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Bipartite Vector Encoding hVEGF and hIL-1Ra for ex Vivo Transduction into Human Islets

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Abstract: Ex vivo gene transfer can improve the outcome of islet transplantation for treating type I diabetes. Earlier we have shown coexpression of human vascular endothelial growth factor (hVEGF) and human interleukin-1 receptor antagonist (hIL-1Ra) after transfection of plasmid DNA encoding these two genes. Due to poor transfection efficiency of plasmid DNA and the better known islet transduction efficiency of adenoviral (Adv) vectors, in this study, we constructed Adv-hVEGF-hIL-1Ra by cloning hVEGF and hIL-1Ra coding sequences and polyA signal under separate cytomegalovirus (CMV) promoters in Adenoquick plasmid (Ad 13.1). There was dose and time dependent expression of these genes after transduction of Adv-hVEGF-hIL1Ra into human islets. The mRNA expression of hVEGF and hIL-1Ra was more than 100 times higher than that of the nontransduced and bipartite plasmid transfected control islets. Transduced islets were viable as evidenced by insulin release upon glucose challenge. Coexpression of hVEGF and hIL-1Ra by islets showed decrease in caspase-3 activity and apoptosis induced by a cocktail of inflammatory cytokines such as TNF-α, IL-1β and IFN-γ. Compared to nontreated or Adv-LacZ transduced islets, transduction of islets with Adv-hVEGF-hIL-1Ra prior to transplantation under the kidney capsules of diabetic NOD-SCID mice reduced the blood glucose levels, and increased serum insulin and c-peptide levels. Immunohistochemical staining of the islet bearing kidney sections at day 20 after transplantation was positive for human insulin, hVEGF and von Willebrand factor. These results indicate that the bipartite Adv vector efficiently expresses both growth factor and antiapoptotic genes, decreases apoptosis and improves the outcome of islet transplantation.

Keywords: Human islets; adenoviral vectors; VEGF; IL-1Ra; islet transplantation

Introduction

Type 1 diabetes is an autoimmune disease that results in the destruction of insulin producing β-cells of the pancreatic islets. Significant progress has been made in the field of islet transplantation where islets are transplanted via the portal veins of type 1 diabetic patients. However, a large number of islet grafts do not secrete insulin, as they fail to revascularize upon transplantation and undergo apoptotic cell death.[1]

Islets have an intricate microvasculature, which is disrupted during the isolation and purification process. Revascularization to the transplanted islets is known to improve the delivery of nutrients and oxygen to the inner core of islets leading to their survival.[2−5] Transduction of islets with an adenoviral (Adv) vector encoding hVEGF or hepatocyte

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growth factor has been shown to promote the formation of new blood vessels, leading to improved islet survival.[3,4,6−8] Apart from revascularization, islet grafts also need to be protected from apoptosis induced by inflammatory cytokines such as interleukin-1 β (IL-1β), tumor necrosis factor (TNF-α) and interferon-γ (IFN-γ) released from resident macrophages and infiltrating immune cells at the transplantation site.[9] Immunosuppressive medications are used simultaneously to protect transplanted islets, but reports of severe side effects induced by immunosuppressive drugs have prompted attention on developing ways to reduce their toxicities.[10] Among various cytokines, IL-1β plays a key role in apoptotic cell death. Inhibition of IL-1β receptor binding using interleukin-1 receptor antagonist (IL-1Ra) resulted in improved islet survival and function.[11−13]

Considering the favorable uses of hIL-1Ra and hVEGF, we recently constructed a plasmid vector encoding these two genes (phVEGFhIL-1Ra) by cloning hIL-1Ra under the cytomegalovirus (CMV) promoter and hVEGF under the elongation factor-1R (EF-1R) promoter in pBudCE4.1 vector, and demonstrated dose and time dependent expression of hVEGF and hIL-1Ra after transfection.[14] Because the transfection efficiency of this bipartite plasmid into human islets was very low and because of the better known transduction efficiency of Adv vectors, in this study, we constructed a bipartite Adv vector encoding hVEGF and hIL-1Ra driven by separate CMV promoters. The use of bipartite vector encoding these genes not only simplifies the amplification and purification process of Adv vectors but also decreases the use of total Adv backbone for transduction, which is expected to minimize the immunogenic and toxic effect of Adv vectors. We determined transduction efficiency, islet function and caspase-3 release upon incubation with an inflammatory cytokine cocktail of TNF-α, IL-1β and IFN-γ. We then transplanted islets under the kidney capsules of NOD-SCID mice after transduction with Adv-hVEGF-hIL-1Ra and determined the blood glucose, insulin and c-peptide levels. Finally, the islet bearing kidney sections of these diabetic mice were stained for human insulin, hVEGF and von Willebrand factor (hvW).

Materials and Methods

Materials. Agarose, tris-borate-EDTA buffer, goat serum, glucose and hematoxylin solution were purchased from Sigma-Aldrich Co. (St. Louis, MO). Fetal bovine serum (FBS) and CMRL-1066 media were purchased from MediaTech Cellgros (Herndon, VA). Human VEGF and IL-1Ra ELISA kits and cytokines IL-1β, TNF-α, and IFN-γ were purchased from R&D Systems (Minneapolis, MN). Human insulin ELISA kits were purchased from Alpco Diagnostics (Windham, NH), and RNA extraction and Caspase-Glo 3/7 assay kits from Promega (Madison, WI). SYBR Green real-time PCR master mix and reverse transcription reagents were purchases from Applied Biosystems (Foster city, CA).

Methods. (1) Construction of Adv-hVEGF-hIL-1Ra. A bipartite Adv vector encoding hVEGF and hIL-Ra genes was constructed with the help of OD 260, Inc. (Boise, ID). Briefly, hIL-1Ra cDNA along with rabbit β-globin polyA was cloned into pE3.1 shuttle plasmid, while hVEGF fragment along with rabbit β-globin polyA was inserted into pE1.2 shuttle plasmid. The pE3.1-hIL-1Ra plasmid was further modified by removing the intron adjoining CMV promoter. These plasmids were transformed in super competent TOP 10 cells and amplified in terrific broth media. pE1.2-hVEGF and pE3.1-hIL-1Ra were digested to excise these expression cassettes for ligation with SfiI digested AdenoQuick 13.1 plasmid simultaneously using T4 DNA

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ligase overnight at 16 °C. The ligated products were packaged into phage lambda particles using MaxPlax packaging extract (Epicenter, Madison, WI). The packaging mixture was used to infect *Escherichia coli* XL-1Blue (Stratagene, La Jolla, CA), and clones were selected on LB supplemented with 25 μg/mL kanamycin and 50 μg/mL ampicillin. The *Pac*I linearized cosmid was transfected into 293 cells to rescue the virus. Three viral plaques were amplified, and their genomic DNAs were extracted. The presence of hVEGF and hIL-1Ra expression cassettes was confirmed by restriction analysis with *Hin*dIII and *Not*I. The clone containing the two genes was transduced to 293 cells, and the viral extract was further transduced into 293 cells, amplified and purified on two CsCl gradients. The bands harvested were dialyzed against GTS buffer (25% glycerol, 25 mM NaCl, 20 mM Tris-HCl, pH 8) and viral suspension was collected, filtered and stored at −70 °C.

(2) Determination of Viral Titer. (a) OD 260-SDS Method. Adv-hVEGF-hIL-1Ra was purified using CsCl density gradient. The purified virus was diluted at different concentrations in the range of 0.1−1.0 in 0.1% sodium dodecyl sulfate (SDS) buffer. The absorbance was recorded at 260 nm. The viral titer was calculated based on the extinction coefficient of 1.1×10^{12} virus particles (VP) per Abs260 unit in the presence of SDS buffer.

(b) Plaque Assay. Freshly confluent 6 cm dishes seeded with 293 cells were infected with various dilutions of Adv-hVEGF-hIL-Ra. Three days later, the cells were split into 10 cm dishes to boost virus plaque formation. The cells were split again into 15 cm dishes 10 days later. The formation of virus plaques was monitored up to 3 weeks after which the infection titer was calculated.

(c) Infectious Viral Titers. The infectious viral titer was also determined by using an Adeno-X rapid titer kit from Clontech. Different dilutions of Adv-hVEGF-hIL-1Ra was taken and transduced to AD-293 cells, using untransduced cells as a control. After 48 h post-transduction, AD-293 cells were fixed and incubated with Anti-Hexon antibody, followed by a secondary horse radish peroxidase (HRP)-conjugated antibody. Then the cells were stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate and positively stained cells were counted under a microscope, and the titer was determined.

(3) Islet Culture and Transduction. Human islets were received from one of the several Islet Cell Resource (ICR) Centers in culture media at 4 °C. On receiving, the islets were cultured in CMRL-1066 medium containing 10% fetal bovine serum (FBS) and 1% penicillin streptomycin. Islets were then transduced with Adv-hVEGF-hIL-1Ra, while nontransfected islets as well as the islets transfected with phVEGF-hIL-1Ra/Lipofectamine complexes (3/1, +/−, 2.5 μg DNA/1000 islets) were used as controls. To determine the optimal multiplicity of infection (MOI) of Adv vectors in terms of plaque forming units (pfu) per cell of an islet which is a compact cluster of 1000 nondividing cells, 1000 islets were incubated with Adv-hVEGF-hIL-1Ra in 300 μL media in a 96 well plate for 12 h, followed by washing and further culturing in 1 mL of media in 24-well plates. Gene expression was determined at day 1 and 3 by quantifying secreted protein in the media by ELISA (R&D Systems). Total protein of the islet cell extracts was also determined using the bicinchoninic acid (BCA) protein assay kit.

(4) Quantitative Real-Time PCR. To determine hVEGF and hIL-1Ra gene expression 3 days after transduction with Adv-hVEGF-hIL-1Ra, the islet pellet was collected for isolation of total RNA using RNAeasy mini isolation kit from Promega. Two hundred fifty nanograms (ng) of extracted RNA was converted into cDNA using MultiScribe reverse transcriptase and random hexamers (Applied Biosystems, Inc.) 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. In all 3.85 μL of cDNA was amplified by real-time RT-PCR. To determine the level of hVEGF expression due to Adv-hVEGF-hIL-1Ra transduction, while excluding the endogenous expression, Adv vector specific reverse primer (5′-TAG CCA GAA GTC AGA TGC TCA AGG-3′) was designed from the PolyA region specific to the vector. The forward primer (5′-TTT CTG CTG TCT TGG GTG CAT TGG-3′) was designed from the hVEGF gene sequence. We also designed both primers from the gene forward (5′-TTT CTG CTG TCT TGG GTG CAT TGG-3′) and reverse (5′-AAG ATG TCC ACC AGG GTC TCG AAT-3′) so that cumulative expression of hVEGF can be determined.

In the case of hIL-1Ra both forward (5′-ATC CAG CAA GAT GCA AGC CCT-3′) and reverse (5′-TTC GTC AGG CAT ATT GGT GAG GCT-3′) primers were designed from the gene sequence (NCBI Accession # AK290898). To determine the level of hIL-1Ra expression excluding its endogenous expression, forward primer was designed from the Adv sequence (5′- AGC CTC ACC AAT ATG CCT GAC GAA −3′) and reverse primer from the gene sequence (5′- TTG CCA TGC AAG AAT GGG AAC AGG-3′) (NCBI Accession # AB021221). 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 for 1 min.

To confirm the amplification specificity, the PCR products were subjected to melting curve analysis. hVEGF and hIL-1Ra gene expression was normalized to 18s rRNA as internal control. Threshold cycle number was compared between Adv-hVEGF-hIL-1Ra with the same parameters using non-transfected islets as controls.

(5) In Vitro Islet Function after Transduction. The in vitro islet function after transduction with Adv-hVEGF-hIL-1Ra was determined by the static incubation method, using nontransduced islets as control. Transfection media was removed and the islets were sequentially incubated at 37 °C for 1 h in the media containing 2.5 mM (basal) and 22 mM glucose (stimulated). After incubation, supernatants were collected and analyzed for insulin release by ELISA (Alpco Diagnostics). Insulin secretion was expressed as μU/mL and the ratio of insulin levels at 22 mM to 2.5 mM glucose was used to calculate the stimulation index.

(6) Cytoprotective Effect of AdVEGF-IL-1Ra. One thousand islets per well of 96 well plates were transduced with 1000 MOI of Adv-hVEGF-hIL-1Ra, Adv-hVEGF or Adv-hIL-1Ra. At 12 h post-transduction, islets were washed and incubated with either fresh media or a cocktail of IL-1β (10 ng/mL), TNF-α (10 ng/mL) and IFN-γ (50 ng/mL). At day 3 post-transduction islets were collected from each well and lysed with lysis buffer to measure the caspase-3 concentration using the Caspase-Glo 3/7 Assay kit (Madison, WI).

We also determined the cytoprotective effect of Adv-hVEGF-hIL-1Ra on islet viability by fluorescence microscopy of islets after transduction with Adv-hVEGF-hIL-1Ra (500, 1000 and 2000 MOI), Adv-hVEGF (1000 MOI), and Adv-hIL-1Ra (1000 MOI) for 12 h, followed by incubation with the cytokine cocktail for 3 days. These islets were stained with propidium iodide (500nM) to identify dying or dead cells under fluorescence microscope (Olympus).

(7) Islet Transplantation Studies. Animal experiments were performed as per the NIH (http://grants1.nih.gov/grants/olaw/ references/phspol.htm) and institutional animal care and use guidelines using approved protocols. To induce diabetic animal model, streptozotocin (STZ) (40 mg/kg) was administered to NOD-SCID mice by intraperitoneal injection for 5 consecutive days. Animals were considered to be diabetic after two consecutive blood glucose measurements $\geq$325 mg/dL using a One Touch Ultra Glucometer. Before transplantation, human islets were transduced by Adv-hVEGF-hIL-1Ra or Adv-LacZ at the dose of 1000 MOI for 12 h and washed with PBS. About 1500 transduced or nontreated islets were transplanted under the left kidney capsules of diabetic mice. The nonfasting glucose levels were measured up to 20 days post-transplantation. The mice were then anesthetized to collect blood to measure serum insulin and c-peptide levels by ELISA; Alpco Diagnostics (Windham, NH). The graft-bearing kidneys were removed to confirm the function of islet grafts by the return to blood glucose levels to $\geq$325 mg/dL for two consecutive days.

(8) Immunohistochemistry and Morphometric Analysis. To determine hVEGF expression and angiogenesis, transplanted mice were sacrificed, kidneys were isolated, washed with PBS, fixed in 4% formaldehyde overnight, and embedded in paraffin as described before.[3] Sections of 5−7 μm thickness were cut and immunostained with rabbit anti-insulin, hVEGF and hvW primary antibodies, respectively. The immunoreactivity was detected using goat antirabbit IgG, H &L chain specific peroxidase conjugate and subsequent incubation with DAB substrate. For morphometric analysis, hVEGF and hvWF-positively immunostained color (brown in each case) was selected for quantification of the relative intensity using NIH Image 1.62 software to determine the mean values, which were subsequently compared between Adv-hVEGF-hIL-1Ra transduced and control islet transplanted diabetic recipient mice.

(9) Statistical Analysis. Statistical significance of the difference between the two groups was determined by unpaired *t* test and between several groups by one-way ANOVA.

Results

Construction of Adv-hVEGF-hIL-1Ra. Adv-hVEGF-hIL-1Ra is a serotype 5 based replication deficient Adv vector that contains a CMV promoter, hVEGF cDNA and rabbit β-globin poly A in the E-1 region and a CMV promoter, hIL-1Ra cDNA and rabbit β-globin poly A in the E-3 region (Figure 1). Both cassettes are oriented from left to right relative to the adenovirus genome and lack the CMV intron that was present in the cosmid pADhVEGF-hIL-1Ra, from which no virus could be recovered. hVEGF and hIL-1Ra coding sequences and polyA signals were placed under the control of a separate CMV promoter cloned into pE1.2 and pE3.1. The resulting vectors were combined in Adenoquick 13.1 to generate a cosmid that contains the entire sequence of recombinant adenovirus. The presence of hVEGF and hIL-1Ra expression cassettes in these vectors was confirmed by restriction digestion with *Hin*dIII and *Not*I. The number of viral particles in the stock solution of Adv-hVEGF-hIL-1Ra was determined by the OD_{260}-SDS method. This gives the total number of 1.2×10^{12} viral particles per mL of the stock solution irrespective of their plaque forming ability. The infectious titer of Adv-hVEGF-hIL-1Ra (8×10^{10} IU/mL) was calculated by plaque assay method. This was further ascertained by performing the adenotiter assay using an Adeno X rapid titer kit and the titer was calculated to be 6×10^9 ifu/mL (infectious units/milliliter). We used infectious units/mL viral titer for all the experiments.

Transduction Efficiency of Adv-hVEGF-hIL-1Ra. Following transduction of islets with Adv-hVEGF-hIL-1Ra, the level and duration of hVEGF and hIL-1Ra proteins increased with increase in MOI. hVEGF and hIL-1Ra protein levels at day 3 post-transduction were over 100 times more than that of the untransduced islets (Figure 2), indicating that Adv-hVEGF-hIL-1Ra transduction would lead to high local concentrations of hVEGF and hIL-1Ra in the immediate vicinity of islets post transplantation. We also transfected islets with the bipartite plasmid DNA encoding these two genes (phVEGF-hIL-1Ra) after complex formation with Lipofectamine, which produced about 100 times lower levels of hVEGF and hIL-1Ra proteins than that of the islets transduced with Adv-hVEGF-hIL-1Ra (Figure 2).

We also determined hVEGF and hIL-1Ra gene expression by real time RT-PCR. The normalized mRNA concentration to that of the control was significantly increased with increase in MOI of Adv-hVEGF-hIL-1Ra (Figure 3). Gene expression at mRNA level of hVEGF and hIL-1Ra was more than 100 times higher than that of untransduced islets. Islets transfected with the bipartite plasmid, phVEGF-hIL-1Ra, also produced very low levels of hVEGF and hIL-1Ra mRNA. Each graph is indicative of the relative increase in expression of hVEGF and hIL-1Ra independently. This shows that Adv-hVEGF-hIL-1Ra can simultaneously express both genes with high

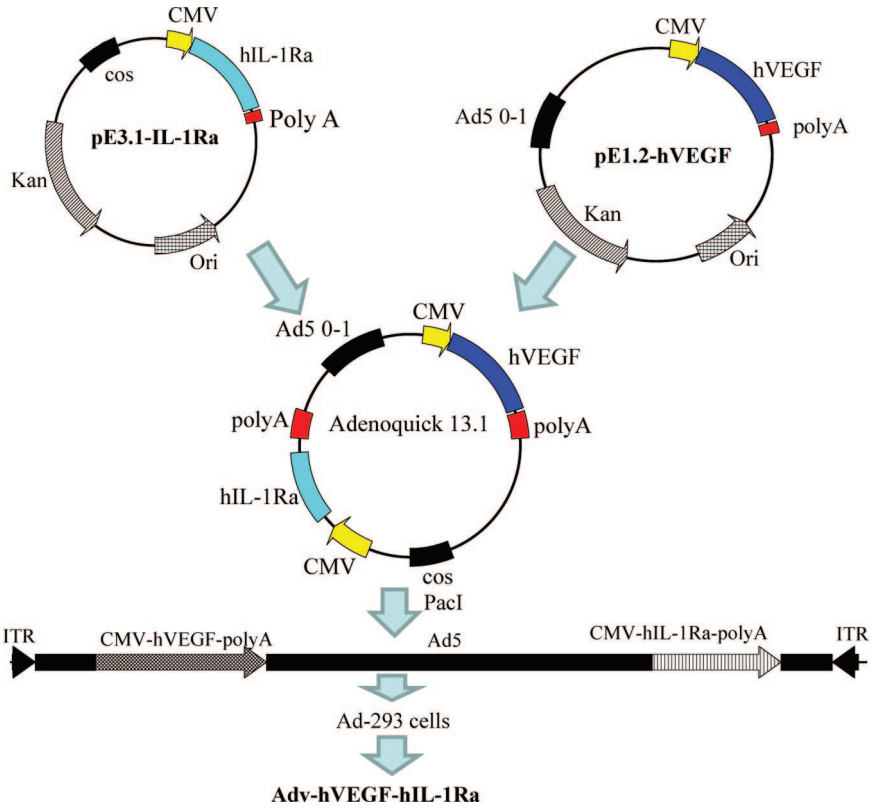

Figure 1. Construction of E1 and E3 deleted bipartite Adv vector by cloning hVEGF and hIL-1Ra into the multiple cloning sites of shuttle plasmids pE3.1 and pE1.2. These expression cassettes were cloned into AdenoQuick plasmid 13.1 to generate a cosmid containing the entire sequence of recombinant adenovirus. After transfection into 293 cells, Adv-hVEGF-hIL-1Ra was produced.

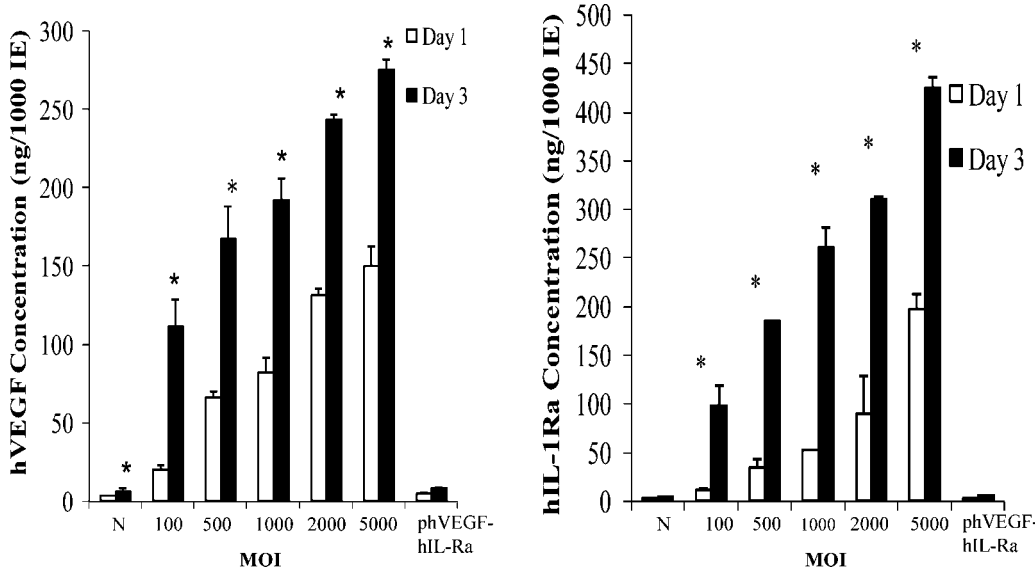

Figure 2. Time profile of hVEGF and hIL-1Ra gene expression at protein levels in human islets transduced Adv-hVEGF-IL-1Ra at different multiplicities of transduction (MOI) 100, 500, 1000, 2000 and 5000 per 1000 islets. Nontransfected islets as well as the islets transfected with phVEGF-hIL-1Ra/Lipofectamine complexes (3/1, +/−, 2.5 μg DNA/1000 islets) were used as controls. ELISA of cell culture supernatants was performed at days 1 and 3. Cumulative expression levels were presented as the mean ± SD of $n = 4$. *$p < 0.05$ compared with untransduced islets using ANOVA.

efficiency. The mRNA levels of hVEGF and hIL-1Ra obtained by using one primer from the gene and the other from the Adv backbone showed dose dependent increase signifying that these genes were being expressed by Adv-hVEGF-hIL-1Ra (data not shown). The mRNA concentrations of each gene were normalized to that of the untrans-

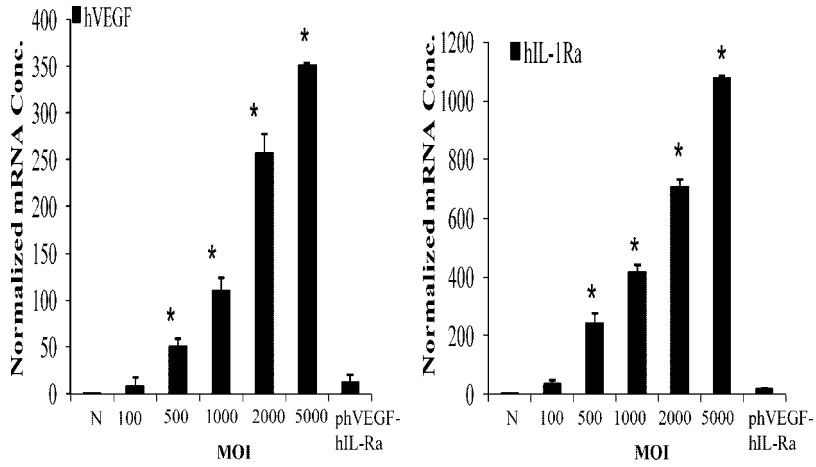

Figure 3. Real-time PCR of human islets at day 3 post-transduction with AdvhVEGF-IL-1Ra at MOI of 100, 500, 1000, 2000 and 5000. Forward and reverse primers were designed from hIL-1Ra and hVEGF genes. Nontransfected islets as well as the islets transfected with phVEGF-hIL-1Ra/Lipofectamine complexes (3/1, +/−, 2.5 μg DNA/1000 islets) were used as controls. Cell pellet was collected at day 3 and processed for mRNA extraction, reverse transcription and real time PCR using SYBR green chemistry. The normalized mRNA values of the different MOI treated islets are plotted. Since different primers were used, no direct comparison can be made, other than demonstration of increase in mRNA levels with increase in MOI. Results are expressed as the mean ± SD of $n = 4$ * $P < 0.05$ compared with control using ANOVA.

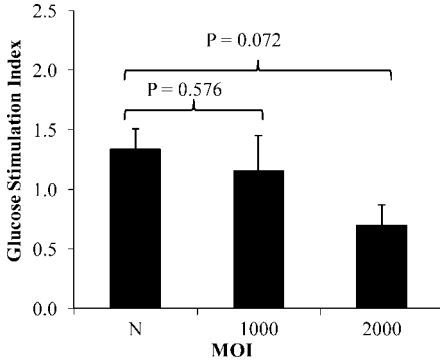

Figure 4. Stimulation index (SI) of human islets with or without cytokine incubation after transduction with Adv-hVEGF-IL-1Ra. Nontransduced (N) islets were used as controls. Results are expressed as the mean ± SD of $n = 3$. *P value compared to untransduced islets.

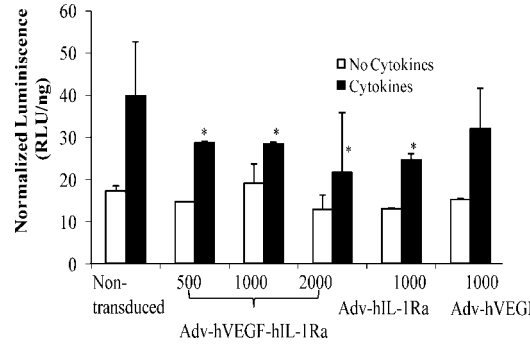

Figure 5. Effect of hVEGF and hIL-1Ra coexpression on caspase-3 activity. Caspase-3 activity in human islets in the absence of or after incubation with the cytokines was determined by measuring the amount of caspase-3 present in the lysate of islets after cell lysis using a Caspase-Glo 3/7 luciferase assay. The islets were transduced with Adv-hVEGF-hIL-1Ra, Adv-hVEGF or Adv-hIL-1Ra prior to incubating with cytokines IL- 1β (10 ng/mL), TNF-α (10 ng/mL) and INF-γ (50 ng/mL). Nontransduced islets were used as control. Mean ± SD of $n = 3$, *p value compared with cytokine treated nontransduced islets using ANOVA.

duced islets. The amplification specificity was confirmed by melting peak analysis, which showed a single predominant peak each for 18 S (85.4 °C), hVEGF (84.5 °C) and hIL-1Ra (87.05 °C), respectively (data not shown).

Effect of Viral Transduction on Islet Function. To determine whether insulin secretion was adversely affected by transduction of islets with Adv-hVEGF-hIL-1Ra, insulin secretion in response to glucose challenge was carried out at day 5 post-transduction. Glucose challenge was determined by quantifying glucose stimulated insulin release at basal level (2.5 mM) and stimulated (22 mM) glucose concentrations. As shown in Figure 4 stimulation index was almost identical to that of untransduced islets when 1000 islets were treated with Adv-hVEGF-hIL-1Ra at the dose of 1000 MOI. However, when 1000 islets were treated with this vector at 2000 MOI, there was some cytotoxicity resulting in decreased stimulation index.

Apoptosis of Islets. Apoptosis of islets leading to their impaired function and subsequent death is an important detriment to islet transplantation. Caspase-3 plays a major role in the apoptosis of islets. Therefore, we determined caspase-3 level in the islets transduced with Adv-hVEGF-hIL-1Ra and incubated with a cocktail of inflammatory cytokines such as IL-1β, TNF-α and IFN-γ. Caspase-3 activity was very high in the untransducted islets treated with the cytokine cocktail compared to that in the absence of the cytokine cocktail (Figure 5). The islets treated with 1000 and 2000 MOI Adv-hVEGF-hIL-1Ra and Adv-hIL-1Ra

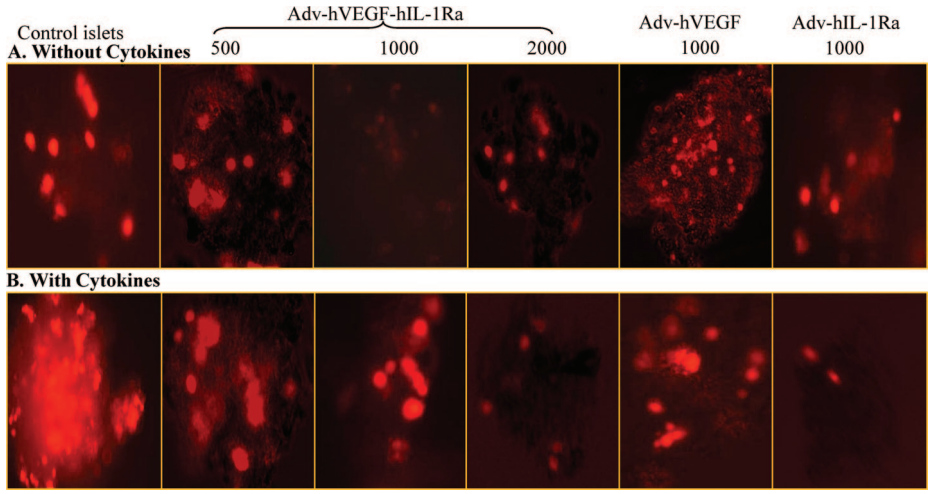

Figure 6. Effect of hVEGF and hIL-1Ra coexpression on islet viability was examined under fluorescence microscopy after propidium iodide staining of intact islets transduced with Adv-hVEGF-hIL-1Ra, Adv-hIL-1Ra and Adv-hVEGF, and then incubated for 3 days with inflammatory cytokines. Red staining represents cell membranes of dying or dead cells.

showed significantly lower levels of caspase-3 activity compared to the untransduced control islets or the islets transducted with Adv-hVEGF at 1000 MOI and incubated in the cytokine cocktail (Figure 5). In contrast, caspase-3 activity of all groups was very low when these islets were not incubated with the cytokine cocktail, suggesting that the Adv vector was nontoxic to islets at these tested doses.

We also determined the cytoprotective effect of Adv-hVEGF-hIL-1Ra on islet viability by fluorescence microscopy after transduction and incubation with the cytokine cocktail. These islets were stained with propidium iodide, which is known to be excluded by viable cells but can penetrate cell membranes of dying or dead cells, as evidenced by red staining. As shown in Figure 6, the number of red cells was significantly lower in all the groups which were not incubated with the cytokine cocktail. However, incubation with the cytokine cocktail significantly increased the number of red cells in case of untransduced control islets. However, transduction of islets with Adv-hVEGF-hIL-1Ra and Adv-hIL-1Ra significantly decreased the number of red cells even after incubation with the cytokine cocktail, suggesting the protective effect of hIL-1Ra gene expression. These results corroborate well with the results shown in Figure 5 for the effect of Adv-hVEGF-hIL-1Ra on caspase-3 activity.

Islet Survival and Function after Transplantation. The effect of hVEGF and hIL-1Ra gene expression on the islet survival and function post-transplantation of 1500 islets transduced with Adv-hVEGF-hIL-1Ra at 1000 MOI under the kidney capsules of NOD-SCID mice was determined in terms of blood glucose, human insulin, and C-peptide levels. Diabetes was partly corrected in higher number of mice receiving Adv-hVEGF-hIL-1Ra transduced islets as compared to those receiving Adv-LacZ transfected and nontreated islets. Adv-hVEGF-AdvhIL-1Ra transduced islets showed much rapid reduction in blood glucose levels to $\leq$220 mg/dL (3 days) than Adv-LacZ transduced or nontreated islets. Mice transplanted with Adv-hVEGF-hIL-1Ra transduced islets had the lowest blood glucose levels, followed by nontreated islets (Figure 7A). Blood glucose levels returned to $\geq$325 mg/dL upon removal of the islet graft-bearing kidney at days 21 after transplantation, confirming that transplanted islets were functional. Upon glucose challenge, both blood insulin and C-peptide levels were significantly higher in mice transplanted with Adv-hVEGF-hIL-1Ra transduced islets compared to all other groups, indicating better islet engraftment and function (Figures 7B and 7C). These results suggest that compared to nontreated or Adv-LacZ transduced islets, coexpression of hVEGF and hIL-1Ra reduced the blood glucose level of mice, and increased the level of blood insulin and c-peptide upon glucose challenge.

Immunohistochemistry. To determine whether hVEGF gene expression after transduction with Adv-hVEGF-hIL-1Ra will promote angiogenesis by human islets, we isolated the kidney bearing Adv-hVEGF-hIL-1Ra transduced islets at 20 days post-transplantation into diabetic NOD-SCID mice for sectioning and staining with polyclonal rabbit hVEGF and vonWillebrand Factor (hvWF) antibodies. Staining with antihuman insulin and untransduced islet sections were used as the controls. The positive staining of hVEGF was higher in the transduced islets compared to untransduced islets (Figure 8), suggesting efficient hVEGF gene expression after transduction with Adv-hVEGF-hIL-1Ra. The positive staining of hvWF factor was more evident in Adv-hVEGF-hIL-1Ra transduced islets, which is an indicator for endothelial cells. Morphometric analysis revealed that Adv-hVEGF-hIL-1Ra transduced islet grafts exhibited higher hVEGF and hvWF-immunostaining intensity than control islet grafts, with relative intensities of 1.45 vs 1 for hVEGF and 1.30 vs 1 for hvWF, respectively. To correlate the degree of islet revascularization with the islet mass in the kidney capsule, we also stained for human insulin. Adv-hVEGF-hIL-1Ra transduced islets displayed significantly higher levels of

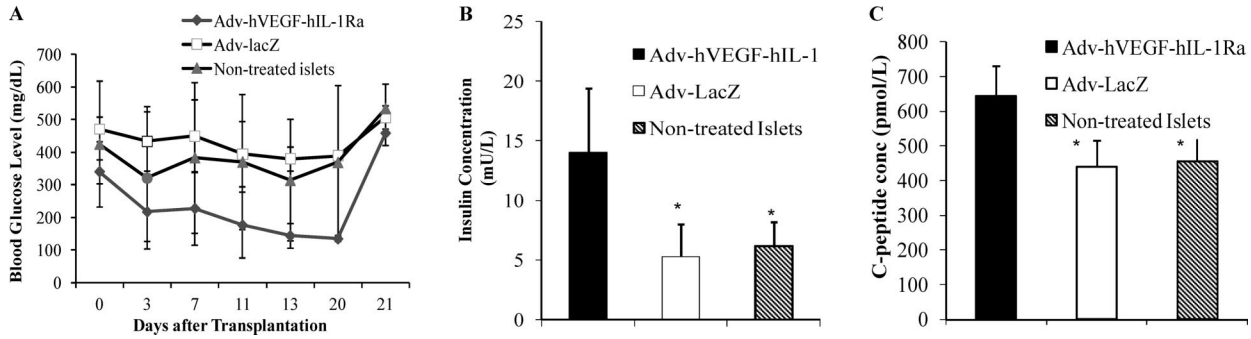

Figure 7. Effect of Adv-hVEGF-hIL-1Ra transduction on the outcome of islet transplantation. Following transduction with Adv-hVEGF-hIL-1Ra at 1000 MOI, 1500 islets were transplanted under the kidney capsule of streptozotocin-induced diabetic NOD-SCID mice. The mice transplanted with Adv-LacZ-transduced and nontransduced islets were used as controls. (A) The nonfasting glucose levels were measured in each animal up to 20 days post-transplantation using a One Touch Ultra Glucometer. Blood glucose levels returned to ≥325 mg/dL upon removal of the islet graft-bearing kidney at days 21 after transplantation. (B) At day 20 post-transplantation, the mice were sacrificed and blood was collected to measure serum insulin by ELISA and C-peptide by ELISA. Data are presented as the mean + SD of $n = 4$, *$p < 0.5$, compared to Adv-hVEGF-hIL-1Ra transduced islet group.

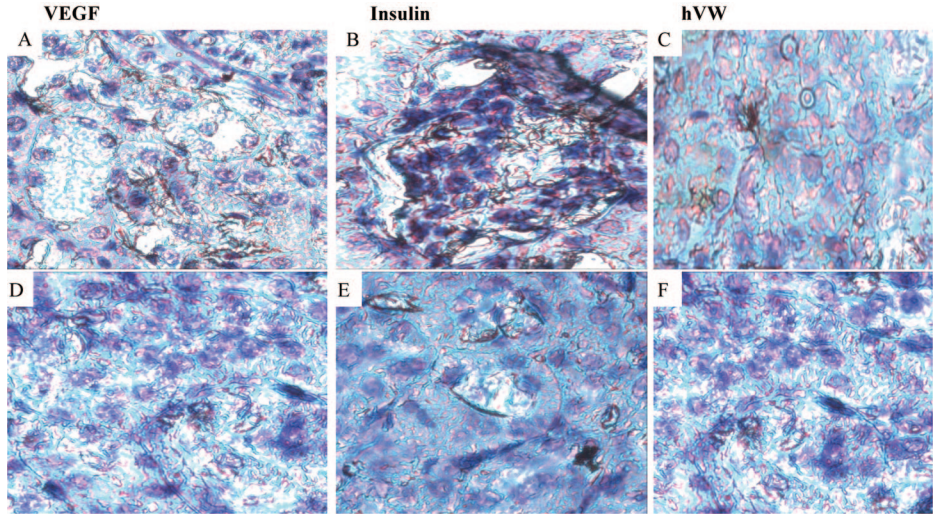

Figure 8. Immunohistochemical staining of islet bearing kidney sections at day 20 post-transplantation of human islets transduced with Adv-hVEGF-hIL-1Ra at 1000 MOI. Rabbit polyclonal anti human VEGF, insulin and vWF were used for detecting human VEGF, insulin and endothelial cells. These pictures were taken at 40× magnification. (A) hVEGF, (B) insulin, (C) vWF staining for islets transduced with Adv-hVEGF-hIL-1Ra; and (D) hVEGF, (E) insulin, (F) vWF staining for control islets.

insulin content than control islet groups (Figure 8B), with relative intensity of 1.3 vs 1. This quantitative difference in insulin content in the kidney capsule between Adv-hVEGF-hIL-1Ra transduced and control islet groups of diabetic recipient mice correlated well with their blood glucose, serum insulin and c-peptide profiles (Figure 7). The positive staining for human insulin also confirmed the functionality of islets. These results indicate the beneficial effect of Adv-hVEGF-hIL-1Ra for the islet survival and function post-transplantation.

Discussion

Isolation and purification of islets disrupt their microvasculature, leading to hypoxia and apoptosis upon transplantation.[15] We and others have shown that hVEGF expression promotes new blood vessel formation and improves the outcome of islet transplantation.[3,14−16] However, effective prevention of islet destruction after transplantation requires not only revascularization but also abrogation of cytokine mediated islet cell death and dysfunction. Islet injury stimulates resident macrophages and infiltrating leukocytes to produce cytotoxic cytokines like IL-1β, TNF-α and IFN-

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γ. Among these cytokines, IL-1β is the key mediator of β cell destruction. Administration of recombinant IL-1Ra protein has been shown to prevent islet dysfunction upon transplantation.[13] Ex vivo transduction of human islets with Adv-hIL-1Ra has been reported to prevent IL-1β induced β cell impairment and activation of islet cell apoptosis.[12] Therefore, we recently transduced human islets with a mixture of Adv-hVEGF and Adv-h IL-1Ra,[16] and demonstrated suppression of cytokine induced iNOS gene expression and NO production.[16,17] Similarly, Bertera et al.[18] reported synergistic effect after simultaneous Adv transduction of mouse islets with two individual genes, such as IL-1Ra and indoleamine 2, 3 dioxygenase or hIL-1Ra and manganese superoxide dismutase (MnSOD).

Transgene expression depends not only on the type of gene carrier, but also on the components of gene expression systems. There are two methods to coexpress two genes: using either internal ribosome entry site (IRES)[19] sequences or two independent promoters.[20] We and others have shown the tendency of low expression of the IRES-dependent second gene relative to the first gene,[21] and thus the need of optimization of the IRES and translation efficiency. Further, adenovirus-based vectors are often constructed using two independent CMV promoters for coexpression of two genes.[22] Therefore, we decided to construct Adv-hVEGF-hIL-1Ra driven by two independent promoters. The use of two independent CMV promoters is unlikely to pose any serious problem because the rate of recombination is very low ($<10^{-4}$/generation) as the virus is nonreplicative.

The objective of this study was to evaluate a bipartite Adv vector for multiple gene delivery to human islets for promoting revascularization and inhibiting inflammatory cytokine-mediated destruction. There are several studies where bipartite Adv vectors have been constructed with one therapeutic gene and another marker gene, especially green fluorescence protein (GFP).[23,24] In this study, we have constructed a replication deficient bipartite Adv vector encoding hVEGF and hIL-1Ra. The use of bipartite vector not only simplifies the amplification and purification process of Adv vectors but also decreases the use of total Adv backbone, compared to the use of two single Adv vectors. This is expected to minimize the immunogenic and toxic effects of Adv vectors. Since we previously measured hVEGF and hIL-1Ra gene expression after transduction of islets with Adv-hVEGF and Adv-hIL-1Ra, respectively,[4,16] in this study we did not measure hVEGF and hIL-1Ra gene expression after transduction of islets with these vectors as controls. However, we used the islets transduced with these vectors for determining apoptosis in terms of caspase-3 as described below.

Microenvironment of islets in a healthy pancreas facilitates β-cell function and survival by efficient circulation of highly oxygenated blood. However, there is an ischemic period immediately after transplantation, leading to hypoxia and consequent apoptotic cell death. VEGF expression is known to be upregulated under hypoxia not only by a hypoxia-inducible factor 1-α (HIP-1 α), but also via increased stability of its mRNA.[25] Unregulated VEGF expression may induce the growth of abnormal blood vessels and hemangiomas in islets,[26] even though VEGF gene expression has been shown to increase β-cell survival and function after transplantation.[27] Therefore, effective regulation of hVEGF gene expression is important for successful transplantation. The erythropoietin (Epo) enhancer and Epo 3-untranslated region (UTR) have been shown to increase VEGF gene expression under hypoxia[28] and thus its application should be explored for improved islet transplantation. Since hypoxia is known to

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upregulate hVEGF gene expression,[29] which promotes angiogenesis[30] and improved vascularization can lead to reduced apoptosis, we did not incorporate Epo enhancer or Epo 3-UTR in our Adv construct, but coexpress hIL-1Ra to protect islets from hypoxia-induced antiapoptotic cell death.[13]

Apoptosis begins during islet isolation process, peaks 2−3 days post-transplantation, and continues for approximately 7−14 days until what remains of the graft has stabilized and become vascularized. Therefore, the use of replication deficient Adv vectors is appropriate. We decided to use Δ*E* 1 and Δ*E* 3 Adv vector because our goal was to achieve transient gene expression, while avoiding other potential side effects and in vivo virus replication.

Several studies have shown little induction of inflammatory responses on islet apoptosis when islets are transduced with Adv vectors at low MOI, which is also true in our studies. We expressed the multiplicity of infection (MOI) of Adv in terms of plaque forming units (pfu) per cell. Since an islet is the compact cluster of 1000 nondividing cells, 1000 MOI/islet means 10 pfu per cell. Therefore, 1000 MOI of replication deficient Adv vector used in this study is safe and does not impair with insulin secretion, as demonstrated in this and our previous studies. Moreover, hIL-1Ra gene expression is known to prevent possible apoptosis by blocking IL-1β. Since islets were transduced in Petri-dishes and free viral particles were removed by washing of transduced islets with PBS, most of the Adv genomes would, therefore, be inside the cells at the time of infusion of transduced islets into the subjects. Since these vectors are replication deficient, we do not expect the host to mount a rigorous immune response. While there will be no free viral particles with the transplanted islets, even direct administration of this dose of Adv vectors have been seen to be nontoxic. Moreover, the local delivery of low and intermediate dose of Adv vectors in humans indicated that these vectors are well tolerated even up to the dose of 10^{11} particles or below.[31,32]Any possible immunotoxicity due to Adv backbone will be countered by hIL-1Ra gene expression which will also protect islets from apoptosis.

To determine hVEGF and hIL-1Ra gene expression, we first transduced human islets with Adv-hVEGF-hIL-1Ra and then monitored transgene expression for 3 days. hVEGF and hIL-1Ra expression over days 1 and 3 showed considerable increase (Figure 2), suggesting the efficacy of Adv-hVEGF-hIL-1Ra. Variation in MOI, promoters and duration of the incubation time are known to affect the transfection efficiency of Adv vectors in various cell types. Therefore, we sought to optimize the transduction conditions of human islets with Adv-hVEGF-hIL-1Ra. hVEGF and hIL-1Ra gene expression increased with increase in MOI from 100 to 5000. However, we decided to infect 1000 islets with this vector at 1000 MOI to avoid any toxic side effects. Unlike the Adv vector, transfection with the bipartite plasmid encoding hVEGF and hIL-1Ra (-hVEGF-hIL-1Ra) produced very little hVEGF and hIL-1Ra (Figure 2) which is consistent with our previous results[14] and is expected since an islet is a cluster of 1000 nondividing cells and difficult to transfect.

Since prolonged incubation with Adv vectors may induce apoptosis and loss of endothelial cells, we decided not to coculture the islets beyond 12 h. We then determined hVEGF and hIL-1Ra gene expression at mRNA level for which we used 18s as an internal control. The mRNA levels of hVEGF and hIL-1Ra increased with increase in MOI (Figure 3), indicating the Adv vector is indeed expressing these two genes as desired. Although hVEGF and hIL-1Ra increased at both protein and mRNA levels with increase in the Adv MOI, these results cannot be directly correlated. Unlike ELISA, we used different primers for different genes (in this case hVEGF and hIL-1Ra) and thus we can only compare the results in relative to the house keeping genes. Also, there is known to be poor correlation between mRNA and protein levels. That is why we have to use housekeeping genes whenever we do real time PCR. Jansen et al. have hypothesized that low correlation of protein and mRNA levels are a result of post-translational modifications.[33]

Transduction process should not affect the normal insulin secretion capacity of human islets, which varies from batch to batch. Therefore, we measured insulin release in response to glucose challenge to assess the islet functional viability. As shown in Figure 4, there was little decrease in the stimulation index when islets were transduced with Adv-hVEGF-hIL-1Ra at the dose of 1000 MOI per 1000 islets. These data indicate that transduction with Adv-hVEGF-hIL-1Ra had only minimal adverse effect on insulin producing β cells of the islets in response to glucose challenge.

Most islet grafts are destroyed due to immune and inflammatory reaction mediated by proinflammatory cytokines. To mimic the in vivo situation of the cytokine mediated insults we incubated islets with a cocktail of IL-1β, TNF-α and INF-γ. Incubation with the inflammatory cytokines induced apoptosis in islets indicated by the increase in caspase-3 (Figure 5), which is a converging point of apoptosis for intrinsic and extrinsic pathways and caspase-6

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and 7 are generated from caspase-3. However, the islets treated with 1000 and 2000 MOI Adv-hVEGF-hIL-1Ra and 1000 MOI of Adv-hIL-1Ra showed significantly lower levels of caspase-3 activity compared to the untransduced islets or the islets transducted with Adv-hVEGF at 1000 MOI and incubated in the cytokine cocktail, possibly due to hIL-1Ra gene expression (Figure 5). Similarly, incubation of islets with the cytokine cocktail significantly increased the number of red cells indicating dying or dead cells (Figure 6), which was much less for the islets transduced with Adv-hVEGF-hIL-1Ra or Adv-hIL-1Ra, even after incubation with the cytokine cocktail. These results are well correlated with the literature where Adv vector encoding IL-1Ra[12] as well as recombinant IL-1Ra[34] have shown to protect islets from inflammatory cytokines. In contrast, there was no increase in caspase-3 after transduction of islets with Adv-hVEGF-hIL-1Ra, Adv-hIL-1Ra or Adv-hVEGF in the absence of inflammatory cytokines, suggesting these Adv vectors are fairly safe at these tested doses.

Effect of Adv-hVEGF-hIL-1Ra transduction on improvement in the survival and function of islets posttransplantation was determined in streptozotocin-induced diabetic NOD-SCID mice. We observed a reduction in blood glucose levels of mice transplanted with Adv-hVEGF-hIL-1Ra transduced islets than that observed with Adv-LacZ-transduced or nontreated islets (Figure 7A). This increase correlated with the higher amount of serum insulin and C-peptide secreted by the islets at day 20 post-transplantation (Figures 7B and 7C). These results indicate that hVEGF and hIL-1Ra coexpression is likely to reduce the islet mass required to achieve normoglycemia. Although we did not examine the apoptosis in graft islet cells, our in vitro data clearly indicated transduction of Adv-hVEGF-hIL-1Ra led to significant reduction of caspase-3 activity, which is the indicator of apoptosis.

Revascularization of islet grafts after transplantation is an important process that influences long-term survival and function of the grafts. Endothelial cells reside within isolated islets and potentially participate in the revascularization process. Immunohistochemical staining in the islet bearing kidney sections at day 20 after transplantation was positive for human insulin, hVEGF and hvWF (Figure 8). 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 the contemporary literature. hvWF is human endothelial cell marker. Therefore, relatively stronger positive staining for hVEGF and hvWF antigens by the Adv-hVEGF-hIL-1Ra transduced islet bearing kidney section groups indicates that blood vessel formation in the transplanted islets was relatively more efficient when islets were transduced with Adv-hVEGF-hIL-1Ra prior to transplantation.

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