

Vascular Endothelial Growth Factor Gene Delivery for Revascularization in Transplanted Human Islets

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Purpose. Islet transplantation is limited by islet graft failure because of poor revascularization, host immune rejection, and nonspecific inflammatory response. Human vascular endothelial growth factor (hVEGF) gene delivery is likely to promote islet revascularization and survival.

Methods. We evaluated gene expression from a bicistronic plasmid encoding hVEGF and enhanced green fluorescent protein (EGFP) (pCMS-EGFP-hVEGF). Glucose responsiveness of islets was evaluated both *in vitro* and *in vivo*, and revascularization in islet graft was evaluated by immunohistochemistry.

Results. After transfection, hVEGF and EGFP expression levels were comparable with original monocistronic plasmids in Jurkat cells but higher and prolonged hVEGF expression in islets transfected with the bicistronic plasmid was observed, possibly as the result of differences in promoter strength and hypoxia response. The 3:1 w/w complexes showed little toxicity to islets at a dose of 5 µg DNA per 2000 islets. On glucose challenge, insulin release from transfected islets as well as secretion from islets after transplantation under the mouse kidney capsules in response to glucose stimulation, increased with time. Immunohistochemical staining of transplanted islets using mouse anti-human insulin, mouse anti-human von Willebrand factor, and rat anti-mouse CD31 antibodies suggests that islets are functional and there is new blood vessel formation.

Conclusions. These findings suggest that transient hVEGF gene expression by the islets may promote islet revascularization and prolong islet survival after transplantation.

KEY WORDS: human islets; bicistronic vector; transfection; cationic liposomes; real-time RT-PCR; immunohistochemistry.

INTRODUCTION

Islet transplantation offers tremendous hope for the treatment of type I diabetes, and there have been reported cases of successful treatment of diabetes using this approach (1). Wide clinical application of this approach, however, is currently limited by host immune rejection, primary nonfunction, and islet cell death after transplantation (2). Apoptotic

death of transplanted islets is further enhanced by hypoxia experienced during initial few days after transplantation (3). To achieve successful islet transplantation, primary nonfunction has to be eliminated. Our previous work has resulted in improved recovery and viability of human islets by optimization of islet culture conditions (4). We have also developed a nonobese diabetic-severe combined immunodeficient (NOD-SCID) mouse model, which allowed more accurate assessment of islet *in vivo* function (4,5).

Islet destruction after transplantation can be prevented by genetically engineering β cells to 1) promote revascularization and 2) intercept apoptotic and inflammatory pathways by expressing desired proteins in the vicinity of the islets (6–8). Although expression of a single therapeutic gene may provide significant improvements in islet function in either aspect (9), it is increasingly being realized that simultaneous expression of multiple therapeutic genes would be a more effective strategy in this clinical setting (10) because islets experience multipronged problems in the initial few days after transplantation. However, as a prelude to the use of two therapeutic genes, it is essential to confirm noninterference of gene expression from bicistronic vectors, absence of any adverse effect on islet function, and acceptable levels of toxicity. Furthermore, one of the two genes should initially be a reporter gene for the optimization of gene transfer process and for easy assessment of transfection efficiency (11). Following these lines, Kapturczak *et al.* (12) have recently developed a bicistronic adeno associated virus vector driven by cytomegalovirus (CMV) enhancer-chicken β-actin hybrid promoter and an internal ribosome entry site element to allow the simultaneous translation of rhodamine and green fluorescent proteins (RFP and GFP) in human and murine pancreatic islets (12).

Because isolated islets can revascularize within approx. 10–14 days after transplantation, we recently investigated the possible use of plasmid DNA based gene delivery approaches for transient expression of human vascular endothelial growth factor (hVEGF) to improve neovascularization (13). VEGF is an endothelial specific growth factor and thus has less propensity to cause unwanted side effects like some other growth factors, e.g., transforming growth factor-β (14). Furthermore, VEGF is also known to enhance vascular permeability *in vivo* (15), which might be valuable to initial islet survival and angiogenesis. More recently, Linn *et al.* (16) have demonstrated that the addition of recombinant VEGF to the culture medium increases proliferation of islet endothelial cells and causes substantial cord formation in a fibrin gel model.

Transfection efficiency and cytotoxicity are dependent on the type, surface charge, and dose of gene carriers used for a given cell type. Commercially available transfection reagents, such as LipofectAMINE® and Superfect®, are known to be toxic to various cell types when used at higher +/- charge ratios and dose. Therefore, we have been working toward the development of nontoxic water-soluble lipopolymers for gene delivery (17,18). Although these lipopolymers are effective in transfecting various cell types, they were not effective in transfecting human islets, probably because of the fact that islets are a cluster of approx. 1000 nondividing cells. Therefore, we decided to use LipofectAMINE® as model cationic liposomes for gene delivery to human islets. To mini-

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mize its toxicity, we formulated it at 3/1 (+/-) charge ratio and transfected islets at a dose of 5 µg DNA/ 2000 islets. In parallel, we are working on the design of suitable novel lipopolymeric gene carriers by incorporating fusogenic molecules, which may be more effective for gene delivery to human islets with little or no toxicity.

In the present study, we constructed a bicistronic vector encoding EGFP and hVEGF and compared gene expression and cytotoxicity with the original monocistronic plasmids. In addition, we also evaluated islet function in comparison with LipofectAMINE®, naked plasmid DNA and nontransfected controls. We determined *in vivo* islet function by measuring human insulin concentrations in the plasma in sets of four NOD-SCID mice transplanted with transfected or control islets under the kidney capsule over a period of 10 days. We also determined *in vivo* islet function and angiogenesis by immunohistochemical staining of transplanted human islets for human insulin, human von Willebrand factor and mouse CD 31.

MATERIALS AND METHODS

Materials

Glycerol, terrific broth, LB agar, ampicillin sodium, agarose, ethidium bromide, DNase, HEPES, type I collagen, isopropanol, ethanol, and chloroform were purchased from Sigma Chemical, Ltd (St. Louis, MO, USA). Dimethyl sulfoxide and glucose were purchased from Aldrich (Milwaukee, WI, USA). Bacterial strain DH5α, *EcoRI*, *HindIII*, *AvaII*, alkaline phosphatase, and T4 DNA ligase were purchased from Promega (Madison, WI, USA). Fetal bovine serum (FBS) and L-glutamine were purchased from Mediatech Cellgro® (Herndon, VA, USA), Penicillin/streptomycin, gentamycin, phosphate-buffered saline (PBS), 0.25% (w/v) trypsin-EDTA, and RPMI 1640 were purchased from GIBCO-BRL (Gaithersburg, MD, USA). QIAgen Endofree purification and Qiaquick gel extraction kits were purchased from QIAGEN (Boulder, CO, USA). ITS media was purchased from Collaborative Biomedical Products (Bedford, MA, USA), which contains the following components: insulin (6.25 µg/ml), transferrin (6.25 µg/ml), selenious acid (6.25 ng/ml), linoleic acid (5.35 µg/ml), and albumin (1.25 mg/ml). Human insulin ELISA kits were purchased from Alpco Diagnostics (Windham, NH, USA). Recombinant Human VEGF₁₆₅ and hVEGF ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA), respectively. LipofectAMINE® and Concert Rapid Plasmid Miniprep Purification Kit were purchased from Invitrogen Corporation (Carlsbad, CA, USA). RNALater and RNA STAT 60 were purchased from Ambion RNA Diagnostics (Austin, TX, USA) and Tel Test Inc. (Friendswood, TX, USA), respectively. SYBR Green-I dye universal PCR master mix, Multi Scribe reverse transcriptase reagents and random hexamers were obtained from Applied Biosystems (Foster City, CA, USA). Paraplast and Fisherbrand Biopsy 2 cassettes for immunohistochemistry were procured from Fisher Scientific (Pittsburgh, PA, USA) and M.O.M. immunodetection kit, avidin/biotin blocking kit and 3,3'-diaminobenzidine substrate kit for peroxidase were purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Mouse anti-human insulin (NCL-insulin) monoclonal antibody was purchased from Novacastra Laboratories, Ltd.

(New Castle, United Kingdom). Rat anti-mouse CD31 (PE-CAM-1) and mouse anti-human von Willebrand factor (vWF) monoclonal antibodies were purchased from DakoCytomation (Carpinteria, CA, USA). Medium-199 1X Earle's Salts was purchased from Irvine Scientific (Santa Ana, CA, USA). Sure-2 supercompetent cells were purchased from Stratagene (La Jolla, CA, USA) and University of Wisconsin (UW) Solution from Viaspan Dupont Pharma (Wilmington, DE, USA).

METHODS

Construction of Bicistronic pCMS-EGFP-hVEGF

pCMS-EGFP-hVEGF was created by cloning hVEGF gene from pCAGGS-hVEGF plasmid into the pCMS-EGFP vector's multiple cloning site using *EcoRI* restriction digestion and sticky end ligation. Briefly, the hVEGF gene fragment was excised from pCAGGS-hVEGF vector using *EcoRI* restriction digestion, separated by gel fractionation, and then purified from the gel using Qiaquick gel extraction kit as per the vendor protocol (Qiagen, Boulder, CO, USA). pCMS-EGFP was simultaneously linearized using a single cutting site of *EcoRI* enzyme and dephosphorylated using alkaline phosphatase. The gene fragment was ligated with the linearized pCMS-EGFP vector at gene to vector molar ratios of 1:1, 1:3, and 1:10 using T4 DNA ligase for 1 h at 37°C. The ligated mixture was used to transform Sure-2 Supercompetent cells (Stratagene, La Jolla, CA, USA). Transformed cells were inoculated on Ampicillin-agar plates at different dilutions (1:1, 1:5, and 1:10) and incubated overnight at 37°C. Single clones were isolated the next day and grown in 10 ml of terrific broth media overnight at 37°C and 125 g. Plasmids were purified from these preparations using Concert Rapid Plasmid Miniprep Purification Kit (Invitrogen Corporation, Carlsbad, CA, USA) and evaluated by double restriction digestion using *HindIII* and *AvaII* enzymes, for single-copy gene insertion in the right orientation. Two of the positive clones were sequenced using ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA, USA), and the one matching the overlapping gene and vector sequence was selected for further studies. The selected *E. coli* clone was grown on large scale in terrific broth media and plasmid purified using Qiagen Endofree Maxi prep kit. The purified plasmid was analyzed by absorbance measurement using UV Spectrophotometer at 260/280 nm and 1.5% agarose gel electrophoresis for purity, integrity and concentration. The ratios of absorbance at 260 nm to 280 nm of the plasmid preparations were in the range of 1.7 to 1.8.

Pancreas Gland Procurement, Islet Isolation, and Purification

Pancreata were obtained from heart-beating cadaveric multiorgan donors with appropriate consent through the efforts of the following agencies: IBS International Bioresearch Center (Tucson, AZ, USA), Tennessee Donor Services, the Mid-South Transplant Foundation, Life Resources Regional Donor Center, and the United Network for Organ Sharing. The pancreata were perfused *in situ* by aortic flushing with University of Wisconsin (UW; Viaspan Dupont Pharma) solution; pancreata were then placed in a container of cold UW

solution and packaged in wet ice for shipping to our Islet Processing Laboratory.

On arrival at our facility, the pancreata were passed into an isolator module (C.B.C. Ltd., Madison, WI, USA), described previously (4). The organ was trimmed and weighed to determine the appropriate size digestion chamber and collagenase volume to use for distension. Collagenase solution was prepared in Hank's balanced salt solution (Mediatech, Inc., Herndon, VA, USA) supplemented with 0.2 mg/ml DNase, 20 mg/dl calcium chloride, and HEPES. The pH was adjusted to 7.7–7.9. Once dissolved, the collagenase was sterile filtered, warmed to 37°C and passed into the isolator module; then, the pancreas was distended intraductally. The chamber was closed and the heating circuit started. The digestion mix was brought up to 35–37°C and maintained at this temperature until tissue collection began, at which time the heating circuit was disabled. Digested tissue was collected into cold islet dilution solution supplemented with 20% human serum albumin. The tissue digest was centrifuged at 400g at 4°C for 5 min.

Islet purification was performed on the COBE 2991 Cell Processor (COBE BCT, Lakewood, CO, USA) using Opti-Prep (Nycomed Pharma AS, Oslo, Norway) and a discontinuous gradient system. After purification, islet tissue was rinsed with islet dilution solution and resuspended for counting in CMRL containing 1% ITS, 1% L-glutamine, 1% penicillin/streptomycin, 16.8 µM/L ZnSO₄ buffered with HEPES to pH of 7.4.

Assessment of Islet Viability and Purity

Dithiocarbazon staining and static incubation were used to assess islet viability. Immediately after isolation, purity was assessed by dithiocarbazon staining, and aliquots were assessed for number and relative size of islets. Counts were normalized to a 150-µm diameter and expressed as islet equivalents (I.E.). *In vitro* viability was determined by static incubation. Aliquots of 200 islet equivalents were sequentially incubated with low glucose (60 mg/dl), high glucose (300 mg/dl), and high glucose plus theophylline (1.8 mg/ml) and again low glucose levels for 1 h each at 37°C in 5% CO₂ incubator. Supernatants were collected and analyzed for insulin release by ELISA (ALPCO Diagnostics, Windham, NH, USA).

Culturing of Human Islets and Jurkat Cells

Aliquots from human islet isolations were cultured in a serum-free media containing 1% ITS, 1% L-glutamine, 1% albumin, and 16.8 µM/L zinc sulfate at 37°C in a 5% CO₂ incubator with media change at weekly intervals. Because islets are nonadherent, nondividing cell clusters, no splitting was needed during their culture. The murine Jurkat T-cell lines were prepared in culture from frozen glycerol stocks by washing them in PBS followed by culturing in 50 ml of RPMI 1640 medium supplemented with 1% L-glutamine, 1% penicillin-streptomycin solution, 1% HEPES buffer, and 10% FBS in 250-cm² flasks at 37°C in a 5% CO₂ incubator with media change every 3 to 4 days and weekly cell splitting.

Transfection and Cytotoxicity of Jurkat Cells

pCMS-EGFP-hVEGF, pCMS-EGFP, and pCAGGS-hVEGF were used for transfection of Jurkat cells at a dose 5

µg DNA equivalent of LipofectAMINE®/pDNA (3:1, w/w) complexes per 2 million cells. The complexes were prepared at 0.1 µg/µl concentration in 5% dextrose medium and incubated at room temperature for 45 min before dilution with serum-free medium for transfection. Jurkat cells were incubated for 6 h with these complexes before addition of 1.7 ml of serum containing media and further incubation at 37°C in 5% CO₂ incubator. Triplicate samples from each set were collected 72 h post-transfection and analyzed for hVEGF mRNA production by real-time reverse-transcription polymerase chain reaction (RT-PCR).

To determine EGFP expression and cytotoxicity, Jurkat cells were analyzed by flow cytometry 36–48 h post-transfection after washing with PBS. Incubation with the membrane impermeable, nuclear staining dye propidium iodide was used to assess % of dead cells as a measure of cytotoxicity. Presence of green fluorescence was indicative of positive transfection whereas red fluorescence indicated dead cells.

Transfection and Viability of Human Islets

Human pancreatic islets were transfected using bicistronic (pCMS-EGFP-hVEGF) as well as both monocistronic plasmids (pCMS-EGFP and pCAGGS-hVEGF) while using nontransfected islets as controls. LipofectAMINE®/pDNA complexes were prepared at 3:1 w/w ratio and used at 2.5 µg pDNA equivalent dose per 1000 islets. After 12 h of incubation, 1.7 ml of fresh media was added and the islets were incubated in 24-well plates at 37°C in 5% CO₂ incubator. Triplicate samples from each group were withdrawn at days 1, 5, and 10, and supernatants were isolated by centrifugation at 1500 rpm for 10 min at room temperature and analyzed for hVEGF concentration by ELISA as per vendor protocol (R & D Systems, Minneapolis, MN, USA).

Viability of human islets after transfection was assessed using 7-amino actinomycin D (7-AAD) reagent to get the % dead cell count by flow cytometry. Islets were washed with PBS and mechanically dispersed to individual cells using 2.5% Trypsin EDTA solution, followed by the addition of 10% FBS for enzyme inactivation. They were washed again with PBS and resuspended in 1× binding buffer (BD Biosciences) at a concentration of 1 × 10⁶ cells per mL. To 100 µl of the resuspended cells was added 5 µl of 7-AAD, incubated for 15 min at room temperature in the dark and analyzed by flow cytometry after addition of 400 µl more of 1× binding buffer.

Quantitative Real-Time RT-PCR for the Evaluation of hVEGF mRNA Expression

Expression of hVEGF in Jurkat cells was assessed at mRNA level by real time RT-PCR. Cells were pelleted 72 h post-transfection by centrifugation at 1500 rpm for 10 min, dispersed in 1 ml of RNALater (Ambion RNA Diagnostics, Austin, TX, USA), and stored at –80°C until further use. Total mRNA was isolated from cryopreserved cells by a guanidine isothiocyanate method using RNA STAT 60 reagent as per the vendor protocol (Ambion RNA Diagnostics). RNA concentration was measured by UV spectrophotometry using GeneQuant pro RNA/DNA Calculator (BioChrom Ltd, UK). One microgram of extracted RNA was converted to cDNA using MultiScribe Reverse Transcriptase Reagent and

random hexamers (Applied Biosystems, Inc., Branchburg, NJ, USA) by incubation at 25°C for 10 min, followed by reverse transcription at 48°C for 30 min and enzyme inactivation at 95°C for 5 min. One hundred nanograms of the extracted cDNA was amplified by real-time PCR using SYBR Green-I dye universal master mix on ABI Prism 7700 Sequence Detection System (Applied Biosystems, Inc., Foster City, CA, USA). The forward and reverse primers used for PCR amplification were derived from the vector and gene component of the plasmid, respectively. Vector-specific forward primer had the sequence 5'-CCT TGG TGA GGT TTG ATC CG-3' for pCAGGS-hVEGF and 5'-TCC ACT TTG CCT TTC TCT CC-3' for pCMS-EGFP-hVEGF; whereas the reverse primers used for both mono- and bicistronic plasmids was hVEGF gene-specific with the following sequence: 5'-CAA GGC CCA CAG GGA TTT T-3' (Gene Bank accession # for hVEGF:AY047581). The PCR conditions included denaturation at 95°C for 10 min followed by 40 cycles of amplification by sequential denaturation at 95°C for 15 s and primer annealing as well as strand extension at 60°C for 1 min. To confirm amplification specificity, the PCR products were subjected to a melting-curve analysis and agarose gel electrophoresis. The expressed hVEGF mRNA level was quantified using standard curve and normalized to the total amount of cDNA used. Threshold cycle number was compared amongst monocistronic (pCAGGS-hVEGF) and bicistronic (pCMS-EGFP-hVEGF) plasmids with same parameters, using non specific plasmid (pCMS-EGFP) and non transfected samples as controls.

***In Vitro* and *In Vivo* Islet Function after Transfection**

The *in vitro* function of human islets after transfection with LipofectAMINE®/pCMS-EGFP-hVEGF complexes was determined by static incubation using islets incubated with LipofectAMINE® alone, pCMS-EGFP-hVEGF alone and 5% dextrose solution (nontransfected control) as controls. Transfection media was removed by centrifugation at 1000 rpm and the islets were sequentially incubated and assessed for insulin secretion by the method outlined above. Insulin level was expressed as ng/mL and the ratio of insulin level at 300 mg/dl glucose to that at 60 mg/dl was used to calculate the stimulation index. We further evaluated *in vitro* islet function after transfection over a period of 10 days.

For *in vivo* islet function, 1500 islet equivalents were transplanted under the left kidney capsule of anesthetized 16-week-old NOD-SCID male mice as described by Gaber *et al.* (4). At 3, 5, and 10 days post-transplantation, overnight fasted NOD-SCID mice were injected peritoneally glucose (2 g/kg of body weight). At 30 min after glucose injection, blood was withdrawn from the tail. Human insulin levels were determined in the plasma in sets of four mice, each transplanted with transfected islets by ELISA (ALPCO Diagnostics). As per the manufacturer, the assay was specific for human insulin with minimal (0.67%) cross-reactivity with mouse insulin. Before transplantation, blood was also withdrawn from the fasted mice at 30 min after glucose injection, and insulin levels were determined.

Immunohistochemistry

To evaluate islet function and angiogenesis, transplanted NOD-SCID mice were sacrificed by cervical dislocation, kid-

neys were isolated, washed with PBS, and fixed in 10% neutral buffered formalin for 12–16 h followed by storage in 70% ethanol at room temperature until further processing. Potentially islet containing region of the kidneys was sectioned transversely and dehydrated by serial incubation in following solvents thrice for 20 min each: 50% ethanol, 70% ethanol, 95% ethanol, 100% ethanol, and then xylene. Sections were then embedded in paraffin for 30 min at 60°C twice, followed by embedding in paraffin in a biopsy cassette and indefinite storage at room temperature. Sections of 5 µm in thickness were then prepared from paraffin embedded blocks on a microtome, floated on lukewarm water, and picked up on adhesive glass slides. Samples were fixed on glass slides overnight in a 37°C oven followed by 2 h of fixation at 60°C. At the time of staining, samples were deparaffinized by incubation in xylene for 5 min thrice and then hydrated in 100% ethanol followed by 95% ethanol, 70% ethanol, and finally in water. Sections were marked with a wax pen and those intended for hvWF detection were treated with proteinase K for 10 min followed by washing by immersion in fresh Tris-buffered saline containing 0.05% Tween 20 (TBS-Tween 20) twice for 5 min each. All sections were then treated with 3% hydrogen peroxide for 5 min to neutralize any endogenous peroxidase activity, followed by washing with fresh TBS-Tween 20 (for mouse anti-human monoclonal von Willebrand factor hvWF primary antibody and negative control) or PBS (for insulin and rat anti-mouse CD31, mCD31, also known as PECAM-1, monoclonal primary antibodies) twice for 5 min each. This was followed by blocking with avidin/biotin and staining procedure as per the vendor protocol, which used horse radish peroxidase-conjugated secondary antibody and 3, 3'-diaminobenzoic acid staining for visualization. Antibody dilutions used were 1:10 for mCD31, 1:25 for hvWF, and 1:50 for mouse anti-human insulin monoclonal antibodies. Right kidney sections (without injected islets) and sections without primary antibody treatment were used as negative control whereas human pancreas sections (for hvWF and hInsulin) and mice kidney (for mCD31) were used as positive controls for method development.

Statistical Analysis

Statistical significance of differences between groups was determined by applying one-way analysis of variance. Statistical significance was set at $p < 0.05$. Results are expressed as the mean \pm S.D.

RESULTS

Construction of Bicistronic Vector

pCMS-EGFP-hVEGF was constructed by cloning the 165 amino acid, secreted isoform of hVEGF gene excised from pCAGGS-hVEGF vector into the polylinker site of pCMS-EGFP vector using the 5'-overhang cohesive ends generated by *EcoR1* digestion of the G/AATTC in both the excised gene and the vector. Cloning reactions using single restriction enzyme for both the gene of interest and the vector often present complications with respect to vector self ligation, gene polymerization during insertion, and orientation of gene insertion. Linearized vector was dephosphorylated using shrimp alkaline phosphatase to prevent self-ligation, which

may lead to vector recircularization. Clone selection was conducted by double restriction digestion using *Hind*III and *Ava*II to rule out any interfering patterns of insert polymerization and incorrect gene insertion. The hVEGF gene has one *Ava*II restriction site, and it generates fragments of size 68 and 508 bp, whereas *Hind*III has no cutting site in the gene. The cutting sites for *Eco*R1, *Hind*III, and *Ava*II are at 1097, (757 and 2414) and (3106, 4273, and 4495) bp in the vector, respectively. The unique cutting site of *Ava*II was used to confirm orientation of the insert. Simple calculations show that presence of a 408-bp fragment in the double digestion would be indicative of hVEGF gene insertion in the right orientation and its relative intensity with respect to other fragments; notably 222-, 692-, 1167-, and 1803-bp fragments; would be indicative of polymerization of insert. Based on the results of restriction digestion four of the 10 clones were propagated, of which two were sequenced using a vector specific primer flanking the gene on the 5'-side. Based on the sequencing data, the right clone was selected and propagated for future applications.

Comparative Gene Expression from Bicistronic Vector

Before transfection into human islets, we transfected our newly constructed pCMS-EGFP-hVEGF bicistronic vector into Jurkat cells. LipofectAMINE® was used for transfection of pCMS-EGFP-hVEGF and pCMS-EGFP into murine Jurkat T-cell lines at 3:1 w/w ratio and 5 µg DNA equivalent per two million cells. The number of EGFP positive cells was comparable for transfection with LipofectAMINE®/pCMS-EGFP and LipofectAMINE®/pCMS-EGFP-hVEGF complexes. Negligible number of EGFP positive cells, as determined by flow cytometry, was seen after transfection with pCMS-EGFP-hVEGF plasmid alone (Fig. 1A). Real-time RT-PCR data showed comparable expression in terms of the proportion of hVEGF transcript in total cellular mRNA for transfection with LipofectAMINE® complexes of both pCMS-EGFP-hVEGF and pCAGGS-hVEGF (Fig. 1B).

Equivalent expression levels were observed for both genes from the bicistronic vector as compared to the monocistronic plasmids. Therefore, the bicistronic plasmid, pCMS-EGFP-hVEGF, could be used instead of separate plasmids to serve both roles simultaneously.

Time Course of Gene Expression in Human Islets

Having obtained positive results with Jurkat cells, we proceeded to test the same formulations in the human islets at a dose of 2.5 µg DNA/1000 islets with the objective to assess comparative levels of secreted hVEGF expression from both monocistronic and bicistronic plasmids over a period of time. The level of secreted hVEGF protein, determined by ELISA, was significantly different for bicistronic and the corresponding monocistronic plasmid, with the level being higher for the bicistronic plasmid (Fig. 2). hVEGF expression levels did not reduce over a period of 10 days. This phenomenon may be explained by the fact that VEGF is a naturally secreted protein by the islets.

Cytotoxicity in Human Islets

LipofectAMINE®/ pCMS-EGFP-hVEGF complexes showed reduced toxicity to human islets at a dose of 5 µg DNA/ 2000 islets as compared to our previous work at 5:1 w/w ratio and 5 µg DNA per 2 million Jurkat cells (13). Time course of islet cytotoxicity was measured by flow cytometry using 7-AAD as a specific probe for dead cell population at days 2, 5, and 7. The islet cytotoxicity profile indicated about 93% viable cell population in the case of transfected samples which remained almost the same at all time points whereas toxicity in nontransfected samples reduced over this period of time.

In Vitro and in Vivo Islet Function after Transfection

In vitro islet function tests by static incubation were carried out after transfection with LipofectAMINE®/pCMS-EGFP-hVEGF, LipofectAMINE® alone, plasmid DNA

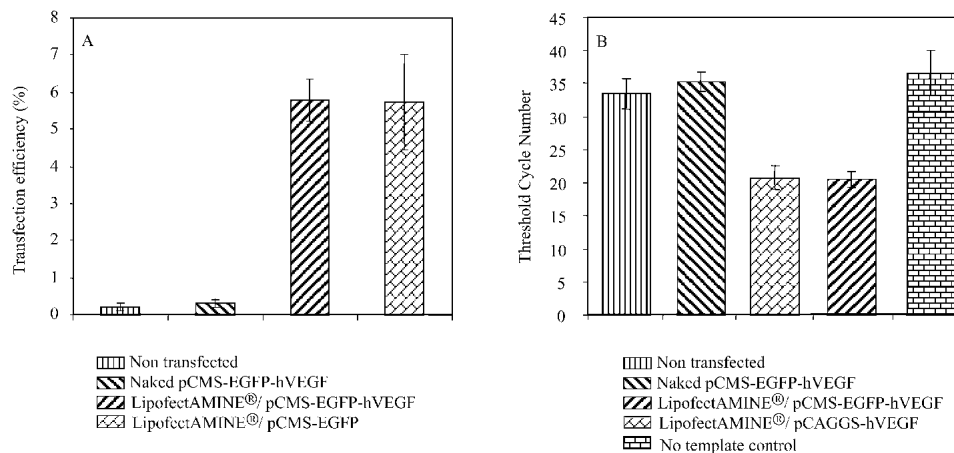


Fig. 1. Comparative expression of EGFP (A) and hVEGF (B) genes from the bicistronic plasmid, pCMS-EGFP-hVEGF, and the corresponding monocistronic plasmids, pCMS-EGFP and pCAGGS-hVEGF, respectively. Although EGFP expression was evaluated at the protein level by measuring % fluorescent cells by flow cytometry, hVEGF expression was measured at mRNA level using real time RT-PCR using the method we had established earlier (13). Importantly, lower cycle number for amplification to the threshold copy number of the target sequence in real time PCR indicates higher level of expression.

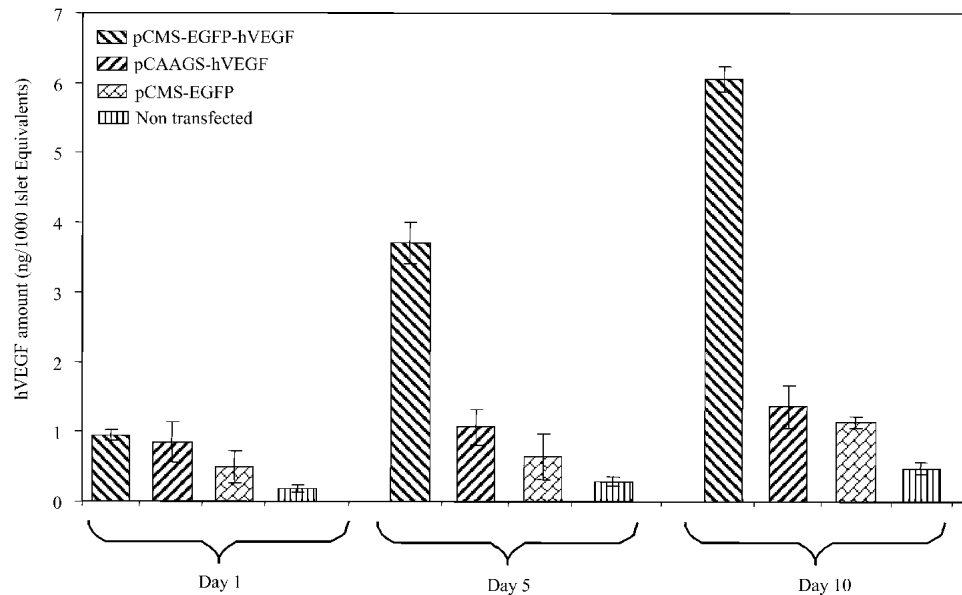


Fig. 2. Time profile of hVEGF expression at protein level in human islets transfected with the bicistronic plasmid, pCMS-EGFP-hVEGF, as compared with the monocistronic plasmid pCAGGS-hVEGF, the unrelated plasmid pCMS-EGFP, and nontransfected controls. ELISA of cell culture supernatants of separate sets of samples was performed at days 1, 5, and 10 to give the cumulative expression levels presented in this figure. The data show consistent gene expression over the time period studied as also the difference in protein expression between pCMS-EGFP-hVEGF and pCAGGS-hVEGF plasmids, despite similar levels of mRNA observed earlier, which is indicative of the effects of promoter/enhancer element differences on transcription.

(pCMS-EGFP-hVEGF) alone and non transfected control (5% glucose). Insulin release by transfected islets after 1h incubation sequentially with media containing basal (60 mg/dl) and stimulated (300 mg/dl) levels of glucose was compared to assess the stimulation index of the islets. This is indicative of the capacity of islets to respond to glucose concentration. As shown in Fig. 3, the stimulation index of islets treated with LipofectAMINE®/pCMS-EGFP-hVEGF complex was comparable with the non transfected islets (3.0 vs. 3.2). The same samples were next incubated in the presence of theophylline,

which is supposed to cause complete exocytosis of insulin vesicles in the islet cells, and then return to basal level of secretion. Although all samples showed decrease in insulin secretion upon reduction in glucose concentration to almost the basal levels, increase in insulin release upon incubation with theophylline was observed with all samples, except, significantly, LipofectAMINE®/pCMS-EGFP-hVEGF complexes. This could be attributed to the endocytotic vesicle mediated uptake of DNA/ liposomal complexes (Fig. 3).

We further evaluated *in vitro* islet function after transfection over a period of 10 days (data not shown) to account for the amount of time islets may have to be cultured after transfection in the clinical scenario. The data indicated maintenance of function over this period of time. The results suggest that transfection with LipofectAMINE®/pCAGGS-hVEGF complexes has only minimal adverse effect on the islet β cell physiological response to changing glucose concentrations. *In vivo* islet function was evaluated by measuring human insulin concentrations in the plasma of NOD-SCID mice transplanted with 1500 transfected or control islets under the kidney capsule, over a period of 10 days (Fig. 4). There was no loss of insulin secretion from transplanted human islet in response to glucose stimulation, which increased with time, at 10 days being the maximum. The results indicated that islet transfection using liposomal carriers and either/both the therapeutic (hVEGF) and marker (EGFP) genes did not significantly affect islet function, and thus can be used for further development.

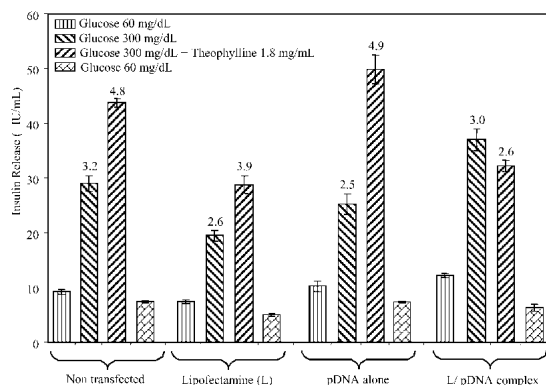


Fig. 3. *In vitro* static incubation test for human islets 72 h post-transfection with the bicistronic plasmid, as compared with lipofectamine or plasmid DNA alone and nontransfected control. Islet function was evaluated by measuring insulin secretion in response to varying levels of glucose concentrations from 60 mg/dl (considered basal stimulation level) to 300 mg/dl (stimulated level) and back to the basal level after theophylline treatment. Increase in secretion of insulin in response to glucose concentration and decrease with reducing concentration was indicative of metabolically healthy and functional islets.

Immunohistochemistry for Evaluation of Islet Function and Angiogenesis

To confirm islet function by insulin staining and to assess angiogenesis and the origin of endothelial cells by staining for

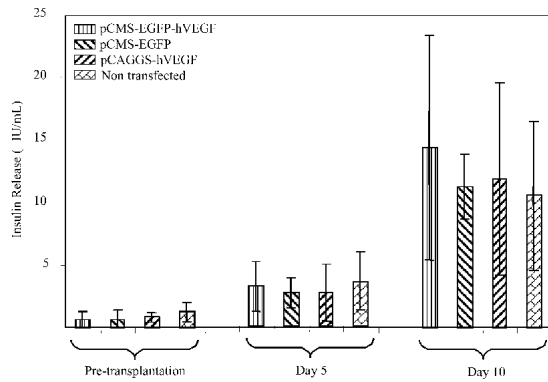


Fig. 4. *In vivo* islet function after transfection with monocistronic and bicistronic plasmids vis-à-vis the non-transfected and untreated controls, was assessed by measuring blood levels of human insulin by ELISA after glucose challenge to the mice. At 5 and 10 day post-transplantation, overnight fasted NOD-SCID mice received glucose intraperitoneally at a dose of 2 g/kg of body weight. At 30 min post-glucose challenge, blood was withdrawn from the tail and analyzed for human insulin by ELISA.

human as well as mice specific endothelial cell markers, we worked toward developing immunohistochemistry techniques whereby kidney sections with transplanted islets in NOD-SCID mice were prepared and evaluated for the expression of human insulin, hvWF, and mouse CD31/ PECAM-1. vWF is a factor VIII-related antigen and mCD31 is platelet endothelial cell adhesion molecules, both of which are known markers of the angiogenic microvessel (19). Anti-hvWF antibodies are expected to stain for blood capillaries derived from human endothelial cells while anti-mCD31 antibodies will identify blood vessels derived from the host endothelial cells. The transplanted human islets stained positive for human insulin (Fig. 5), hvWF (Fig. 6), and mCD31 (Fig. 7) with both monocistronic (Figs. 5A, 6A, and 7A) and bicistronic (Figs. 5B, 6B, and 7B) hVEGF plasmids. Islets were positive for insulin staining at both days 3 and 5 after transplantation, suggesting islets were functional. Because hvWF stains endothelial cells specifically in human and is an indicator of early angiogenesis

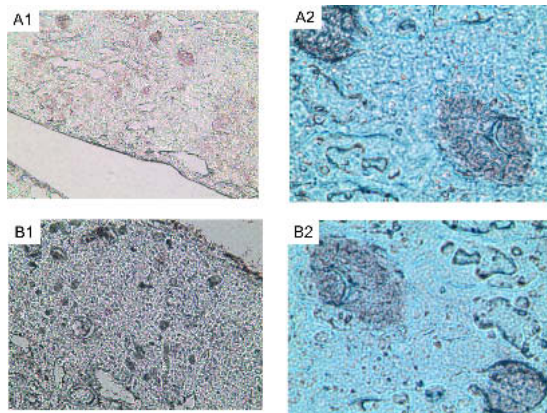


Fig. 5. Immunohistochemical staining of islets for human insulin was done after sacrificing the NOD-SCID mice implanted with 1500 human islets under the kidney capsules. Islets were transfected *ex vivo* with monocistronic (A) and bicistronic (B) hVEGF plasmids complexed with LipofectAMINE®. The sections were stained for human insulin, as seen in the images (40×, A1 and B1; and 400×, A2 and B2, magnification images).

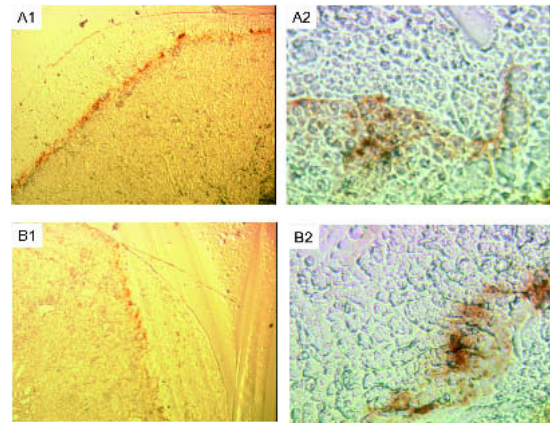


Fig. 6. Immunohistochemical staining for hvWF, an early indicator of angiogenesis, after sacrificing the NOD-SCID mice implanted with 1000 human islets under the kidney capsules. Islets were transfected *ex vivo* with (A) pCAGGS-hVEGF complexed with LipofectAMINE® (40×, A1; and at 400×, A2, magnification images). Image A1 indicates the stretch of adrenal cortex under the kidney capsule with the positively stained region, indicative of the injected islets and A2 represents a high magnification image of one section of the region; and (B) pCMS-EGFP-hVEGF complexed with LipofectAMINE®. Although image B1 represents the stretch of islets under the kidney capsule, image B2 is representative of one section of this region.

(20), the results shown in Fig. 6 suggest that the islets indeed undergo neoangiogenesis after transplantation under the mouse kidney capsules. Positive staining of mCD31 (Fig. 7) suggests that both human endothelial cells of the islets and mouse endothelial cells of the surrounding tissues participate in the revascularization process.

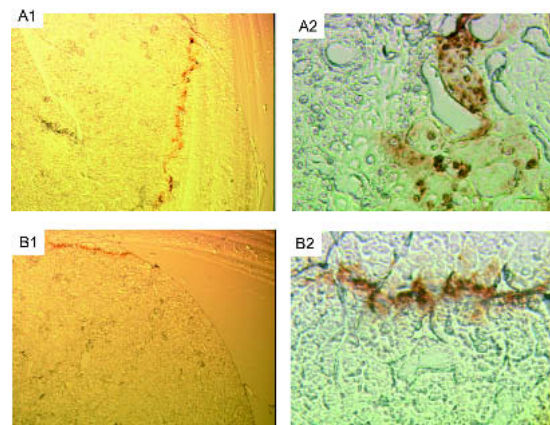


Fig. 7. Staining for mouse CD31 (mCD31, also known as PECAM-1), an endothelial cell marker, after sacrificing the NOD-SCID mice implanted with 1500 human islets under the kidney capsules. Islets were transfected *ex vivo* with pCMS-EGFP-hVEGF complexed with LipofectAMINE® (40×, A; and at 400×, B, magnification images). Image A1 indicates the stretch of adrenal cortex under the kidney capsule with the positively stained region, indicative of the injected islets, A2 represents a higher magnification image of one section of this region; and (B) pCMS-EGFP-hVEGF complexed with LipofectAMINE®, whereas image B1 represents the stretch of islets under the kidney capsule, image B2 is representative of one section of this region. Positive staining for mCD31 and of hvWF (Fig. 6) suggests that both human endothelial cells of the islets and mouse endothelial cells of the surrounding tissues contributed to the revascularization process.

DISCUSSION

Both gene carrier development and gene manipulation are important for the success of islet transplantation using gene therapy. Because islets are a cluster of about 1000 non-dividing cells, gene delivery to islets remains a challenge. We and others are developing various gene carriers (17,21,22), that may be used for gene transfer to islets after some improvements. Because islets are transfected *ex vivo* in cell culture conditions before transplantation into the recipient, the use of a targeting ligand may not be necessary because islets are already isolated free from other cell types and tissues. Because an islet is a cluster of about 1000 nondividing cells, gene delivery to islets remains a challenge. As we reported previously (13), the pattern of transgene expression is heterogeneous; some cells within the islets were highly positive for GFP whereas the majority displayed lesser expression for GFP. Moreover, transgene expression was seen mainly in the peripheral islet cells. Our results are in good agreement with the work of Benhamou *et al.* (23,24), who reported that transfection of islets with viral and nonviral vectors is predominantly in the peripheral cells, especially with larger islets, possibly because of the relative inaccessibility of the central core cells to the vector.

Islets normally have a rich blood supply, but when isolated from the pancreas by collagenase digestion, islets become dependent upon diffusion of oxygen and nutrients from the islet periphery. Islet survival and long-term function after transplantation is often antagonized by the lack of re-establishment of capillary networks within the islets, which also exacerbates immune destruction of transplanted islets (25). Moreover, diabetes is known to impair angiogenesis, as seen by the reduction in neoangiogenesis in NOD mice caused by the increase in VEGF secretion in the ischemic tissues (26). Vasir *et al.* also reported that angiogenesis is delayed in diabetic recipients and thus islets transplanted into the diabetic milieu do less well than those placed into a normoglycemic environment (27). With this interest in mind, we have worked toward the evaluation of a bicistronic plasmid vector pCMS-EGFP-hVEGF for multiple gene delivery to human pancreatic islets. The expression of EGFP as a reporter gene in addition to hVEGF as a functional transgene from this bicistronic vector could aid in the easy assessment of transfection efficiency and the optimization of gene transfer process.

To determine hVEGF gene expression, while excluding hVEGF expression resulting from endogenous production or stimulation by the plasmid CpG motifs, we designed a forward primer from the plasmid vector (pCAGGS or pCMS), and the reverse primer from hVEGF gene. Because the plasmid vectors of hVEGF and EGFP genes are different, two different forward primers and separate standard calibration curves for real time PCR experiment, one specific for pCMS and the other for pCAGGS vector, were used. In the real time PCR experiment, lower cycle number for amplification to the threshold copy number of the target sequence indicates higher level of expression. Figure 1B shows rapid amplification of hVEGF for LipofectAMINE®/pCMS-EGFP-hVEGF and LipofectAMINE®/pCAGGS-hVEGF transfected cells, compared to that of naked pCMS-EGFP-hVEGF and non-transfected cells. As expected, there was little or no amplified hVEGF for the cells transfected with LipofectAMINE®/

pCMS-EGFP. Amplification specificity was confirmed by melting curve analysis of all the PCR products, with a single predominant distinct melting temperature (T_m) of 84.3°C, which is in a good agreement with our recently published work (13) and the work of Simpson *et al.* (28) who measured retinal VEGF mRNA by real time RT-PCR using SYBR Green I.

Concentration of hVEGF protein was estimated in islet samples transfected using ELISA over a period of 10 days. The results indicated persistent hVEGF expression, which was higher than the control, unrelated plasmid, pCMS-EGFP. Because the half-life of VEGF is known to be only a few minutes (29), we speculate that the prolonged hVEGF expression was caused by hypoxia or the plasmid DNA was relatively stable due to the complex formation with cationic liposomes. The results shown in Fig. 2 indicate higher level of hVEGF gene expression from the bicistronic plasmid as compared to the monocistronic control. Expression for both plasmids was consistent over the 10-day period. Higher expression of VEGF from the bicistronic plasmid was attributed to the transfection being under the control of the CMV promoter instead of the chicken β -actin promoter in the monocistronic plasmid, which may be more active in the islets. Jeong *et al.* (30) also reported that a vector with CMV promoter/enhancer induces more potent *in vivo* gene expression than do other vectors with viral or cellular promoter.

Fig. 2 also shows relatively small but significant levels of hVEGF secretion from LipofectAMINE®/pCMS-EGFP-transfected islets, which is likely the result of endogenous production and/or immunostimulation by CpG motifs of bacterial plasmids and cationic liposomes. VEGF and its receptors, flt-1 and flk-1, are known to be expressed by islets and upregulated under hypoxic conditions (31). Because 2000 I.E. islets were incubated in a 1.5-ml Eppendorf tube containing 300 μ l of media, these islets most likely experienced hypoxia. Moreover, isolated islets *in vitro* are completely dependent upon diffusion of oxygen and nutrients from surrounding media (32). We believe the islets during and post-transfection are exposed to a lower oxygen concentration and this may be one of the possible reasons for a modest increase in the amount of secreted hVEGF (Fig. 2).

We have previously reported that transfection efficiency of LipofectAMINE/pCAGGS-hVEGF complexes into human islets is low and dependent on the incubation period of islets with the complexes. The optimal incubation period was found to be approx. 12 h (13). There are also reports of low transfection of lipofectin/pDNA, LipofectAMINE®/pDNA, DOSPA:DOPE liposome/pDNA, and polyethylenimine (PEI)/pDNA complexes into intact mouse, rat, and porcine islets. Unlike the 3:1 w/w ratio LipofectAMINE®/pCMS-EGFP-hVEGF complexes used in the present study, Benhamou *et al.* (24) used relatively large amount of cationic lipid (DOTAP, complexes were formulated 12/1 DOTAP/pDNA weight ratio). These authors also suggested that islet transfection is predominantly in the peripheral cells due to the relative inaccessibility of the central core cells to the vector.

Benhamou *et al.* (24) and Saldeen *et al.* (33) did not observe any significant improvement in reporter gene expression in intact porcine islets when transduced with adenoviral vector at low multiplicity of infection compared with cationic liposomes (DOTAP). However, relatively higher transfection was observed when these islets were dispersed by trypsiniza-

tion. Easy transfection of dispersed islet cells, as opposed to intact islets is attributable to the greater number of cells becoming exposed to the transfection agents after dispersion. Rodriguez *et al.* (34) reported that gene gun technology could be used to enhance the cellular uptake and gene expression of gold-coated DNA particles into intact islet cells. However, the use of dispersed islets as well as the gene gun is not very desirable, since these methods are likely to damage the plasma membranes of human islets.

Lakey *et al.* (22) evaluated different cationic liposomes, such as LipofectAMINE®, DOTAP, and DOSPER, for β -gal gene delivery to intact canine islets. However, these authors did not use any therapeutic genes and human islets, and assessed transfection efficiency based on β -gal staining. In contrast, we determined the transfection efficiency into human islets using flow cytometry for EGFP and ELISA and real-time RT-PCR for hVEGF, respectively. The design of forward and reverse primers specific for the plasmid vector and hVEGF gene, respectively allowed us to determine hVEGF transgene expression while excluding the endogenously produced hVEGF, which is not possible for β -gal staining.

Any molecular manipulation performed in pancreatic islets should not affect their normal insulin secretion capability. Therefore, we measured insulin release in response to glucose stimulation to assess the islet functional viability. As shown in Fig. 3, the stimulation index of insulin secretion by the islets measured as a ratio of insulin secretion response to stimulated, higher, glucose concentration as compared to lower, basal, concentration was found to be comparable for the LipofectAMINE®/pCMS-EGFP-hVEGF transfected islets as compared to nontransfected islets. Furthermore, reduction of glucose concentration back to basal level has resulted in reduction in insulin response to basal level in all cases, indicating that the islets do remain dynamically responsive to glucose concentration. These data indicated that transfection with LipofectAMINE®/pCMS-EGFP-hVEGF complexes had only minimal adverse effect on the islet β cell response to glucose challenge. In contrast to our results, Lakey *et al.* (22) reported a sharp decrease in the stimulation index of the canine islets when transfected with LipofectAMINE®/pCMV- β -Gal complexes than those of the nontransfected canine islets. This discrepancy is likely caused by the fact that these authors transfected islets at a dose of 6 μ g DNA per 500–1000 canine islets, even though they formulated the complexes at 3:1 w/w ratio (i.e., 6 μ g DNA and 12 μ l of 2 μ g/ μ l LipofectAMINE®). However, in the present study, islets were transfected with LipofectAMINE®/pCAGGS-hVEGF complexes at a dose of 2.5 μ g DNA/1000 I.E. and 3:1 weight ratio.

In the immediate post-transplantation period, islets are known to be quite vulnerable because of the decrease in vascular density and often undergo disruption of structure and loss of function (35,36). Human islets were transplanted under the left kidney capsule of NOD-SCID mice because they lack T- and B-cells but still are capable of mounting tissue inflammatory response. This model, therefore, is well suited for evaluating primary islet function (4). As shown in Fig. 4, the plasma concentration of human insulin in the mice transplanted with transfected or non-transfected human islets is significantly low. However, insulin concentration increased with time for all the groups, being the maximum at day 10—the last day of testing. Our results are in good agreement with the work reported by Davalli *et al.* (35), who reported de-

crease in graft insulin content to only 10 to 30% of pretransplanted values. No significant difference in insulin concentration was found in the mice receiving islets transfected with LipofectAMINE®/ pCAGGS-hVEGF, LipofectAMINE®/ pCMS-EGFP-hVEGF, 5% glucose as well as fresh islets.

Revascularization of islet grafts after transplantation is an important process that influences long-term survival and function of the grafts (37). Neoangiogenesis is important for the survival and function of transplanted islets. Endothelial cells and/or their progenitors reside within isolated islets and potentially participate in revascularization process (16).

Although it would be much better to quantitatively determine the formation of new blood vessels by immunostaining of blood vessels, the primary objective undertaken in this study was to confirm the origin of endothelial cells in the new blood vessels formed viz. whether they originate from the endothelial cells of the donor islets or the recipient kidneys. To the best of our knowledge, this kind of study has not yet been reported with islet transplantation. Hence, we focused on the method development and qualifying the source of endothelial cells in the newly formed blood vessels in transplanted islets. Because the vascular density of transplanted islets has been shown to be lower than endogenous pancreatic islets (36), it is important to quantify the number of blood vessels of transplanted islets with or without gene therapy treatment. This experiment along with the efficacy of gene therapy treatment on reducing the islet mass required for controlling blood glucose concentration is currently underway, and will be reported in a subsequent publication.

Immunohistochemical staining in the kidney sections at day 5 after transplantation was positive for human insulin, human factor VIII-related antigen (also known as hvWF), and mouse-specific platelet endothelial cell adhesion molecule-1 (PECAM-1, also known as CD31). The positive staining for insulin, which stains pancreatic β cells, corroborates the blood insulin data and confirms that islets are indeed functional after transplantation, which is in good agreement with contemporary literature. hvWF and mCD31 are human and murine endothelial cell markers, respectively. Therefore, positive staining for these two antigens indicate blood vessel formation in the transplanted islets and the surrounding tissue of the host arising from both human and mice endothelial cells.

Islet revascularization is known to depend on the duration of islet culture before transplantation as well as the time after transplantation. Mendola *et al.* (38) observed, for example, that when fresh islets were transplanted under the kidney capsule, they rapidly acquired new endothelial cell lining, as demonstrated by positive staining for factor VIII related antigen, at day 5, whereas 1-week cultured islets failed to become fully vascularized even at day 7 post-transplantation. They further demonstrated this as being the result of the loss of intra-islet endothelial cell staining pattern in cultured islets as compared to freshly isolated islets, with even overnight cultured islets causing loss of endothelial cell lining. In our studies, we used islets that were cryopreserved for 16 weeks before transfection/transplantation and observed vascularization at day 5. Although these results do indicate the positive effects of VEGF gene delivery on islet revascularization, we plan to further investigate the degree of vascularization at different time points after transplantation.

Immunologic reaction is one of the major concerns in developing gene therapy approaches for islets and that viral vectors may cause various kinds of immunologic and nonimmunologic adverse reactions that have been limiting their application in gene therapy. The use of immunocompetent mouse model may present a better picture in terms of such adverse reaction with our gene therapy protocols. However, in this study we used NOD-SCID mouse model, as we are transplanting human pancreatic islets in mice tissue, which may lead to immunologic reactions in the host tissue due to cross-species transplantation *per se* (4,13,39). The immunologic reaction thus elicited will not be reflective of the nature of gene carriers but would rather result from the tissue antigens themselves. Hence, we have been using NOD-SCID mice in our studies. We plan to evaluate this aspect of our gene therapy protocols in syngenic islet transplantation, i.e., transplanting mice islets in mouse tissues of the same species, which will give us a better idea of immunologic reactions originating from the vector.

In summary, we constructed and evaluated a bicistronic plasmid vector, pCMS-EGFP-hVEGF for its transfection efficiency compared with the monocistronic counterparts (pCMS-EGFP and pCAGGS-hVEGF) both in rapidly dividing Jurkat cell lines and nondividing human islets. We further evaluated the time course of cytotoxicity and gene expression from this vector and have demonstrated that islet function was not affected by transfection both *in vitro* and *in vivo*. Immunohistochemical staining of transplanted islets indicated that the revascularization process was initiated by both host and donor derived endothelial cells.

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