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Featured Article

Co-Expression of Vascular Endothelial Growth Factor and Interleukin-1 Receptor Antagonist Improves Human Islet Survival and Function

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Purpose. *Ex vivo* gene therapy approaches can improve the outcome of islet transplantation for treating type I diabetes. We have previously shown the improvement in islet function and vascularization following *ex vivo* transfection for human vascular endothelial growth factor (hVEGF) gene expression. In this study, we tested the hypothesis that co-expression of two genes, which target different challenges faced by islets post-transplantation, supplement each other to improve the survival and function of islets. We determined whether there is an additive effect of hVEGF and human interleukin-1 receptor antagonist (hIL-1Ra) gene expression in human islets.

Materials and Methods. Human islets were co-infected with adenoviral vectors encoding hVEGF and hIL-1Ra. Islets were then incubated with a cocktail of inflammatory cytokines (IL-1β + TNFα + IFNγ), and islet viability and function were determined. *In vivo* function was evaluated by transplanting islets under the kidney capsules of streptozotocin-induced non-obese diabetic severe combined immunodeficient (NOD-SCID) mice.

Results. Infection of human islets with Adv-hVEGF and/or Adv-hIL-1Ra inhibited expression of inducible nitric oxide synthase (iNOS), decreased the production of nitric oxide (NO), and prevented the loss of *in vitro* glucose-stimulated insulin response and viability. Moreover, co-expression 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.

Conclusions. Our results indicated that co-expression of genes that target different insults to transplanted islets can improve the outcome of islet transplantation better than either gene alone.

KEY WORDS: adenovirus; diabetes; gene therapy; interleukin-1 receptor antagonist; transplantation; vascular endothelial growth factor.

INTRODUCTION

Human islet transplantation is a potential treatment for type I diabetes. However, a large number of islets (>90,000 IE) are required to obtain insulin independence in clinical islet transplantation, requiring two to four cadaveric pancreata (1,2). This is because of the limited survival and function of transplanted islets in the host tissue (3). In this context, *ex vivo* gene therapy is a potential strategy to modify the biochemical environment in the immediate vicinity of the transplanted islets.

Islet isolation and purification processes disrupt the intra-islet microvasculature (4), which needs to be rebuilt and well-connected to the host perfusion system to prevent hypoxic cell death in the core of islets. Thus, increasing revascularization has a role in improving their survival and function post-transplantation (5–8). We and others have shown that hVEGF expression promotes new blood vessel formation and improves the outcome of islet transplantation (5,9,10). We previously transfected islets with Lipofectamine/pCAGGS-hVEGF complexes preceding transplantation under the kidney capsules of NOD-SCID mice. We demonstrated that hVEGF gene expression promotes new blood vessel formation after islet transplantation, with the involvement of endothelial cells of both the host (mouse) and the donor (human). Even though the occurrence of revascularization of the transplanted islets could be seen by immunohistochemical staining, transfection efficiency was very low (5). Therefore, we decided to replace our non-viral systems with a bicistronic vector encoding green fluorescent protein (GFP) and hVEGF. We observed high levels of hVEGF gene expression, with little adverse effect on the cellular and functional viability of human islets (7). Our results are in good agreement with the work of Zhang *et al.* (9), who reported that *ex vivo* infection of murine islets with

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ABBREVIATIONS: Adv, adenoviral; hIL-1Ra, human interleukin-1 receptor antagonist; hVEGF, human vascular endothelial growth factor; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NOD-SCID, non-obese diabetic severe combined immunodeficient.

Adv-hVEGF followed by transplantation under the kidney capsules resulted in elevated VEGF expression and enhanced islet revascularization.

Effective prevention of islet destruction after transplantation requires not only revascularization of islets, but also abrogation of cytokine-mediated islet cell death and dysfunction triggered by immune and inflammatory reactions. Islet injury stimulates resident islet macrophages and passenger leukocytes to produce cytotoxic cytokines IL-1β, TNFα, and IFNγ. These lead to islet dysfunction and death by NFκB/iNOS/NO pathway (Fig. 1) (11,12). Inhibition of IL-1β receptor binding using human interleukin-1 receptor antagonist (hIL-1Ra) has been shown to prevent suppression of islet function and NO production in mouse (13), rat (14), and human (15) islets. In this study, we determined whether co-expression of hVEGF and hIL-1Ra has a beneficial effect in improving islet survival and function.

MATERIALS AND METHODS

Adenoviral Vectors

Replication deficient serotype 5 Adv vectors encoding hIL-1Ra (gift from Dr. Paul Robbins; University of Pittsburgh, PA) and hVEGF (Invivogen) were chosen to achieve transient gene expression, while minimizing potential side effects and *in vivo* replication. Advs were propagated by transfection of human embryonic kidney 293 cells and quantitated by plaque assay. Titers of amplified Advs were observed in the range of 1×10^8 to 8×10^9 plaque forming units (pfu) per milliliter.

Islet Culture, Transfection, and Gene Expression

Human islets were received from one of the several Islet Cell Resource (ICR) Centers through ICR Services for Basic Science Applications, in culture media at 4°C. On arrival at our facility, islets were cultured overnight in Memphis-Serum Free Media (M-SFM) composed of CMRL 1066 medium containing 1% ITS, 1% L-glutamine, 1% pencillin/streptomycin, 1% albumin, and 16.8 μM zinc sulfate (pH 7.40 ± 0.05) at 37°C. After overnight culture, islets were visualized under the microscope for morphological integrity, percent purity and viability by trypan blue exclusion and used for experiments immediately.

Islets were transfected using Adv-hIL1-Ra, using blank adenovirus (Adv-BL)-transfected and non-transfected islets as controls. To determine the optimal multiplicity of infection (MOI) of Adv in terms of pfu per islet equivalent (IE), 1,000 IE were incubated with diluted Adv in 300 μl media in a 96-well plate for 12 h, followed by washing and further culture in 2 ml media in 24-well plates. Gene expression was monitored at days 1, 3, 5, 7 and 10 by quantifying secreted protein in the media by ELISA (R&D Systems). At day 10, cell pellet was collected and total RNA was isolated by guanidine isothiocyanate method using RNA STAT60 reagent (Tel-Test, Friendswood, TX). One μg of isolated

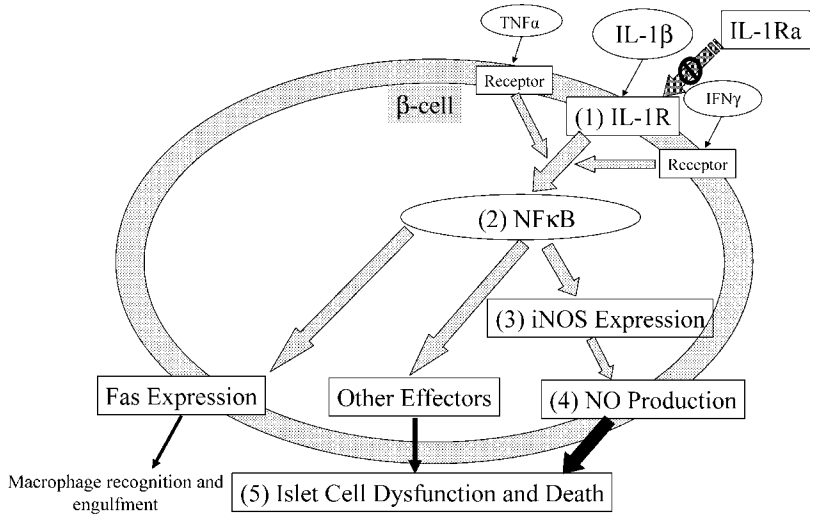

Fig. 1. Intracellular cascade overview model of islet cell death pathways induced by inflammatory cytokines. Interleukin-1β (IL-1β) activates IL-1 receptors (IL-1R) (*Step 1*) which initiates an intracellular cascade of biochemical events. Its effect is potentiated by tumor necrosis factor α (TNFα) and interferon γ (IFNγ). These pathways lead to downstream activation of nuclear factor κB (NFκB) expression (*Step 2*) followed by its nuclear translocation and activation of target gene transcription. Upregulation of inducible nitric oxide synthase (iNOS) (*Step 3*) and expression of cytotoxic nitric oxide (NO) (*Step 4*) predominantly mediates cytotoxic effects on islets (*Step 5*). Other pathways *e.g.,* generation of reactive oxygen species (ROS) also contribute to islet cell dysnfuction and death. Still other pathways, *e.g.,* Fas expression, mediate destruction of transplanted islets by host immune cells. Upstream inhibition of cytokine-mediated NFκB/iNOS/NO pathway by IL-1Ra antagonism of IL-1β activation of IL-1R can improve the outcome of islet transplantation.

RNA was then reverse transcribed using MultiScribe reverse transcriptase reagents and random hexamers (Applied Biosystems, Foster City, CA). The cDNA (100 ng) was amplified by real-time PCR (5–7) using forward primer from hIL-1Ra (5′-AGAGCTCGTTTAGTGAACCGTAAG-3′) and reverse primer from the vector (5′-ACAGGAAGAGGAGGAGA GTGATTA-3′; pAdlox; Gene Bank #U62024, Amplicon size: 113 bp). Primers for hVEGF were described earlier (7). Gene expression was normalized to 18S rRNA as internal control using following primers: Fwd: 5′-ACATCCAAGGAAGG CAGCAG-3′; Rev: 5′-TGATCGTCTTCGAACCTCCG-3′ (Gene Bank #K034032; Amplicon size: 602 bp).

Effect of hIL-1Ra and/or hVEGF expression on cytokine-mediated NO production, iNOS expression, and islet function was determined in (1) non-transfected islets, (2) Adv-BL transfected islets at 1,000 MOI, (3) Adv-hVEGF+Adv-BL transfected islets at 500 MOI each, (4) Adv-hIL-1Ra+Adv-BL transfected islets at 500 MOI each, and (5) Adv-hVEGF+Adv-hIL-1Ra transfected islets at 500 MOI each, using non-transfected islets without cytokine treatment as control. At 24 h post-transfection, islets were incubated with the cytokine cocktail of IL-1β (75 U/ml) + TNFα (1,000 U/ml) + IFNγ (1,000 U/ml) (16–18). At 96-h post-cytokine incubation, human iNOS mRNA levels were determined by real-time RT-PCR using following primers and normalized to 18S rRNA expression: Forward: 5′-TTTCCTTACGAGGC GAAGAA-3′, Reverse: 5′-TCAGAGCGCTGACATCTCC-3′ (Gene Bank #L09210, Amplicon size 69 bp).

Nitrite Production and Insulin Release

Human islets (2000 IE per sample) were treated as above and incubated with cytokine cocktail in 250 μl media for NO assay and 2 ml for static incubation assay. NO production was determined in undiluted media by measuring the concentration of nitrite, a stable, non-volatile metabolite of NO, by colorimetry using Griess assay (Promega). Islet function was assessed by determining the stimulation index by *in vitro* static incubation assay. Islets were suspended in a 10 ml plastic column (Bio-Rad) with overnight hydrated (in PBS) Sephadex G10 followed by washing and incubation in 60 mg/dl (basal) and 300 mg/dl (stimulated) glucose-containing media for 1 h each, after initial equilibriation with the basal medium for 1 h. Samples were analyzed for the amount of secreted human insulin by ELISA (Alpco Diagnostics). Stimulation index was determined as the ratio of amount of insulin secreted at 300 mg/dl and 60 mg/dl glucose concentrations.

Nucleosome Concentration and Caspase Assay

Apoptotic and necrotic cell death levels in transfected and cytokine-treated samples, prepared as above, were determined by measuring relative nucleosome concentration in cytoplasmic lysates and cell culture supernatants, respectively, by Cell Death Detection ELISA^{Plus} kit (Roche). Role of cytokine composition on the cytoprotective effect of Adv-hIL-1Ra was determined by incubating Adv-BL or Adv-hIL-1Ra transfected islets with IL-β alone, TNFα + IFNγ, or IL-1β + TNFα + IFNγ, using no cytokine treatment group as a control, followed by determination of nucleosome concentration.

Total caspase activity in groups of 250 transfected and non-transfected islets incubated with cytokines, as above, was determined using Homogeneous Caspases Assay Kit (Roche), alongwith positive, negative and background controls and R-110 standards (free Rhodamine 100) in various concentrations. Substrate was incubated with cell lysate for 12 h at 37°C followed by determination of level of cleaved, free R-110 by fluorimetry. Results were expressed as the mean ± S.E. of the relative units of caspase activity per milligram of total protein, determined by BCA assay (Pierce).

Fluorescence Microscopy

To visualize islet viability in descriptive terms, islets, treated as above, were washed with PBS, resuspended in 100 μl media, stained with a freshly prepared solution of 4 μM Calcein AM and a 2 μM solution of ethidium homodimer-1 (EthD-1) in PBS (Invitrogen), and visualized under fluorescence and confocal microscopes (Carl Zeiss).

Islet Function Post-transplantation

To determine the effect of *ex vivo* transfection of islets on *in vivo* islet function, 2000 human islets were infected as above and at 24 h of infection, washed, resuspended in 100 μl PBS, and transplanted under the kidney capsule of diabetic NOD-SCID mice (age: 6–8 weeks; weight 21–24 g; sex: male; source: Jackson Labs), as described before (19). These mice were rendered diabetic by intraperitoneal injection of streptoxotocin (125 mg/kg). Blood glucose levels >500 mg/dl on days 5 and 7 after streptozotocin injection were considered diabetic. Animals that did not become diabetic received another injection of streptozotocin on day 7. Islets were transplanted 1–3 weeks post-diabetes induction. Insulin (Humulin U100, Eli Lilly) was injected at a dose adjusted to blood glucose level and weight, once a day for the first 7 days to support transplanted islets. At 3 week post-transplantation, mice were challenged with 10 g/kg intraperitoneal glucose after overnight fasting, followed by blood glucose, insulin, and C-peptide determination at 30 min post-injection. Blood levels of human insulin were determined by ELISA (Alpco Diagnostics; 0.7% cross reactivity with murine insulin) and C-peptide by RIA (Diagnostic Products Corporation). This experiment was repeated thrice (n = 5).

Statistical Analysis

Statistical significance of the difference between two groups was determined by unpaired t-test and between several groups by one-way ANOVA using SPSS Software.

Animal Care and Use

All animal experiments were performed as per NIH (http://grants1.nih.gov/grants/olaw/references/phspol.htm) and institutional animal care and use guidelines using approved protocols.

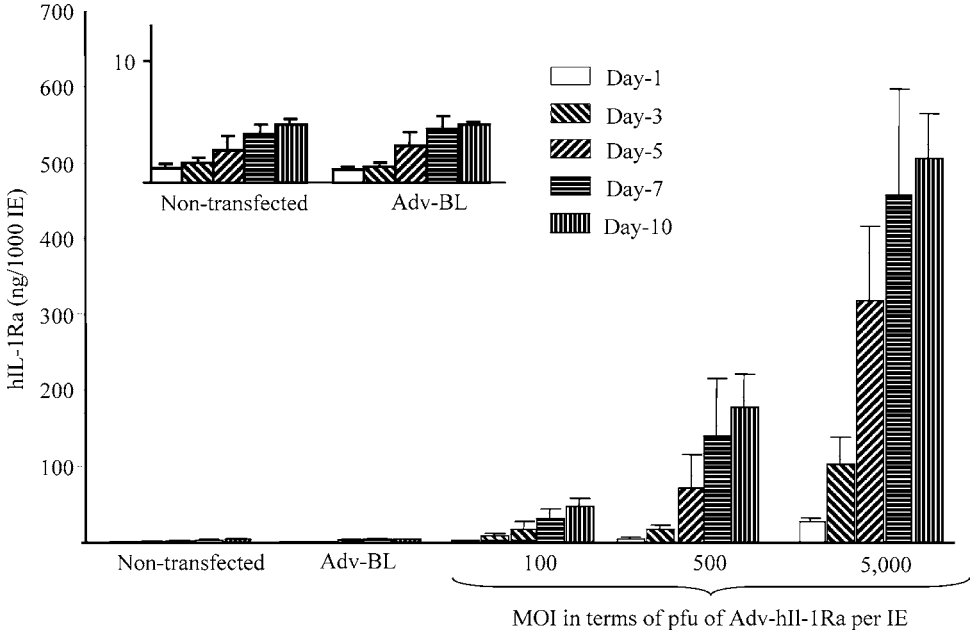

Fig. 2. Effect of multiplicities of infection (MOI) of Adv-hIL-1Ra on human Interleukin-1 Receptor antagonist (hIL-1Ra) gene expression from human islets. MOI represent plaque forming units (pfu) of adenovirus used per islet equivalent (IE). Blank adenovirus (Adv-BL) infected islets and non-infected (NT) islets were used as controls. Supernatants were collected on days 1, 3, 5, 7, and 10 and analyzed for the expression of hIL-1Ra by ELISA using the vendor protocol (R&D Systems, Minneapolis, MN).

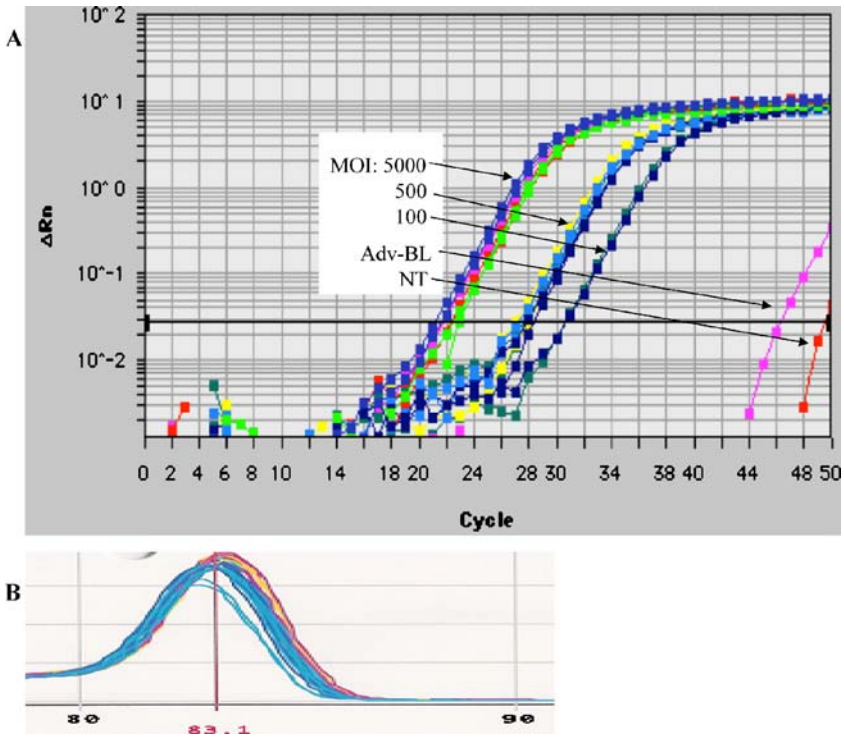

Fig. 3. Real time RT-PCR of human islets at day 10 post-infection with different MOIs of Adv-hIL-1Ra (A) and melting curve of amplified transcripts (B). Forward primer was designed from the gene and the reverse primer from the vector to exclude the detection of endogenously expressed hIL-1Ra. Adv-BL infected islets and non-infected (NT) islets were used as controls. Cell pellet was collected at day 10, processed for mRNA extraction, reverse transcription and real time PCR using SYBR Green I chemistry.

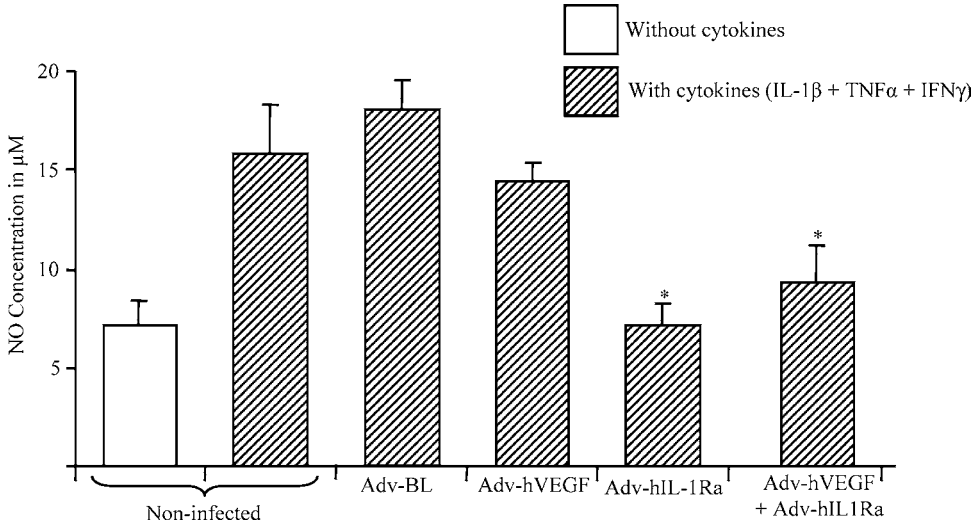

Fig. 4. Effect of Adv-hVEGF and/or Adv-hIL-1Ra infection on nitric oxide (NO) production by human pancreatic islets upon incubation with cytokine cocktail, consisting of IL-1β, TNFα, and IFNγ, for 4 days at 37°C in humidified 5% CO_2 incubator. Non-infected and Adv-BL infected islets were used as controls. NO production was determined in the form of nitrite in the cell culture supernatant by Griess assay and presented as mean ± SE of $n = 3$ as picomoles of total nitrite released by 250 islet equivalents (IE). *, $p < 0.05$ compared with Adv-BL-infected islets with cytokine treatment.

RESULTS

Infection Efficiency of Adv-hIL-1Ra

The level and duration of hIL-1Ra protein expression at days 1, 3, 5, 7, and 10 post-infection (Fig. 2) showed a linear increase with viral dose ($R^2 > 0.99$). At day 10, cumulative hIL-1Ra secretion from Adv-hIL-1Ra infected islets was ~1,000 times that of control, mock-transfected islets, indicating that Adv-hIL-1Ra infection would lead to significantly high local concentrations of hIL-1Ra in the immediate vicinity of islets post-transplantation.

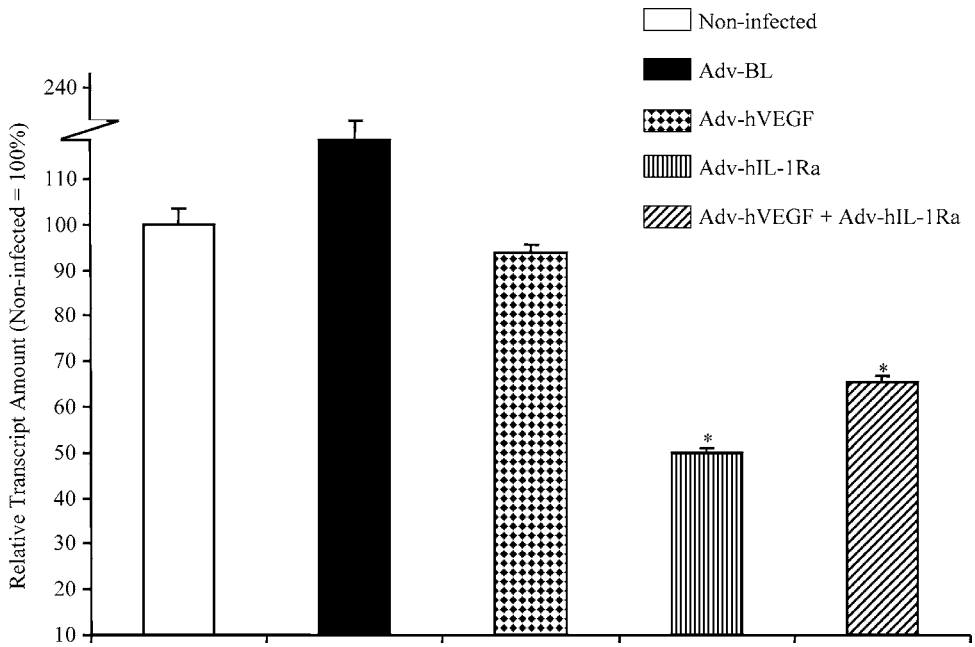

Fig. 5. Effect of infection with Adv-hVEGF and Adv-hIL-1Ra on inducible Nitric Oxide Synthase (iNOS) gene expression in human islets. Following infection, islets were incubated with inflammatory cytokines (IL-1β, TNFα, and IFNγ). Non-infected (NT) islets and islets infected with blank adenovirus (Adv-BL) were used as controls. Expression of iNOS was determined by real-time RT-PCR using SYBR Green I chemistry as described in 'Materials and Methods' and normalized to the expression level of 18S rRNA as the housekeeping gene. Gene expression level in terms of relative amount of transcript (non-infected group normalized to 100%) was calculated for $n = 3$ and presented as Mean ± SE. *, $p < 0.05$ compared with Adv-BL-infected islets with cytokine treatment.

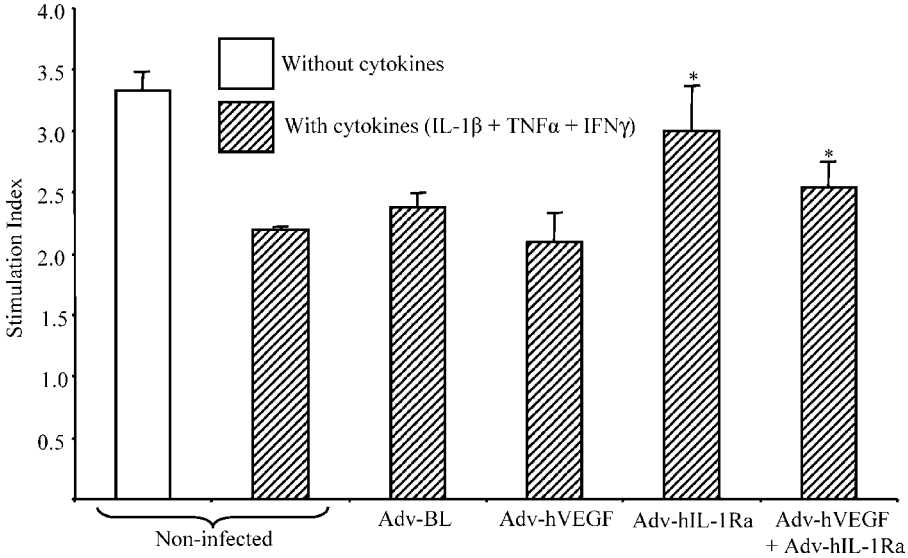

Fig. 6. Stimulation Index (SI) of human islets with or without cytokine incubation after infection with Adv-hVEGF and/or Adv-hIL-1Ra. Non-infected (NT) islets and islets infected with blank adenovirus (Adv-BL) were used as controls. SI was determined as the ratio of the amount of insulin secretion at 300 mg/dl concentration of glucose in the media to that at 60 mg/dl concentration upon incubation for 1 h period. Insulin concentrations were determined by insulin enzyme immunoassay (EIA) using vendor protocol (Alpco Diagnostics, Wandham, MA). Presented results are representative of three replications of this experiment. *, $p < 0.05$ compared with Adv-BL-infected islets with cytokine treatment.

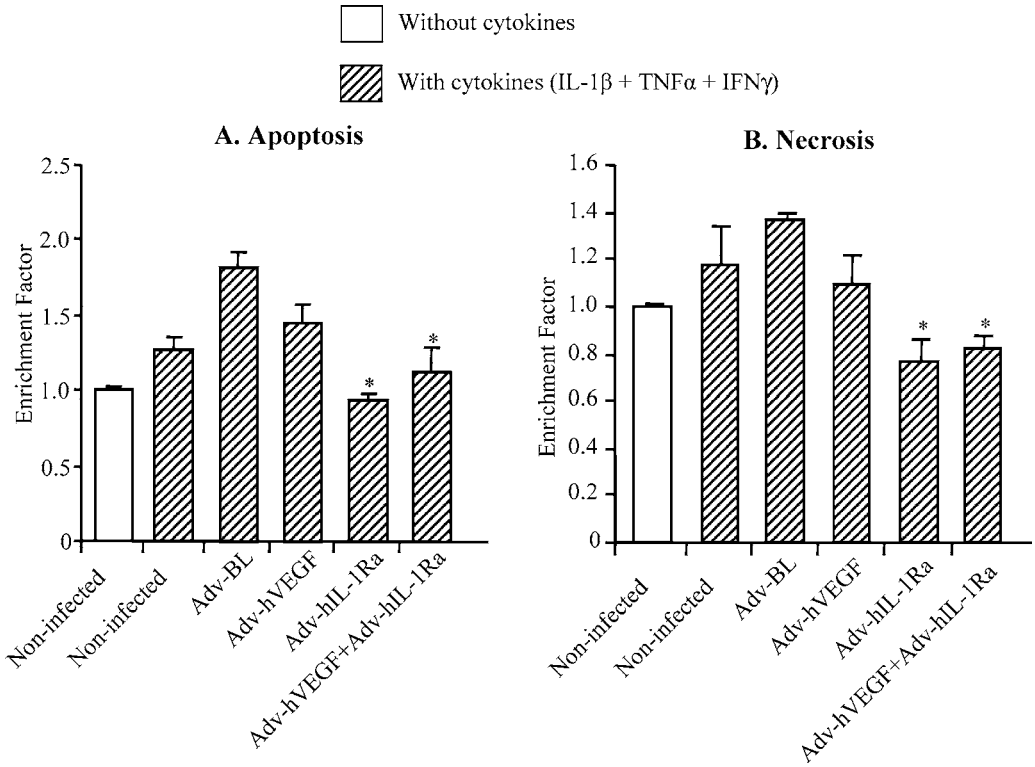

Fig. 7. Apoptotic (A) and necrotic (B) cell death in the islets without or with cytokine exposure for 4 days, after infection with Adv-hVEGF and/or Adv-hIL-1Ra. Non-infected (NT) islets and islets infected with blank adenovirus (Adv-BL) were used as controls. Relative cell death was determined by measuring nucleosome concentration in the cell lysates or culture supernatants for apoptosis and necrosis, respectively, by ELISA using vendor protocol (Cell Death Detection ELISA^{Plus}, Roche Applied Sciences, Indianapolis, IN). Data were normalized to untreated controls and expressed as a ratio of nucleosome concentration, called enrichment factor. Reported values are Mean ± SE of $n = 4$. *, $p < 0.05$ compared with Adv-BL-infected islets with cytokine treatment.

Messenger RNA levels of hIL-1Ra were determined by real-time RT-PCR. To exclude the detection of endogenous hIL-1Ra (5–7), we used forward primer from hIL-1Ra gene sequence and reverse primer from the vector backbone. As shown in Fig. 3A, there was an increase in hIL-1Ra mRNA expression with increase in the MOI of Adv-hIL-1Ra, while Adv-BL infected and non-infected islets showed very little hIL-1Ra expression ($C_T > 44$). Amplification specificity was confirmed by melting curve analysis (Fig. 3B), which showed a single, predominant peak of 83.1°C. The extent of 18S rRNA (20) amplification was same for all samples (not shown).

NO and iNOS Levels

Cytoprotective effect of hVEGF and/or hIL-1Ra expression was determined in terms of NO levels. Mock transfected islets produced detectable levels of NO (6.94 ± 1.32 μM) in the absence of cytokine treatment, but increased significantly (15.72 ± 1.84 μM) upon cytokine addition. Pre-transfection of islets with Adv-hIL-1Ra prevented this increase (7.24 ± 0.68 μM), but infection with Adv-hVEGF did not (14.48 ± 0.92 μM). Infection of islets with a mixture of Adv-hVEGF and Adv-hIL-1Ra showed only a moderate increase (9.24 ± 1.36 μM) upon cytokine incubation (Fig. 4).

Expression of iNOS mRNA in the presence of cytokines was several fold higher than without cytokines (not shown). In the cytokine treated samples, Adv-hIL-1Ra infection significantly reduced iNOS expression, as compared to non-infected controls (Fig. 5). Adv-hVEGF had little effect on iNOS expression, which decreased significantly upon infection with a mixture of Adv-hVEGF and Adv-hIL-1Ra. These observations correlated well with the reduction in NO production.

Islet Function

Incubation of islets with inflammatory cytokines reduced the stimulation index of islets, as compared to islets without cytokine treatment (Fig. 6). Infection of islets with Adv-hVEGF did not inhibit cytokine-mediated impairment of insulin secretion. In contrast, infection with Adv-hIL-1Ra prevented this impairment, resulting in almost identical stimulation index to that of non-infected islets without cytokine treatment. The co-infected islets had only a moderate decrease in islet function, indicating persistence of cytoprotective effects of Adv-hIL-1Ra.

Apoptotic and Necrotic Cell Death

Islet cell death following both cytokine incubation and adenoviral infection can proceed via either apoptotic or necrotic pathway. Apoptotic cells have nucleosome accumulation in the cytoplasm, while necrotic cells undergo membrane disruption leading to nucleosome release in the cell culture medium. Hence, apoptotic and necrotic cell death

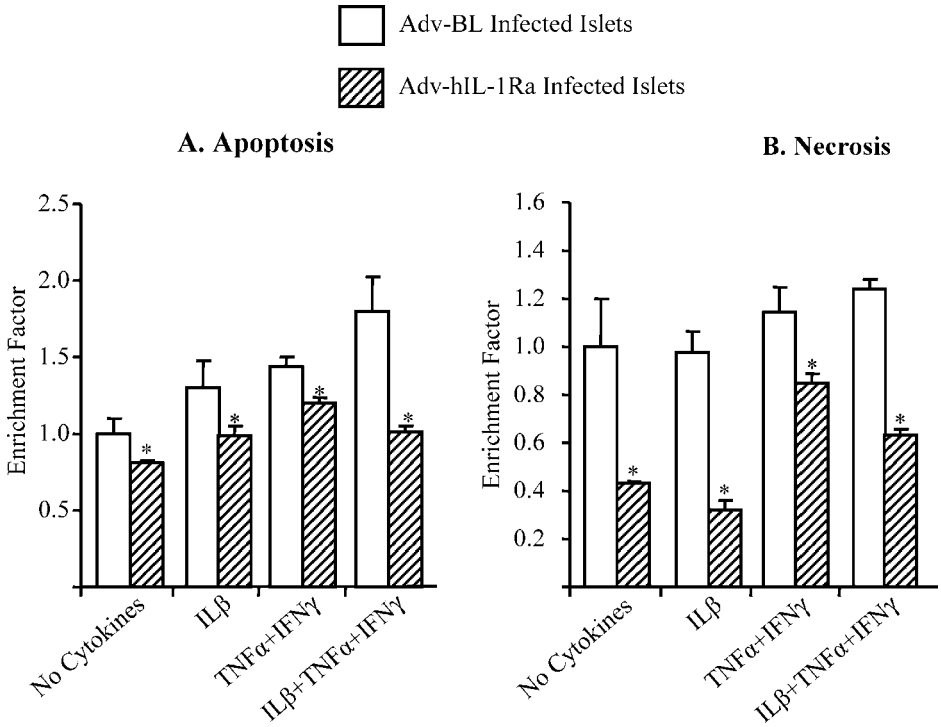

Fig. 8. Apoptotic (A) and necrotic (B) cell death in the islets after Adv-BL and Adv-hIL-1Ra infection followed by incubation with IL-1β, (TNFα + IFNγ), or (IL-1β + TNFα + IFNγ) using no cytokine incubation samples as control. At 24 h of infection, islets were incubated with cytokines as above for 6 days followed by determination of relative cell death by measuring nucleosome concentration in the cell lysates or culture supernatants for apoptosis and necrosis, respectively. Data were normalized to controls and expressed as a ratio of nucleosome concentration, called enrichment factor. Reported values are Mean ± SE of $n = 5$. *, $p < 0.05$ compared with corresponding Adv-BL-infected islets.

were determined in terms of relative nucleosome concentration in the cytoplasmic fraction of islet cell lysates and cell culture supernatants, respectively. Nucleosome concentration was expressed as enrichment factor (EF), which is a ratio of absorbance in the test samples to non-infected, non-cytokine-treated islets and could vary from 0 to 3 in these experiments. Thus, higher EF indicated greater cell death, relative to background levels (EF = 1) (Figs. 7 and 8).

We observed an increase in islet apoptosis (by 28%) and necrosis (by 17%) after cytokine treatment, which was further increased upon Adv-BL infection (Fig. 7). Also, apoptosis was higher than necrosis. Expression of hIL-1Ra significantly reduced both apoptosis and necrosis, and its effect persisted upon co-expression with hVEGF. Since hIL-1Ra only antagonizes IL-1β actions on its receptors, we investigated whether Adv-hIL-1Ra infection counteracted TNFα and IFNγ mediated potentiation of IL-1β actions on the islets. Apoptotic islet cell death was increased by 30% upon incubation with IL-1β alone and by 44% with TNFα+IFNγ (EF of 1.30 ± 0.17 and 1.44 ± 0.07, respectively, Fig. 8A). Incubation with all three cytokines together showed greater cell death (80% higher than control) than either group alone (EF 1.80 ± 0.23). Although IL-1β is expected to be more cytotoxic than TNFα + IFNγ, higher apoptosis was observed in the TNFα+IFNγ group. This was attributed to the release of endogenous IL-1β by the islets. Thus, Adv-hIL-1Ra infection reduced of TNFα + IFNγ induced apoptosis by 24%. Islet cell death induced by IL-1β with TNFα and IFNγ was greater than IL-1β alone. In both cases, infection with Adv-hIL-1Ra reduced the level of cell death to control levels (Fig. 8A). Similar observations were noted for necrosis.

While hVEGF and hIL-1Ra gene expression could partly suppressed apoptosis and necrosis compared to blank adenoviral (Adv-BL) infected islet groups, this beneficial effect was minimal when islets were incubated with IL-1β, IFN-γ and TNF-α and compared with non-infected islet groups (Figs. 7 and 8), suggesting that infection of islets with Adv adversely affects human islets.

Total Caspase Activity

Caspases not only play a central role in apoptotic cell death, but are also associated with immune responses. Therefore, we determined total caspase activity in infected and non-infected islets at 4 days post-incubation with cytokines using a substrate solution consisting of tetrapeptide Asp-Glu-Val-Asp (DEVD) coupled to Rhodamine-110. Upon cell lysis and cleavage by cytoplasmic caspases, free rhodamine is released, which is quantitated florimetrically. As shown in Fig. 9, the total caspase activity of cell extracts increased with the addition of cytokine cocktail, which increased further with Adv-BL transfection. hIL-1Ra expression reduced caspase activity to ~30% of non-infected islets without cytokine treatment. Furthermore, co-expression of hIL-1Ra and hVEGF showed persistence of beneficial effect of hIL-1Ra expression.

Islet Viability by Fluorescence Microscopy

The viability of islets after incubation with inflammatory cytokines for 4 days, with or without infection with Adv-hVEGF and/or Adv-hIL-1Ra, was visualized using Calcein^{AM} and EthD-1. Calcein^{AM} is a membrane permeable lipophilic ester of polyanionic calcein. Intracellularly, it is cleaved by esterases in live cells to membrane impermeable, green fluorescent calcein. In contrast, EthD-1 enters cells only

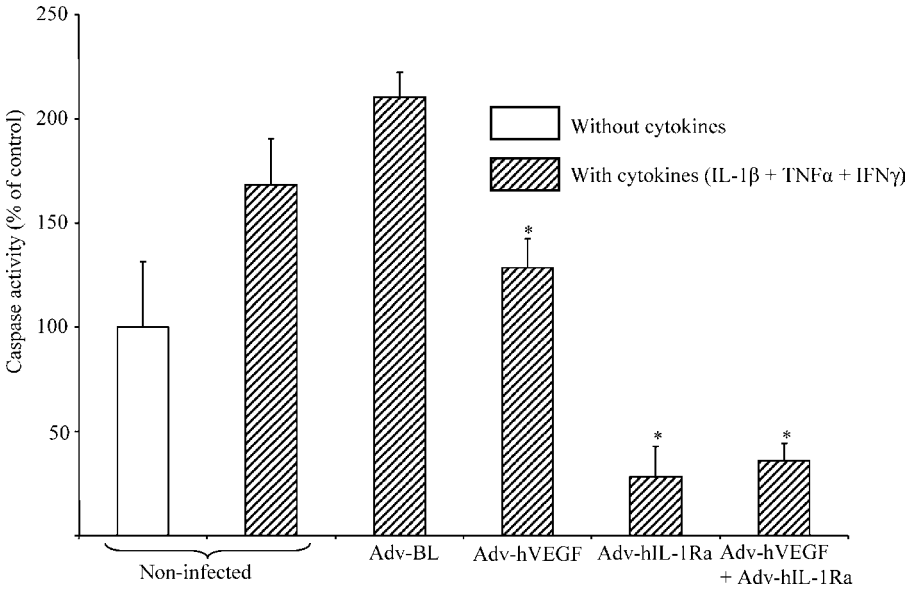

Fig. 9. Effect of transgene expression on the activation of caspases in human islets. Islets were infected with Adv-hVEGF and/or Adv-hIL-1Ra using Adv-BL and non-infected islets as controls. Following infection, islets were incubated with inflammatory cytokines (IL-1β, TNFα, and IFNγ) for 4 days at 37°C in humidified 5% CO_2 incubator. Thereafter, islets were lysed and relative total caspase activity was determined by fluorimetric assay, and expressed as nanomolar of free rhodamine—which corresponds to the amount of substrate cleaved by total caspases. Reported values are Mean ± SE of $n = 3$. *, $p < 0.05$ compared with Adv-BL-infected islets with cytokine treatment.

those cells with damaged cell membranes and shows intense red fluorescence, which is indicative of dead or apoptotic cells. Thus, staining of islets with a mixture of both these dyes in optimized concentrations permits visualization of both live and dead cells in the same islet. Images of representative single islets are presented in Fig. 10, with the same islet imaged in green and red fluorescence mode. Non-infected islets without cytokine incubation showed high viability, which was significantly reduced upon cytokine incubation. Infection of islets with Adv-hVEGF and Adv-hIL-1Ra was seen to have beneficial effect on islet viability, which persisted in the co-infected group.

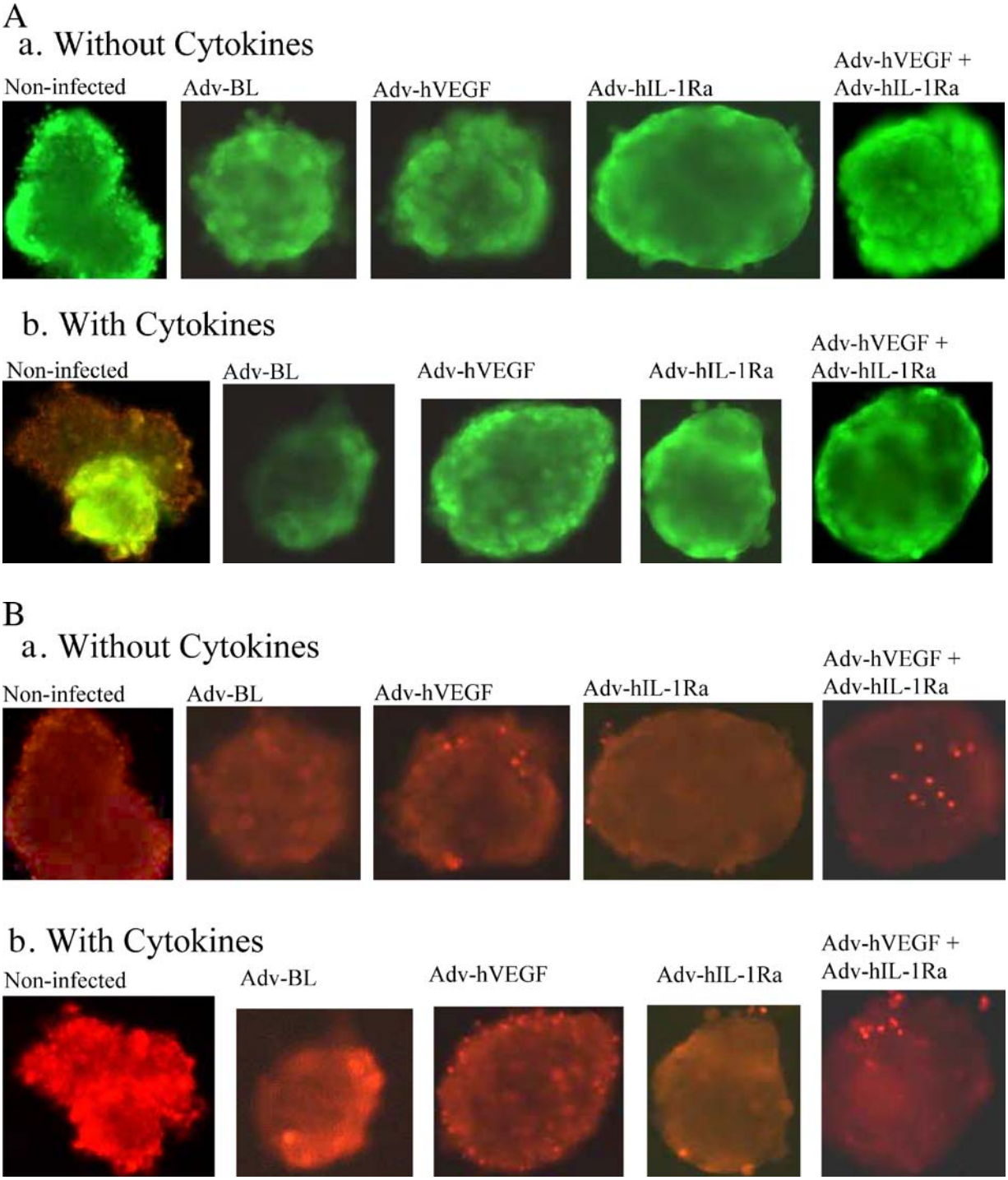

Fig. 10. Effect of transgene expression on islet viability was examined visually using Calcein^{AM} and Ethidium homodimer-1 (EthD-1 staining of intact islets after infection with Adv-hVEGF and/or Adv-hIL-1Ra using Adv-BL and non-infected islets as controls, followed by 4 days of incubation with inflammatory cytokines. Representative images of intact islets indicating live (A) and dead (B) cells are shown for all test groups.

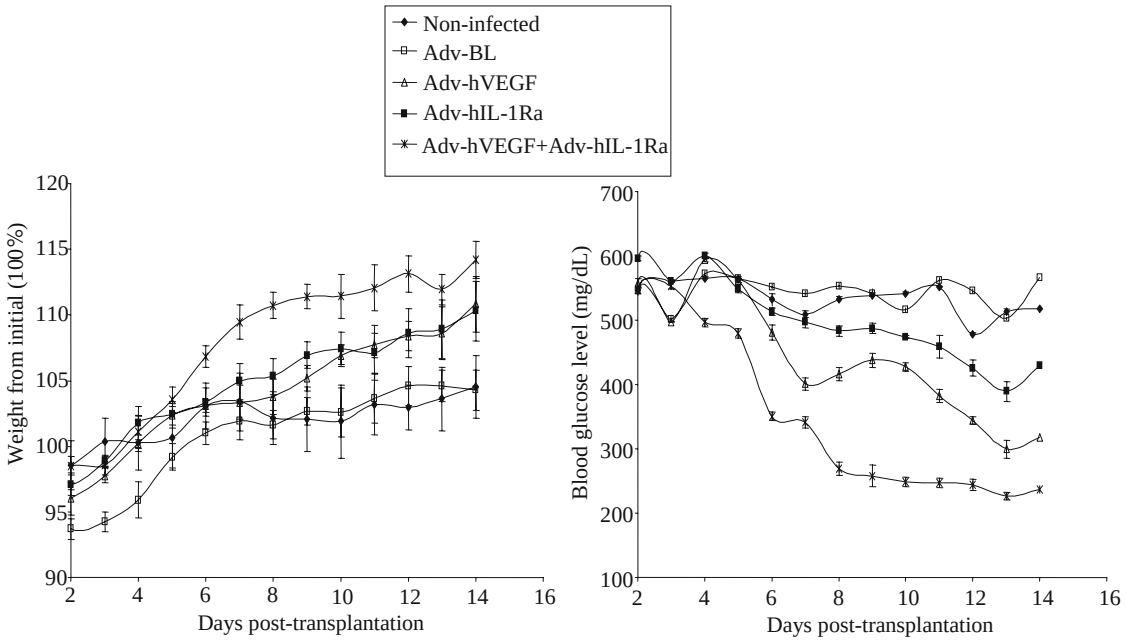

Fig. 11. Effect of islet infection on mice weight (A) and blood glucose (B) levels post-transplantation. Two thousand islets were transplanted under the kidney capsule of streptozotocin-induced diabetic NOD-SCID mice after infection with Adv-hIL-1Ra and/or Adv-hVEGF using Adv-BL and non-infected islets as controls. Mice were monitored daily for weight and blood glucose levels. Weight was calculated as percent of initial weight and blood glucose concentration was measured by glucometer strips. Data are presented as Mean ± SE of percent weight change from initial (A) and blood glucose concentration in milligrams per deciliter (B) for $n = 5$ mice per group.

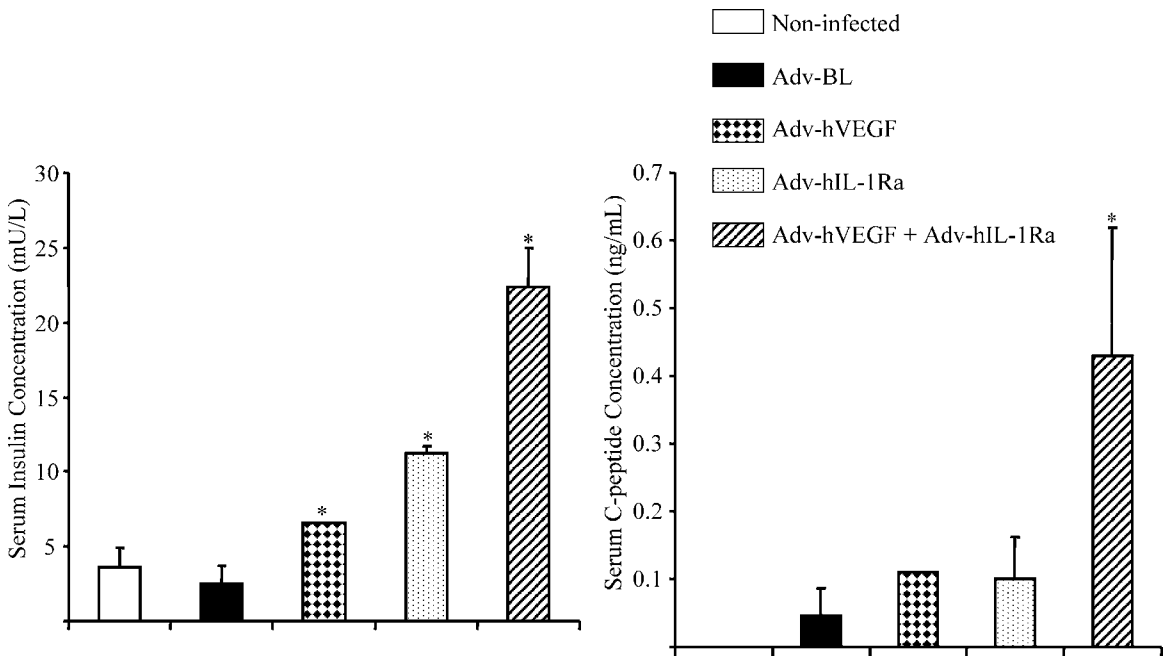

Fig. 12. Effect of islet infection on islet function in terms of human insulin (A) and C-peptide (B) expression by the transplanted islets. Two thousand islets were transplanted under the kidney capsule of streptozotocin-induced diabetic NOD-SCID mice after infection with Adv-hIL-1Ra and/or Adv-hVEGF using Adv-BL and non-infected islets as controls. Three weeks post transplantation, mice were challenged with 300 μl intraperitoneal injection of 50% dextrose solution after overnight fasting, followed by blood sampling 30 min later. Blood insulin and C-peptide levels were determined using enzyme linked immunoassay (EIA) and radioimmunoassay (RIA), respectively, using vendor protocols (Alpco Diagnositcs, Wintham, NH for Insulin EIA and Diagnostic Products Corporation, Los Angeles, CA for C-peptide RIA). Data are presented as Mean ± SE of $n = 4, 4, 1, 3$, and 3 for non-infected, Adv-BL, Adv-hVEGF, Adv-hIL-1Ra and Adv-hVEGF + Adv-hIL-1Ra infected groups, respectively. *, $p < 0.05$ compared with corresponding Adv-BL-infected islets.

Islet Survival and Function After Transplantation

Effect of gene transfer on islet survival and function post-transplantation was determined in terms of (i) weight, (ii) blood glucose, (iii) insulin, and (iv) C-peptide levels (Figs. 11 and 12). In addition, (v) time required for reduction of blood glucose was noted. Although none of the infected groups led to normoglycemia, diabetes was partly corrected in higher number of mice receiving Adv-hIL-1Ra and/or Adv-hVEGF infected islets as compared to those receiving Adv-BL infected and non-infected islets. Adv-hVEGF+Adv-hIL-1Ra infected islets showed more rapid reduction in blood glucose levels to <500 mg/dl (4–6 days) than Adv-hIL-1Ra (8–10 days) infected islets. Weight of mice receiving Adv-hIL-1Ra+Adv-hVEGF infected islets increased at a higher rate than all other groups (Fig. 11A). In terms of blood glucose levels, mice transplanted with Adv-hVEGF+Adv-hIL-1Ra infected islets had the lowest ambient blood glucose levels, followed by Adv-hVEGF and Adv-hIL-1Ra infected, which were better than non-infected and Adv-BL infected islets (Fig. 11B). Upon glucose challenge, both blood insulin and C-peptide levels were significantly higher in mice transplanted with Adv-hVEGF+Adv-hIL-1Ra infected islets compared to all other groups, indicating better islet engraftment and function (Fig. 12).

DISCUSSION

Addressing different challenges to islets post-transplantation is required to improve the success of islet transplantation. Recent studies used the expression of more than one gene targeting the same pathway to evaluate their possibly synergistic effects (21,22). Thus, Bertera *et al.* co-infected mouse islets with Adv vectors expressing indoleamine-2,3-dioxygenase, manganese superoxide dismutase (MnSOD), and IL-1Ra (21); while Mysore *et al.* generated transgenic mice expressing extracellular SOD with or without human copper/zinc SOD, and/or cellular glutathione peroxidase (22). These studies, however, focused on expressing multiple genes that target islet destruction by a single pathway, *i.e.*, oxidation stress (22) and immune/inflammation (21).

In this study, we have investigated the possible additive effect of expressing genes that intercept two very different pathways of islet destruction, viz., revascularization and cytokine-mediated destruction. More importantly, we targeted the expression of secreted proteins from the islets. This is because Adv-mediated co-expression of two genes in human islets infects only a limited number of cells (~24%), of which only a fraction (~14%) get infected with both genes (21). This limits the use of co-expression of non-secreted proteins, since both proteins must be expressed in the same cell to have synergistic action. In our studies, we selected two secreted proteins, in which case the total amount secreted by islets is more important, thus obviating the issues of expression in all cells and co-expression of both proteins in the same cell. Furthermore, total amount of protein secreted by human islets upon co-infection with two Adv vectors was similar to single Adv-infection at the same MOI (not shown). This was also shown by Bertera *et al.* in terms of percentage of cells expressing a given gene upon single *vs.* co-infection (21).

In line with our previous results with Adv-hVEGF (7), Adv-hIL-1Ra infection resulted in a dose and incubation time dependent gene expression (Figs. 2 and 3). To mimic the *in vivo* situation of cytokine-mediated insults, we incubated islets with a cocktail of IL-1β + TNFα + IFNγ (16–18). Incubation with these cytokines increased NO production by ~2-fold (Fig. 4). Expression of iNOS in the absence of cytokines was several fold lower (not shown), though NO levels were only ~2 fold different. This reflected the role of other forms of NOS in NO production in islets. Effect of cytokines on increase in NO production varies with experimental conditions and cell type *e.g.,* cell lines (23,24), or mouse (25,26), rat (11,27), and human islets (11,28). In cytokine treated samples, iNOS expression increased ~2-fold upon incubation with Adv-BL as compared to non-infected islets. Adv-hIL-1Ra infection significantly reduced the amount of iNOS mRNA to ~50% of non-infected control islets (Fig. 5). In terms of NO release and iNOS expression, hIL-1Ra showed a greater cytoprotective effect than hVEGF (Figs. 4 and 5), reflecting predominant role of IL-1β (28). Islets infected with both Adv-hVEGF and Adv-hIL-1Ra showed intermediate level of NO production and iNOS expression. These results also correlated with islet function (Fig. 6). Our results are in good agreement with the literature where adenoviral vector encoding IL-1Ra (15) as well as recombinant IL-1Ra (29) has been shown to suppress NO production and IL-1β induced impairment of β-cell function.

Visualization of islet viability and apoptosis using calcein^{AM} and EthD-1 also indicated cytokine mediated toxicity, protection by hIL-1Ra expression, and persistence of beneficial effect upon hVEGF and hIL-1Ra dual expression (Fig. 10). While these results do provide a visual idea of islet cell death among different treatment groups, they are not definitive because of limited dye penetration to the core of islets. We verified this fact by confocal imaging of intact islets, which showed darkened core (not shown). Furthermore, the overlapping fluorescence from the three-dimensional cluster of cells leads to the observation of cumulative effects. These phenomena were also observed by Ylipaasto *et al.* (30) and Delaney *et al.* (31). Also, Liu *et al.* (29) showed that cytokines induced significantly less apoptotic staining in islets isolated from iNOS$^{-/-}$ rats, indicating the central role of iNOS/NO pathway in islet damage.

We determined the relative level of apoptosis and necrosis in human islets incubated with cytokines cocktail at 48 h (not shown), 96 h (Fig. 7), and 6 days (not shown) by estimation of nucleosome concentration. Although increase in incubation time had an effect on islet viability, the EF was similar at different time points, since EF is calculated as a ratio of test *vs.* control samples. Also, the absolute EF values were lower than those expected with healthy, dividing cell lines because of high nucleosome concentrations in control islets. This could be due to the quality of islets, cold storage and shipment of isolated islets, or hypoxic cell death experienced by cells in the core of islets during culture. Nevertheless, the variability within replicas ($n = 5$) were low (SEM < 20%), allowing us to compare the effect of Adv infection between different groups. Cytokines induced higher apoptosis than necrosis, which was also noted by Liu *et al.* (29) in mouse islets and Delaney *et al.* (31) in human islets.

Also, hIL-1Ra expression reduced both apoptosis and necrosis, and this effect persisted upon its co-expression with hVEGF. These results correlated with total caspase activity in cells. Because of unknown differences in relative importance of different caspases (29), we determined total caspase activity in the islets. We observed increased total caspase activity in islets incubated with cytokines, which increased further upon infection with Adv-BL (Fig. 9). While hVEGF expression reduced casapase activity which was attributed to its effects on the protection and proliferation of endothelial cells, expression of hIL-1Ra reduced it even further, and this beneficial effect persisted in the co-infection group.

Effect of Adv-hVEGF and/or Adv-hIL-1Ra infection on improvement in the survival and function of islets post-transplantation was determined in streptozotocin-induced diabetic NOD-SCID mice. We observed an increase in the weight of mice and reduction in blood glucose levels of mice transplanted with Adv-hVEGF + Adv-hIL-1Ra transfected islets, which was higher than that observed with Adv-hVEGF and Adv-hIL-1Ra alone (Fig. 11). This increase correlated with the higher amount of insulin and C-peptide secreted by the islets in response to intraperitoneal glucose challenge at 3-weeks post-transplantation (Fig. 12). Lack of complete recovery from diabetes in our studies could be due to islet damage during the process of cold storage and shipment of human islets from the isolation facility to our laboratory (transplantation of ~3 day old islets). Also, these diabetic mice already had blood glucose levels more >600, which might have also contributed to this failure of complete recovery from diabetes. Although complete recovery from diabetes was not observed, these results nevertheless underscore the beneficial effect of co-expression of genes targeting different pathways of islet destruction.

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