to isolated rat islet cells. The intracellular mechanisms through which hypoxia regulates the transcription and expression of VEGF are complex (56–58). More recent studies have shown that hypoxia-associated upregulation in VEGF mRNA results from not only increased tran-

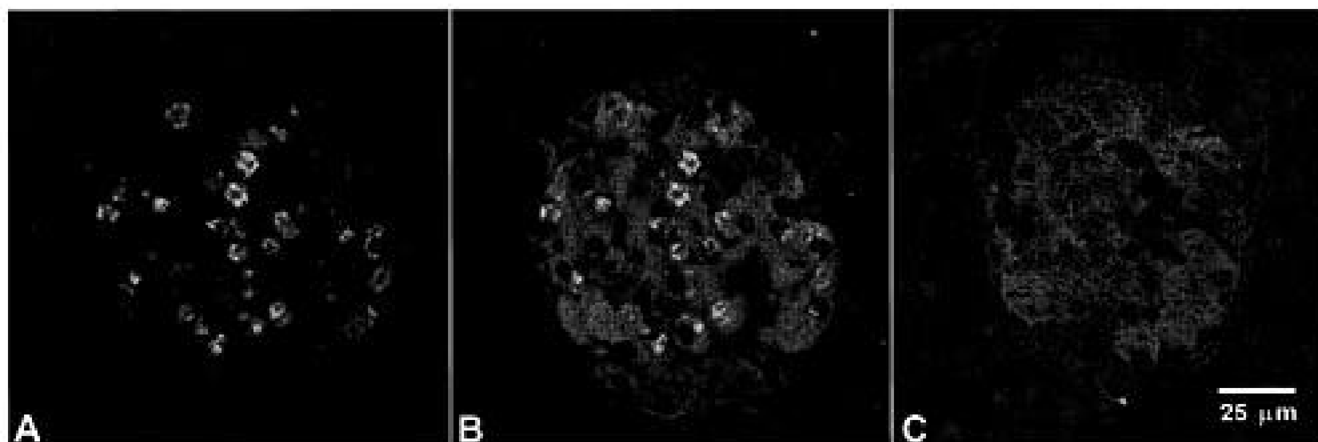

FIG. 6. Immunostaining for VEGF and insulin in islets of Langerhans in normal adult rat pancreas. VEGF immunostaining was diffusely distributed in the whole islet as seen in (*A*) showing VEGF alone and (*B*) the same image overlayed with insulin staining. *C*: Negative control of an adjacent section double-stained with insulin and VEGF antibody preabsorbed with immunogenic peptide; VEGF immunostaining was not detectable.

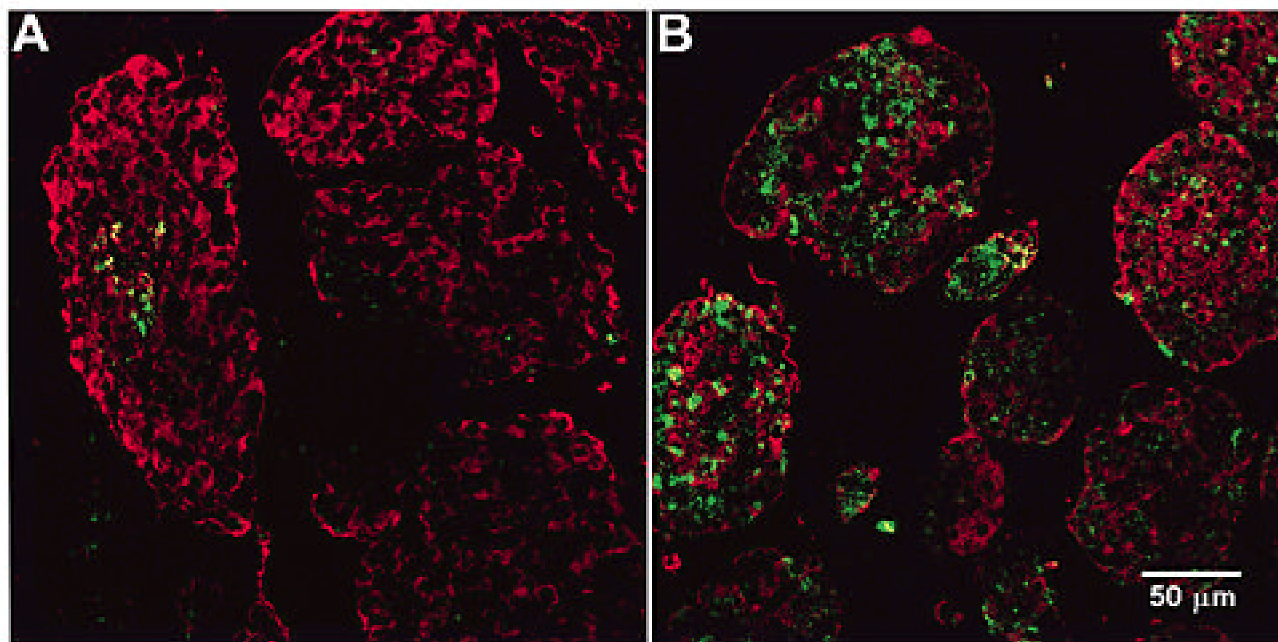

FIG. 7. Double-immunostaining for VEGF (green) and insulin (red) in freshly isolated islets (*A*) and in islets cultured in normoxic conditions for 24 h (*B*). In freshly isolated islets, VEGF immunoreactivity was detected in only a few β-cells located in the center of large islets. In contrast, strong VEGF immunostaining was observed in most of the islets cultured for 24 h in normoxic culture conditions.

scription but also stabilization of VEGF mRNA due to binding with hypoxia-induced proteins (29).

In glioma tumors, in situ hybridization has demonstrated the presence of high levels of VEGF mRNA in the vicinity of ischemic and necrotic areas of the tumors, suggesting that hypoxia may be a potent inducer of VEGF expression (26). Our studies showed that when isolated islets were cultured for 24–48 h in normoxic culture conditions, large islets had central necrosis, which was even more severe after exposure to hypoxic culture conditions (Fig. 3). These conditions were associated with the increased upregulation of VEGF mRNA gene expression. In our studies, after 48-h exposure to normoxic culture, islets had a threefold increase in VEGF mRNA expression compared with freshly isolated islets. Islets subjected to hypoxia showed a similar 3.8-fold increase in VEGF mRNA when compared with freshly isolated islets. Although hypoxia might have been expected to give even more VEGF expression, death of an increased portion of islet core could have resulted in fewer cells capable of producing VEGF. With immunochemistry, strong VEGF immunostaining was seen in areas of islet cells adjacent to the central core to the periphery, as islet cells in this region are probably subjected to physiological hypoxia, which may stimulate the hypoxic-induced expression of VEGF mRNA (Fig. 7*B*). On the other hand, β-cells in the core of the islets may be subjected to severe hypoxia, which may threaten the viability of these cells and impair their VEGF protein synthesis, storage, and secretion. In culture, islets tend to form clumps and small islets trapped in the middle of such clumps tend to be subjected to severe hypoxia. It is likely that on such occasions even islets with diameter <100 µm may show positive immunostaining for VEGF, as shown in a small islet in Fig. 7*B*. Taken together, these results indicate that when islets are placed in culture even in normoxic conditions, hypoxia in the central portion of islets, especially large islets, is the major stimulus for VEGF gene expression. Similar results were reported by Gorden et al. (55). Although hypoxia seems to be the likely stimulus for the increased expression of VEGF in islets, there is a difficulty interpreting the complex events occurring in the different zones of whole islets in vitro.

Therefore, we extended these studies to dispersed islet cells that could be subjected to more uniform oxygen pressures. In both normoxic and hypoxic culture conditions, >98% of these islet cells were viable. Here both increased VEGF gene expression and increased levels of VEGF protein in the culture media, presumably from increased constitutive secretion, were seen and were clearly due to hypoxia. In addition, these results confirmed the findings of others that increased VEGF mRNA expression in cell lines and tissues correlated with amounts of secreted VEGF protein (58–60). The analysis of the islet cell pellets after recovery from Matrigel matrix indicated that >90% of the cells were β-cells. With immunofluorescence, strong VEGF staining could be demonstrated in β-cells and in non–β-cells (Fig. 8), an observation also reported by other investigators (61), although others have found VEGF only in β-cells (62). These findings suggest that all islet cells may act as a source of VEGF. Fibroblasts are known to produce VEGF, but because so few were found during this short culture period, their contribution to the secreted VEGF was probably negligible. In the intact pancreas, VEGF immunostaining was found in islets, suggesting that continued low-level secretion of VEGF may maintain the fenestrated rich microvasculature of islets. It was interesting that very little VEGF immunostaining was detected in exocrine pancreas.

Both VEGF receptors are expressed in adult and fetal pancreatic islets (55,63). At least some of these receptors must be on endothelial cells contained in islets, but the finding

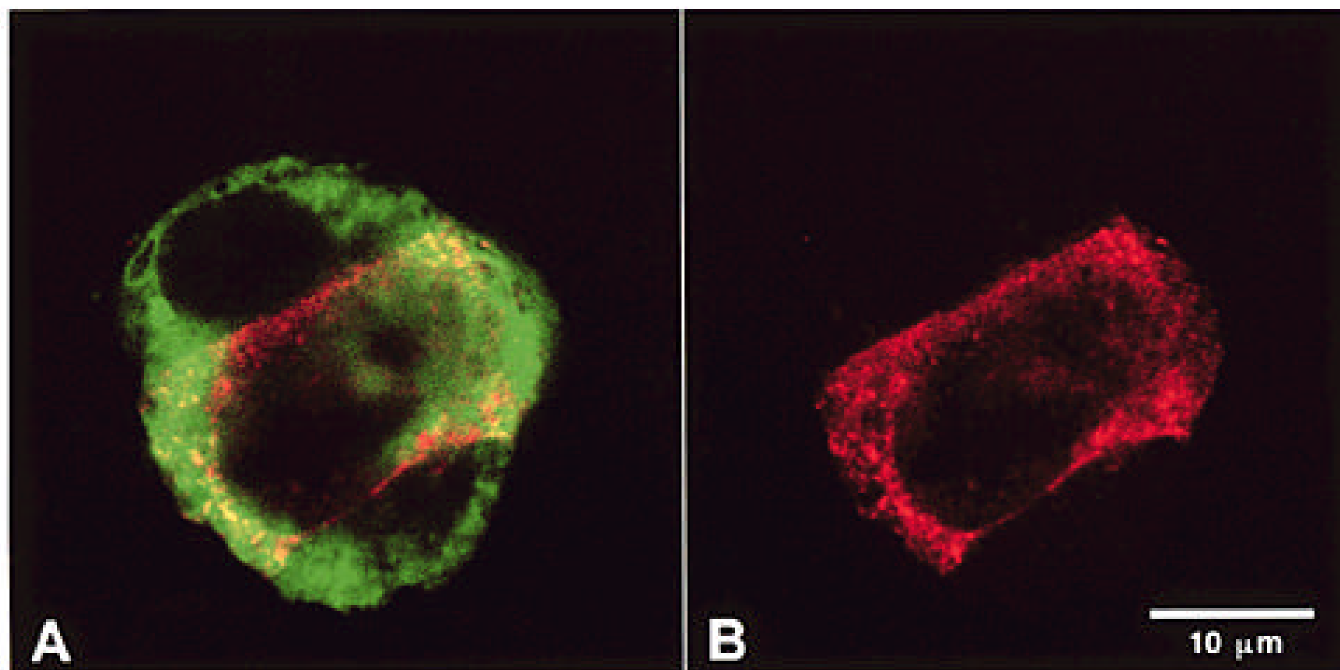

FIG. 8. Double-immunostaining for VEGF (green) and insulin (red) in dispersed islet cell cluster cultured on Matrigel matrix after exposure to a final 24-h hypoxic culture period (1% O_2), as described in METHODS. Strong VEGF immunoreactivity was observed in all three cells of the islet cell cluster (*A*) with the central cell staining positive with insulin antibody (*B*) surrounded by two non–β-cells.

that β-cell replication could be stimulated by VEGF suggests that islet cells themselves may contain receptors (38). As part of VEGF's role in angiogenesis in vivo (12,16,23), its receptor expression can be upregulated in areas of ischemia (64). The revascularization of transplanted islets, which has been well characterized as occurring over a period of 3–14 days (10,11,65,66) may depend on increased secretion of VEGF by ischemic islets and upregulation of VEGF receptors in the adjacent host tissue.

In conclusion, we demonstrated that VEGF mRNA expression and secretion are increased in islet cells exposed to hypoxic conditions. Because similar hypoxia is likely to exist during the first few days of transplantation, it seems likely that VEGF is a major stimulus initiating the growth of new blood vessels into the graft and the development of their fenestrations. Moreover, the presence of VEGF in normal islets suggests it may be important for maintenance of normal islet vasculature.

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REFERENCES

1. Lacy PE: Status of islet cell transplantation. *Diabetes Rev* 1:76–92, 1993
2. Stagner JI, Samols E: The induction of capillary bed development by endothelial cell growth factor before islet transplantation may prevent islet ischemia. *Transplant Proc* 22:824–828, 1990
3. Samols E, Stagner JI, Nakagawa A: Intra-islet and islet-acinar portal systems: relevance to transplantation. *Diabetes Nutr Metab* 5 (Suppl. 1):3–7, 1992
4. Davalli AM, Scaglia L, Zangen DH, Hollister J, Bonner-Weir S, Weir GC: Vulnerability of islets in the immediate post transplantation period. *Diabetes* 45:1161–1167, 1996
5. Lifson N, Lassa CV, Dixit PK: Relation between blood flow and morphology in islet organ of rat pancreas. *Am J Physiol* 249:E43–E48, 1985
6. Bonner-Weir S, Orci L: New perspectives on the microvasculature of islets of Langerhans in the rat. *Diabetes* 31:883–889, 1992
7. Weir GC, Bonner-Weir S: The islets of Langerhans: the puzzle of intraislet interactions and their relevance to diabetes (Perspective). *J Clin Invest* 85:983–987, 1990
8. Stagner JI, Mokshgundam S, Samols E: Hormone secretion from transplanted islets is dependent upon changes in islet revascularization and islet architecture. *Transplant Proc* 27:3251, 1995
9. Dionne KE, Colton CK, Yarmush ML: Effect of hypoxia and insulin secretion by isolated rat and canine islets of Langerhans. *Diabetes* 42:12–21, 1993
10. Menger MD, Jager S, Walter P, Feifel G, Hammersen F, Messmer K: Angiogenesis and hemodynamics of microvasculature of transplanted islets of Langerhans. *Diabetes* 38 (Suppl. 1):199–201, 1989
11. Vajkoczy P, Olofsson AM, Lehr HA, Leiderer R, Hammersen F, Arfors KE, Menger MD: Histogenesis and ultrastructure of pancreatic islet graft microvasculature: evidence for graft revascularization by endothelial cells of host origin. *Am J Pathol* 146:1397–1405, 1995
12. Dvorak HF, Lawrence FB, Detmer M, Dvorak AM: Vascular permeability factor/endothelial growth factor, microvascular hyperpermeability, and angiogenesis: review. *Am J Pathol* 146:1029–1039, 1995
13. Brier G, Risau W: The role of vascular endothelial growth factor in blood vessel formation. *Trends Cell Biol* 6:454–466, 1996
14. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW: The vascular endothelial growth family: identification of a fourth molecular species and characterization of alternative splicing of RNA. *Mol Endocrinol* 5:1806–1814, 1991
15. Klagsbrun M, D'Amore PA: Regulators of angiogenesis. *Annu Rev Physiol* 53:217–239, 1991

16. Folkman J, Shing Y: Angiogenesis. *J Biol Chem* 267:10931–10934, 1992
17. Folkman J, Klagsbrun M: Angiogeneic factors. *Science* 235:442–443, 1987
18. Ferrara N, Henzel WJ: Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun* 161:851–858, 1989
19. Plouet J, Schilling J, Gospodarowicz D: Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. *EMBO J* 8:3801–3806, 1989
20. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J: Tumor vascular permeability factor stimulates endothelial growth and angiogenesis. *J Clin Invest* 84:1470–1478, 1989
21. Conn G, Soderman DD, Schaeffer M, Wile M, Hatcher VB, Thomas KA: Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line. *Proc Natl Acad Sci U S A* 87:1323–1327, 1990
22. Thieme H, Aiello LP, Takagi H, Ferrara N, King GL: Comparative analysis of vascular endothelial growth factor receptors on retinal and aortic vascular endothelial cells. *Diabetes* 44:98–103, 1995
23. Ferrara N, Houch KA, Jakeman LB, Leung DW: Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr Rev* 13:18–32, 1992
24. Senger DR, Van de Water L, Brown LF, Nagy JA, Yeo KT, Berse B, Jackman RW, Dvorak AM, Dvorak HF: Vascular permeability factor (VPF, VEGF) in tumor biology. *Cancer Metastasis Rev* 12:303–324, 1993
25. Aiello LP, Bursell S-E, Clermont A, Duh E, Ishii H, Takagi C, Mori F, Ciulla TA, Ways K, Jirousek M, Smith LEH, King GL: Vascular endothelial growth factor–induced retinal permeability is mediated by protein kinase C in vivo and suppressed by an orally effective β-isoform–selective inhibitor. *Diabetes* 46:1473–1480, 1997
26. Shweiki D, Itin A, Soffer D, Keshet E: Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359:843–845, 1992
27. Goldberg MA, Schneider T: Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. *J Biol Chem* 269:4355–4359, 1994
28. Levy AP, Nina SL, Wegner S, Goldberg MA: Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem* 270:13333–13340, 1995
29. Levy AP, Levy NS, Goldberg MA: Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. *J Biol Chem* 34:19761–19766, 1996
30. Aiello LP, Northrup JM, Keyt BA, Takagi H, Iwamoto MA: Hypoxic regulation of vascular endothelial growth factor in retinal cells. *Arch Ophthalmol* 113:1538–1544, 1995
31. Senger DR, Perruzzi CA, Feder J, Dvorak HF: A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. *Cancer Res* 46:5629–5632, 1986
32. Dvorak HF, Sioussat TM, Brown LF, Berse B, Nagy JA, Sotrel A, Manseau EJ, Van De Water L, Senger DR: Distribution of vascular permeability factor (vascular endothelial growth factor) in tumors: concentration in tumor blood vessels. *J Exp Med* 174:1275–1278, 1991
33. Tisher E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA: The human gene for vascular endothelial growth factor. *J Biol Chem* 266:11947–11954, 1991
34. Shima DT, Kuroki M, Deutsch U, Ng Y-S, Adamis AP, D'Amore PA: The mouse gene for vascular endothelial growth factor: genomic structure, definition of the transcriptional unit, and characterization of transcriptional and post-transcriptional regulatory sequences. *J Biol Chem* 271:3877–3883, 1996
35. Quinn TP, Peters KG, De Vries C, Ferrara N, Williams LT: Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. *Proc Natl Acad Sci U S A* 90:7533–7537, 1993
36. Millauer B, Witzigmann-Voos S, Schnurch H, Martinez R, Moller NP, Risau W, Ullrich A: High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. *Cell* 72:835–846, 1993
37. de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT: The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* 255:989–991, 1992
38. Oberg-Welsh C, Sandler S, Andersson A, Welsh M: Effects of vascular endothelial growth factor on pancreatic duct cell replication and the insulin production of fetal islet-like cell clusters in vitro. *Mol Cell Endocrinol* 126:125–132, 1997
39. Efrat S, Linde S, Kofod H, Spector D, Delannoy M, Grant S, Hanahan D, Baekkeskov S: Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. *Proc Natl Acad Sci U S A* 85:9037–9041, 1988
40. Ralph P, Nakoinz I: Antibody-dependent killing of erythrocyte and tumor targets by macrophage-related cell lines: enhancement by PPD and LPS. *J Immunol* 119:950–954, 1977
41. Mauel J, Defendi V: Infection and transformation of mouse peritoneal macrophages by simian virus 40. *J Exp Med* 134:335–350, 1971
42. Sutton R, Peters M, McShane P, Gray DWR, Morris PJ: Isolation of rat pancreatic islets by ductal injection of collagenase. *Transplantation* 42:689–691, 1986
43. Takagi H, King GL, Aiello LP: Identification and characterization of vascular endothelial growth factor receptor (flt) in bovine retinal pericytes. *Diabetes* 45:1016–1023, 1996
44. Chomczynski P: A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* 15:532–537, 1993
45. Liang P, Pardee AB: Differential display of eukaryotic messenger RNA by means of a polymerase chain reaction. *Science* 257:967–971, 1992
46. Masiakowski P, Breathnach R, Bloch J, Gannon F, Krust A, Chambon P: Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell lines. *Nucleic Acids Res* 10:7895–7903, 1982
47. Zangen DH, Bonner-Weir S, Lee CH, Latimer JB, Miller CP, Habener JF, Weir GC: Reduced insulin, GLUT 2, and IDX-1 in β-cells after partial pancreatectomy. *Diabetes* 46:258–264, 1997
48. Lorenzo A, Razzaboni B, Weir GC, Yanker BA: Pancreatic islet cell toxicity of amylin associated with type-2 diabetes mellitus. *Nature* 368:756–760, 1994
49. Scopsi L, Wang B-L, Larsson L-I: Non-specific immunocytochemical reactions with certain neurohormonal peptides and basic peptide sequences. *J Histochem Cytochem* 43:1469–1475, 1986
50. Kletzien RF, Harris PK, Foellmi LA: Glucose-6-phosphate dehydrogenase: a "housekeeping" enzyme subject to tissue-specific regulation by 7–21 hormones, nutrients, and oxidant stress. *FASEB J* 8:174–181, 1994
51. Graven KK, Troxler RF, Kornfeld H, Panchenko MV, Farber HW: Regulation of endothelial cell glyceraldehyde-3-phosphate dehydrogenase expression by hypoxia. *J Biol Chem* 269:24446–24453, 1994
52. Risau W: Mechanisms of angiogenesis. *Nature* 386:671–674, 1997
53. Yoshida A, Anand-Apte B, Zetter BR: Differential endothelial and proliferation to basic fibroblast growth factor and vascular endothelial growth factor. *Growth Factors* 13:57–64, 1996
54. Minchenko A, Bauer T, Salceda S, Caro J: Hypoxic stimulation of vascular endothelial growth factor expression in vitro and in vivo. *Lab Invest* 71:374–379, 1994
55. Gorden DL, Mandriota SJ, Montesano R, Orci L, Pepper MS: Vascular endothelial growth factor is increased in devascularized rat islets of Langerhans in vitro. *Transplantation* 63:436–443, 1997
56. Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL: Activation of vascular endothelial growth factor gene transcription by hypoxia inducible factor 1. *Mol Cell Biol* 16:4604–4613, 1996
57. Mukhopadhyay D, Tsiokas L, Zhou X-M, Foster D, Brugge JS, Sukhatme VP: Hypoxic induction of human vascular endothelial growth factor expression through c-Src activation. *Nature* 375:577–581, 1995
58. Takagi H, King GL, Robinson GS, Ferrara N, Aiello LP: Adenosine mediates hypoxic induction of vascular endothelial growth factor in retinal pericytes and endothelial cells. *Invest Ophthalmol Vis Sci* 37:2165–2176, 1996
59. Jian-Wei G, Adair TH: Hypoxia-induced expression of VEGF is reversible in myocardial vascular smooth muscle cells. *Am J Physiol* 273:H628–H633, 1997
60. Warren RS, Yuan H, Matli MR, Gillet NA, Ferrara N: Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. *J Clin Invest* 95:1789–1797, 1995
61. Rooman I, Schuit F, Bouwens L: Effect of vascular endothelial growth factor on growth and differentiation of pancreatic ductal epithelium. *Lab Invest* 76:225–232, 1997
62. Kuroda M, Oka T, Oka Y, Yamochi T, Ohtsubo K, Mori S, Watanabe T, Machinami R, Ohnishi S: Co-localization of vascular endothelial growth factor (vascular permeability factor) and insulin in pancreatic islet cells. *J Clin Endocrinol Metab* 80:3196–3200, 1995
63. Christofori G, Naik P, Hanahan D: Vascular endothelial growth factor and its receptors, flt-1 and flk-1, are expressed in normal pancreatic islets and throughout islet cell tumorigenesis. *Mol Endocrinol* 9:1760–1770, 1995
64. Banai S, Shweiki D, Pinson A, Chandra M, Lazarovici G, Keshet E: Upregulation of vascular endothelial growth factor expression induced by myocardial ischaemia: implications for coronary angiogenesis. *Cardiovasc Res* 28:1176–1179, 1994
65. Menger MD, Vajkoczy P, Leiderer R, Jager S, Messmer K: Influence of hyperglycemia on microvascular blood perfusion of pancreatic islet isografts. *J Clin Invest* 90:1361–1369, 1992
66. Mendola JF, Conget I, Manzanares MJ, Corominola H, Vinas O, Barcelo J, Gomis R: Follow-up study of the revascularization process of purified rat islet β-cell grafts. *Cell Transplant* 6:603–612, 1997

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