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iNOS Gene Silencing Prevents Inflammatory Cytokine-Induced β-Cell Apoptosis

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Abstract: Human islet transplantation has great potential as an effective means of treating insulin-dependent diabetes mellitus. Upregulation of inducible nitric oxide synthase (iNOS) and subsequent product of radical nitric oxide (NO) impair islet β-cell function. Therefore, we hypothesize that iNOS gene silencing will prevent β-cell death and improve the survival and function of islets. Small interfering RNA duplex (siRNA) inhibited rat iNOS gene expression and NO production in rat β-cell lines (INS-1E) in a dose- and sequence-dependent manner. iNOS gene silencing also protected these β-cells from inflammatory cytokine-induced apoptosis and increased their capacity to secret insulin. Three siRNA sequences against human iNOS were then designed and transfected into human islets. Although there was dose- and sequence-dependent iNOS gene silencing and NO production in human islets, the effect of iNOS gene silencing on apoptosis of islets was only moderate, as evidenced by 25–30% reduction in caspase 3 activity and in the percentage of apoptotic cells. Since an islet is a cluster of 200–1000 cells, the transfection efficiency of lipid/siRNA complexes into human islets was only 21–28%, compared to effective transfection efficiency (>90%) in β-cell lines. Nevertheless, these results suggest that siRNA may penetrate beyond the periphery into a larger percentage of an islet mass than previously thought.

Keywords: siRNA; islet transplantation; iNOS; β cells; apoptosis

Introduction

Human islet transplantation is a promising therapeutic strategy for treating type I diabetes mellitus. Despite recent success, islet transplantation still lags behind primarily because a large number of transplanted islets do not function. This results in the need for multiple islet infusions for each patient. Most islet grafts are destroyed due to immune and inflammatory reaction mediated by proinflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). These proinflammatory cytokines induce a series of intracellular inflammatory signal cascades, leading to β-cell death, probably due to apoptosis, necrosis, and other processes.[1] The stimulation of inducible nitric oxide synthase (iNOS) expression and consequent production of radical nitric oxide (NO) as well as of the other radicals, such as peroxynitrite ($OONO^-$) and superoxide (O_2^-), represent a pivotal step in these processes.[2–5] The inhibition of iNOS has shown significant protection of β-cells

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from proinflammatory cytokine-induced damage.[6–8] Therefore, it is reasonable to suggest that iNOS might be a potential target for improving the outcome of human islet transplantation.

RNA interference (RNAi) is a promising therapeutic strategy which utilizes an evolutionary conserved biologic process to induce sequence-specific, post-transcriptional gene silencing by small interference RNA (siRNA).[9] siRNA-mediated gene silencing in rodent β-cells and pancreatic islets has been shown to inhibit a target gene.[10,11] In mouse insulinoma cells, Burkhardt et al.[12] demonstrated that siRNA targeting Fas, another important mediator of β-cell death, was able to silence Fas gene expression and inhibit Fas-mediated β-cell damage in response to inflammatory cytokines. Keeping these promising findings in mind, we recently tested the effect of chemically synthesized siRNAs against iNOS and NF-κB on gene expression, NO production, and apoptosis in insulin producing β-cell line (INS-1E) and demonstrated that silencing of the iNOS gene is more effective than than silencing NF-κB.[13] There are also conflicting reports on the role of NF-κB on pancreatic β-cell death.[14–16] McCabe and O'Brien used lentiviral vector-based shRNA delivery to suppress IL-1β-mediated induction of iNOS expression, resulting in significant protection against the cytotoxic effects of IL-1β exposure.[6,17] However, these authors used rat insulinoma (RIN-r) cell lines and not human islets. Moreover, they did not determine the functional viability of RIN-r cells by measuring glucose-stimulated insulin release. Taking care of the usefulness of iNOS gene silencing in islet transplantation, in this study, we compared transfection efficiency and effect of iNOS gene silencing on apoptosis in INS-1E cells and intact human islet, which is a cluster of 200–1000 cells and hard to transfect.

This report implicated the pivotal role of iNOS in cytokine-induced β cell death. We demonstrate the possibility of using iNOS gene silencing as a strategy to improve the outcome of human islet transplantation.

Table 1. Small Interfering RNA (siRNA) Sequences of Rat iNOS (NM_012611)

	siRNA sequence	coding region[a]
224	S:[b] 5′-CUACCAAGGUGACCUGAAAGA [dT][dT]-3′	224–244
	AS:[c] 5′-UCUUUCAGGUCACCUUGGUAG [dT][dT]-3′	
675	S: 5′-GAAGCCGUAACAAAGGAAAUA [dT][dT]-3′	675–695
	AS: 5′-UAUUUCCUUUGUUACGGCUUC [dT][dT]-3′	
898	S: 5′-CCUCGGAUAUCUCUUGCAA [dT][dT]-3′	898–916
	AS: 5′-UUGCAAGAGAUAUCCGAGG [dT][dT]-3′	
2225	S:5′-GUUCGAUGUUCGAAGCAAACA [dT][dT]-3′	2225–2245
	AS: 5′-UGUUUGCUUCGAACAUCGAAC [dT][dT]-3′	

[a] ORF region. [b] S, sense. [c] AS, antisense.

Materials and Methods

Fetal bovine serum (FBS), sodium pyruvate, and CMRL 1066 medium were purchased from Mediatech, Inc. (Herndon, VA). Penicillin/streptomycin, phosphate-buffered saline (PBS), 0.25% (w/v) trypsin-EDTA, and RPMI-1640 medium and OptiMEM-I reduced serum medium were purchased from GIBCO-BRL (Gaithersburg, MD). 2-Mercaptoethanol was obtained from Sigma Aldrich (St. Louis, MO). Recombinant IL-1β, TNF-α, and IFN-γ were purchased from R&D Systems (Minneapolis, MN). siRNA against rat iNOS (Gene Bank no. NM_012611, Table 1) and human iNOS (Gene

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Table 2. Small Interfering RNA (siRNA) Sequences of Human iNOS (NM_000625)

	siRNA sequence	coding region[a]
511	S:[b] 5′-CGGCAUGUGAGGAUCAAAA[dT][dT]-3′	511–529
	AS:[c] 5′-UUUUGAUCCUCACAUGCCG[dT][dG]-3′	
539	S: 5′-GCGGGAUGACUUUCCAAGA [dT][dT]-3′	539–557
	AS: 5′-UCUUGGAAAGUCAUCCCGC[dT][dG]-3′	
691	S: 5′-GCUAUCGAAUUUGUCAACC[dT][dT]-3′	691–709
	AS: 5′-GGUUGACAAAUUCGAUAG [dT][dT]-3′	

[a] ORF region. [b] S, sense. [c] AS, antisense.

Bank no. NM_000625, Table 2), control siRNA, fluorescein-labeled siRNA, and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA). All primers used for real time RT-PCR were obtained from Integrated DNA Technology (Coralville, IA). TUNEL assay kit was from BD Pharmingen (Franklin Lakes, NJ). Caspase 3 cellular activity assay kit was purchased from EMD Biosciences, Inc. (San Diego, CA). RNeasy Mini kit and RNase-Free DNase Set were purchased from Qiagen (Valencia, CA). Multiscribe reverse transcription kit was from Applied Biosystems (Foster City, CA). SYBR Green-I dye universal PCR master mix and other reagents for real time RT-PCR were from Roche (Indianapolis, IN). Griess assay kit was from Promega (Madison, WI). BCA protein assay kit was from Pierce Chemical Co. (Rockford, IL).

Cell Culture and Transfection. Rat insulin producing β-cells (INS-1E cells), a kind gift from Professor Claes B. Wolheim (University Medical Center, Geneva, Switzerland), were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in complete medium composed of RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 U/mL of penicillin, and 100 μg/mL of streptomycin.

INS-1E cells were seeded 24 h before transfection in a 24-well plate (5×10^5 cells/well) for all of the experiments except for the TUNEL assay, for which we used 12-well plates (10^6 cells/well). siRNAs were transfected into INS-1E cells after complex formation with Lipofectamine 2000 according to the manufacturer's instructions. Briefly, siRNA molecules and Lipofectamine 2000 were mixed together in OPTMEM I reduced serum medium at a ratio of 30 nmol/1 mg (siRNA/ Lipofectamine 2000), and complex formation was allowed to proceed for 30 min at room temperature before addition into INS-1E cells. At 12 h post-transfection, media containing lipid/siRNA complexes were replaced with fresh media.

To determine the transfection efficiency and effect of iNOS gene silencing on protection of human islets from proinflammatory cytokines, we redesigned three siRNA sequences targeting different regions of human iNOS mRNA (Table 2) and used for transfection into human islets. These islets were received from one of the several Islet Cell Resource (ICR) Centers through ICR Services for Basic Science Applications. On arrival at our facility, islets were cultured at 37 °C in CMRL 1066 medium supplemented with 10% FBS. Human islets were seeded in a 24-well plate (2000IE/well) 2 h before transfection and were transfected with siRNA after complex formation with Lipofectamine 2000 as described above.

Fluorescence Microscopy and Flow Cytometry. To determine the transfection efficiency, INS-1E cells and human islets were transfected with fluorescein-labeled siRNA after complex formation with Lipofectamine 2000. At 12 h post-transfection, cells were visualized under fluorescence microscopy. To determine the percentage of transfected cells, INS-1E cells were washed with 1× PBS, trypsinized, and suspended in 1× PBS. Since human islets are a cluster of cells, they were treated with trypsin-EDTA for 10 min at 37 °C followed by vigorous pipeting to create a single-cell suspension. Then the cell suspensions were directly introduced to a FACSCalibur flow cytometer (Becton, Dickinson, NJ) equipped with a 488 nm argon ion laser. The FL-1 emission channel was used to monitor the fluorescein, and results from 10000 fluorescent events were obtained for analysis. Cells without transfection were served as negative controls.

Real Time RT-PCR. Expression of rat iNOS gene in INS-1E cells and that of human iNOS in human islets was determined at the mRNA level by real time RT-PCR. Following treatment, total RNA was extracted with an RNeasy Mini KIT and treated with DNase by on-column digestion (RNase-Free DNase Set). RNA concentration was determined by spectrophotometer (NanoDrop). A 170 ng portion of total RNA was converted into cDNA using multiscribe reverse transcriptase reagents and random hexamers at a 10 μL reaction system. cDNA (2 μL) was used as a template and analyzed by SYBR Green-I dye universal PCR master mix on a LightCycler 480 Instrument. The primers used for real-time PCR were as follows: rat iNOS[13] (forward) 5′-ACCAGAGGACCCAGAGACAAGC-3′, (reverse) 5′-TCCAGGCCATCTTGGTGGCAAA-3′ (amplicon size 208 bp); rat 18S rRNA[18] (forward) 5′-CGG CTA CCA CAT CCA AGG AA-3′, (reverse) 5′-GCT GGA ATT ACC GCG GCT-3′(amplicon size 186bp); human iNOS[19] (forward) 5′-ACGTGCGTTACTCCACCAACA-3′, (reverse) 5′-CATAGCGGATGAGCTGAGCA-3′ (amplicon size 102bp); human ribosomal protein S19 (human S19) (forward) 5′-GCTTGCTCCCTACCGATGAGA-3, (reverse) 5′-ACCCCG-GAGGTACAGGTG-3′ (amplicon size 73bp). To assess the specificity of the amplified PCR product, the melting curve analysis was performed on the LightCycler 480 Instrument. The results of the iNOS mRNA level were compared by calculating the C_P value and normalized by the reference

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genes (rat 18S or human S19). Four independent experiments were performed, and the results were expressed as a percentage of control.

Griess Assay. Nitric oxide (NO) is rapidly oxidized in culture medium into nitrite, which accumulates in the sample and can be easily correlated with NO production. Therefore, nitrite concentration was determined using the Griess assay (Promega, Madison, MI). Fifty microliters of cell culture supernatant was added to a 96 well-plate and mixed with 50 μL of 1% sulfanilamide in 5% phosphoric acid solution and incubated for 5 min at room temperature in the dark. Then, 50 μL of 0.1% *N*-1-naphthylethylenediamine dihydrochloride (NED) aqueous solution was added to each well. The plate was incubated for an additional 10 min, and absorbance was measured at 560 nm using a microplate reader. To determine the nitrite concentration in each sample, a standard curve was prepared using nitrite standard solution and culture medium as matrix.

Effect of iNOS Gene Silencing on Apoptotic Cell Death. To determine whether iNOS gene silencing inhibits apoptosis of β cells due to inflammatory cytokines, INS-1E cells (10^6 cells/well in a 12-well plate) were transfected with siRNA against iNOS as well as with control siRNA. The cells were incubated with a cocktail of TNF-α (50 pg/mL), IL-1β (5 ng/mL), and IFN-γ (50 ng/mL) for 48 h, followed by visualization under microscopy.

Caspase-3 Activity Assay. The cellular caspase-3 activity of human islets was measured with a Caspase-3 cellular activity assay kit. Human islets were seeded at a density of 2000IE/well in a 24-well plate. After treatment, cells were collected and lysed with the lysis buffer. Ten microliters of the cell lysate solution was added in 40 μL of assay buffer and mixed with 50 μL of caspase-3 substrate (Ac-DEVD-pNA) at a half-volume 96-well plate. The mixture was kept at 37 °C, and absorbance at 405 nm was read continuously using a spectrophotometer every 5 min for 1 h. The absorbance versus time for each sample was plotted, and the activity was calculated from the slope of the linear curve. To normalize the activity, total protein concentration of each sample was also measured with BCA protein assay kit.

TUNEL Assay. To gain insights on the mechanism of β cell death, a terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assay with the APO-DIRECT kit was used to detect apoptotic cells. Following transfection and incubation with a cytokine cocktail, INS-1E cells and human islets were made to single cell suspension as described above. The cells were fixed in 1% paraformaldehyde in PBS (pH 7.4) and then treated with ice-cold 70% ethanol and stained with FITC-dUTP and then with propidium iodide. The intensity of fluorescence was measured by a flow cytometer and analyzed using CELLQUEST software (BD Bioscience). Nontransfected cells without cytokine treatment were served as a negative control. Three sets of independent transduction experiments were carried out for each assay.

Assessment of Human Islet Function Post-Transfection. The ability of human islets to respond to glucose stimuli was used to determine the effect of transfection of Lipofectamine 2000/siRNA complexes on human islet function. Following transfection, islets were incubated with a cocktail of inflammatory cytokines including TNF-α (50 pg/mL), IL-1β (5 ng/mL), and IFN-γ (50 ng/mL) for 48 h. Then, islets were challenged with two different concentration of glucose (60 and 300 mg/dL) for 1 h at 37 °C. Supernatants were then collected for measuring insulin secretion by human insulin ELISA Kit (Alpco Diagnostics, Windham, NH). Results were normalized by measuring the total protein using a BCA assay kit. Stimulation index was calculated by measuring insulin release by islets 1 h after incubation with media containing basal (60 mg/dL) and stimulated (360 mg/dL) levels of glucose.

Statistical Analysis. The difference between groups was determined by unpaired *t*-test, and a P value of ≤ 0.05 was considered statistically significant. Results are expressed as the means $\pm$ standard deviation (S.D.).

Results

Transfection Efficiency of Lipid/siRNA Complexes in Rat β Cells. We used fluorescein-labeled siRNA at concentrations of 50, 100, and 150 nM to quantitatively assess the incorporation of siRNA into rat β cells. After 12 h of incubation with Lipofectamine 2000/fluorescein-labeled siRNA complexes, cells were observed under fluorescence microscopy. Figure 1A demonstrates that these cells were effectively transfected with fluorescein-labeled siRNA. The photographs show green cells with distinct spots speckled their surface and in the nucleus, suggesting enhanced cellular uptake and nuclear translocation. To quantitatively determine the transfection efficiency, cells were analyzed by flow cytometry. As shown in Figure 1B, the transfection efficiency increased from 75.9% to 95.9% with an increase in siRNA concentration from 50 to 150 nM. The average fluorescence intensity also increased accordingly from 2.48×10^4 to 6.72×10^4 (data not shown).

Effect of siRNA Sequence on iNOS Gene Silencing and NO Production on Rat β Cells. Figure 2A demonstrates the effect of siRNA sequences on iNOS gene silencing. All four siRNA sequences were able to silence iNOS expression, and siRNA-iNOS-898 showed silencing around 50% of iNOS expression. Figure 2B shows the effect of siRNA sequence on the NO production by rat β-cells. Compared with control siRNA, all four siRNAs targeting different regions of rat iNOS mRNA showed inhibition of NO production by rat β-cells. Among them, siRNA-iNOS-898 was the most potent one, which reduced the NO production by 60%. Therefore, siRNA 898 was used for further studies on rat β-cells. We also noticed that there was almost no change in the NO production in the control siRNA-treated group (Figure 2B), which indicates the inhibition of NO production was due to the specific silencing of iNOS mRNA and not due to the nonspecific effects.

Effect of iNOS Gene Silencing on Rat β-Cell Death. To determine whether iNOS gene silencing inhibit apoptosis of rat β-cells induced by inflammatory cytokines, INS-1E

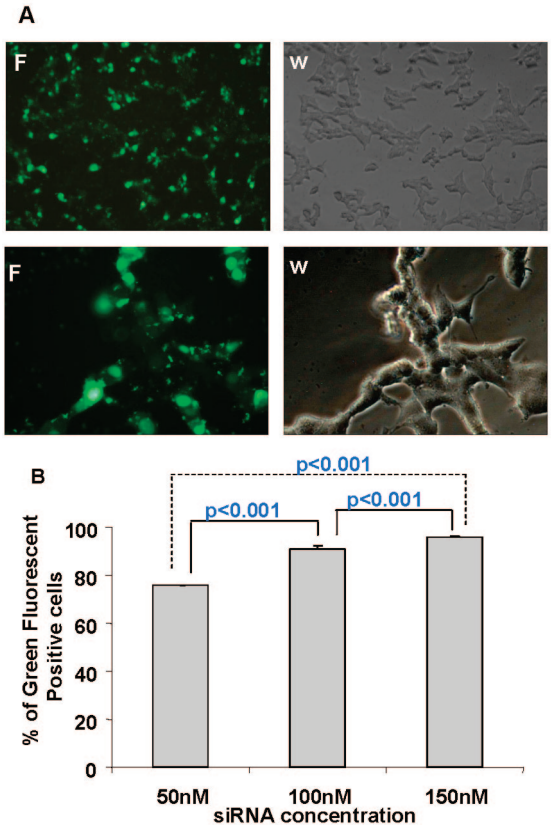

Figure 1. Transfection efficiency of siRNA into rat insulin producing β-cells. INS-1E cells were transfected with fluorescein-labeled siRNA, after complex formation with Lipofectamine 2000 at siRNA concentrations of 50, 100, and 150 nM. At 12 h after transfection, cells were visualized under fluorescence microcopy and analyzed with flow cytometry. (A) Fluorescent images showing fluorescein-labeled siRNA incorporation into INS-1E cells. Left panels show cells under fluorescent light and right panels under visible light. Upper panel is low magnification (100×), and lower panel is high magnification (400×). (B) Percentage of green fluorescence positive cells. Results are the mean ± SD of triplicates. The *P* value of <0.05 was considered statistically significant.

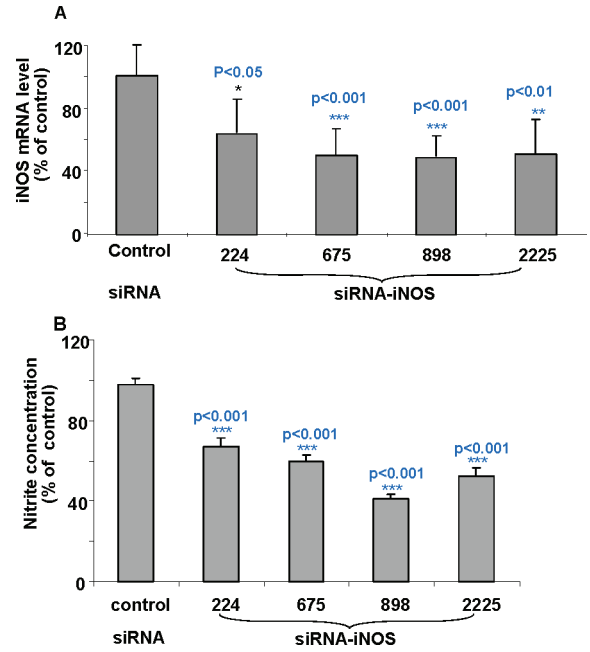

Figure 2. Effect of siRNA sequences on iNOS gene expression (A) and NO production (B) in rat β cells. Following transfection of INS-1E cells with 150 nM siRNA-iNOS complexed with 5 μg/mL of Lipofectamine 2000, cells were treated for additional 12 h with the cytokine cocktail of IL-1β (50 pg/mL), TNF-α (5 ng/mL), and IFN-γ (50 ng/mL). Cells not treated with the cytokines were used as negative control. iNOS expression is expressed as the amount of iNOS mRNA measured by real time RT-PCR. Nitrite concentration is expressed as percentage of positive control. Results are the mean ± SD of triplicates and were normalized with live cells. The *p* value of <0.05 was considered statistically significant.

cells (10^6/well in a 12-well plate) were transfected with siRNA against rat iNOS as well as the control siRNA. Then the cells were incubated with cytokine cocktail (IL-1β 50 pg/mL, TNF-α 5 ng/mL, and IFN-γ 50 ng/mL) for 48 h. As visualized under microscopy, almost all the cells transfected with the control siRNA were aggregated and round up, indicating cell death, probably due to apoptosis (Figure 3A). In contrast, only a small part of the cell treated with siRNA-iNOS were aggregated, indicating only a small fraction of the cells were undergoing apoptosis.

One of the later steps in apoptosis is DNA fragmentation, which results from the activation of endonucleases during the apoptotic process. Therefore, the analysis of DNA fragmentation with TUNEL assay is a useful approach to get information regarding cell apoptosis. As shown in Figure 3B, cells with no cytokine treatment showed 0.8% apoptotic cells (Figure 3B1) and cells transfected with control siRNA treated with cytokines showed 42.3% apoptotic cells (Figure 3B2). The cells transfected with siRNA against iNOS and treated with cytokines showed 12.3% apoptotic cells (Figure 3B3), clearly demonstrating that iNOS gene silencing can help protect β cells from proinflammatory cytokines.

Transfection Efficiency of Lipid/siRNA Complexes into Human Islets. After confirming the beneficial effect of iNOS gene silencing on rat β-cells from proinflammatory cytokines, we used fluorescein-labeled siRNA at doses of 100 and 400 nM to determine the transfection efficiency in human islets under fluorescence microscopy. Figure 4A demonstrates that human islets were transfected with fluorescein-labeled siRNA. While the untreated control human islets showed only weak background fluorescence, human islets transfected with fluorescein-labeled siRNA showed distinct spots speckled throughout their surface with some concentrations noted at the edges and over their interior surface. These images clearly demonstrated that siRNA was introduced into human islets in culture, but we could not determine whether siRNA had penetrated into the interiors of human islets. Thus, transfected human islets were analyzed by flow cytometry upon dispersion

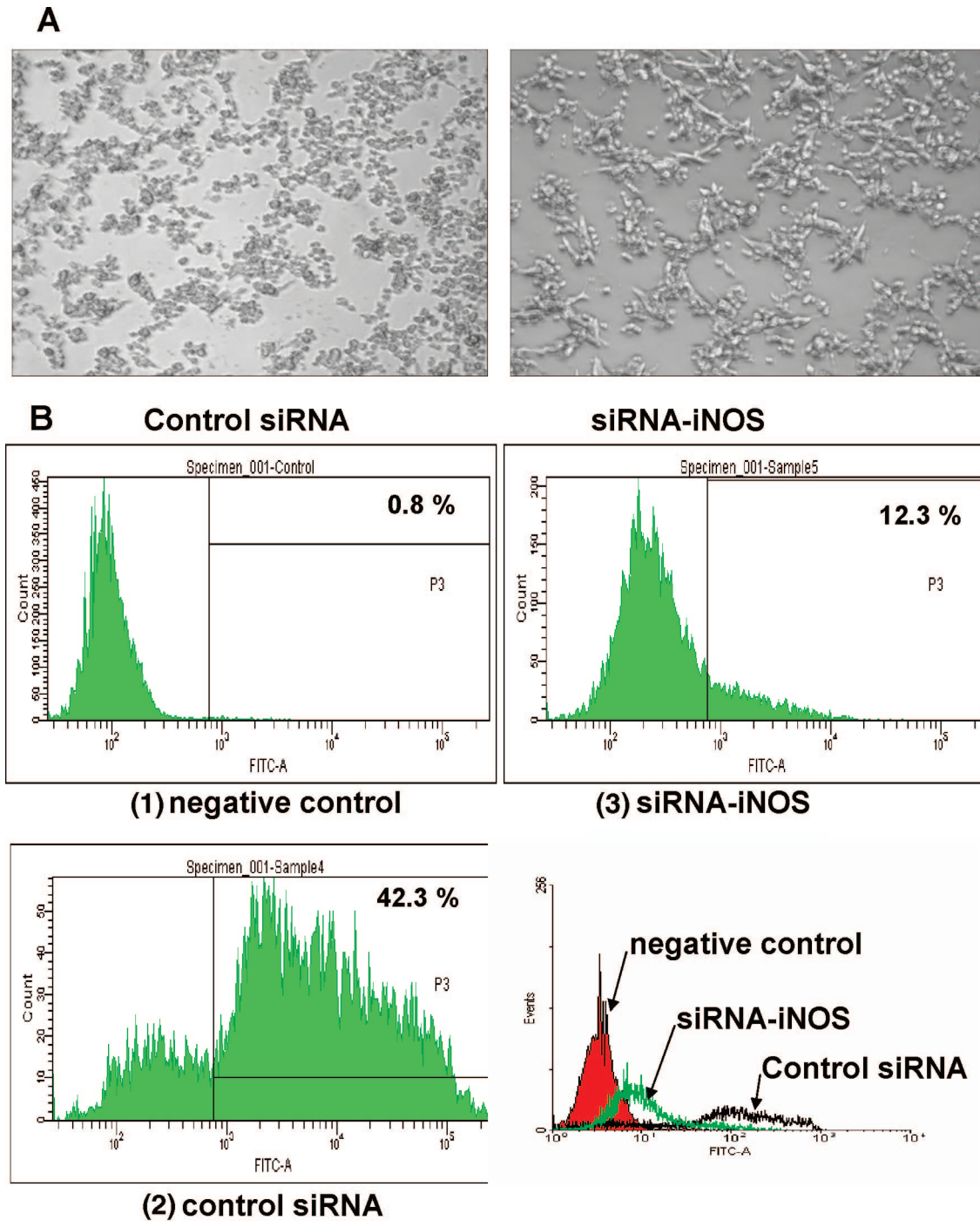

Figure 3. Effect of iNOS gene silencing on rat β-cell death. Following transfection of INS-1E cells with siRNA-iNOS-898 as well as the control siRNA, the cells were incubated with a cytokine cocktail of IL-β (50 pg/mL), TNF-α (5 ng/mL), and IFN-γ (50 ng/mL) for 48 h, then visualized under microscopy (A) and analyzed by TUNEL assay (B). (A) Left panel, most cells treated with the control siRNA were aggregated and round up, indicating cell death; right panel, only a part of the cell treated with siRNA-iNOS were aggregated and indicating only a small fraction of the cells were undergoing apoptosis. (B) TUNEL assay of INS-1E cells treated with siRNA-iNOS-898 as well as control siRNA. Negative control, cells with no cytokine treatment; siRNA-iNOS, cells treated with siRNA-iNOS-898 and cytokine; control siRNA, cells treated with control siRNA and cytokine. The values show the percentage of positive cells.

into single cells after treatment with trypsin-EDTA for 10 min at 37 °C followed by vigorous pipeting. Both the fluorescent images for siRNA dose titration and the more quantitative FACS data help document how much siRNA was incorporated into islet cells. Almost 21.5% and 28.3% islet cells incorporated fluorescein-labeled siRNA when transfected with siRNA at a concentration of 100 and 400 nM, respectively (Figure 4B).

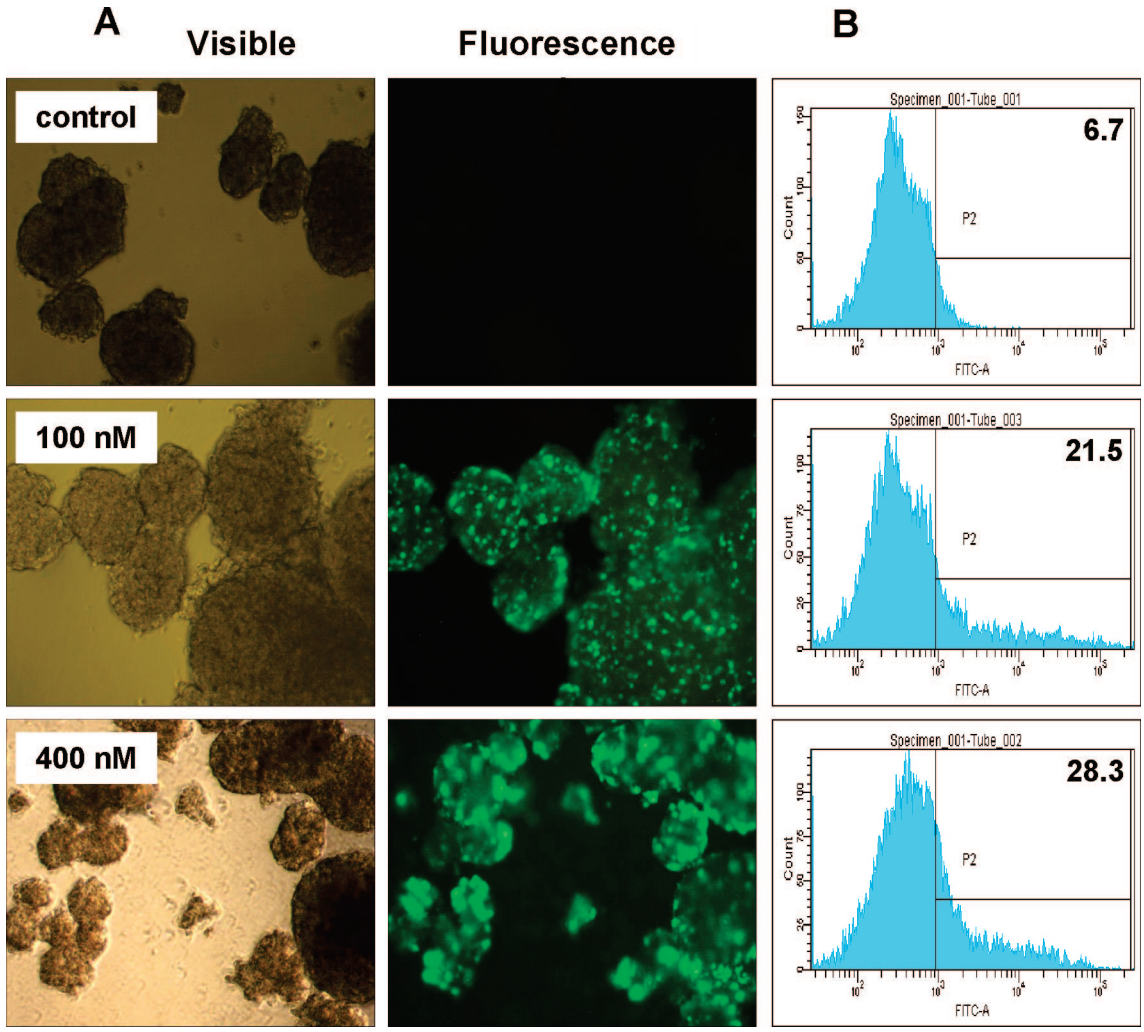

Figure 4. Transfection efficiency of siRNA into intact human islets. Fluorescein-labeled siRNA were transfected into human islets and visualized under fluorescence microscope at 12 h post transfection. (A) Fluorescent images showing fluorescein-labeled siRNA incorporation into intact human islets. Left panels show human islets under visible light, and right panel under fluorescence. (B) Flow cytometry analysis showing percentage of green fluorescence positive cells.

Effect of siRNA Sequences on iNOS Gene Silencing in Human Islets. Following screening of the siRNA sequences using INS-1E cells and demonstrating the beneficial effect of iNOS gene silencing on the protection of β-cells from inflammatory cytokines, we determined the effect of iNOS gene silencing in human islets. Since there is not 100% homogeneity in rat iNOS mRNA (Gene Bank Accession no. NM_012611) and human iNOS mRNA (Gene Bank Accession no. NM_000625), we redesigned three siRNA sequences targeting different regions of human iNOS and used for transfection into human islets. Quantitative real-time RT-PCR was used to measure the human iNOS gene expression level using SYBR green chemistry with human iNOS specific primer and human S19 as a reference gene. Figure 5A shows that all three siRNAs tested were able to reduce iNOS gene expression levels in human islets. Among all of the siRNAs tested, siRNA-iNOS-691 was the most potent one, which reduced iNOS expression level by 50%. Therefore, siRNA-iNOS-691 was used for further evaluation. Compared to the control siRNA group, there was a significant decrease in NO production when human islets were transfected with lipid/siRNA-iNOS-691 complexes (Figure 5B). This result indicates that the inhibition of NO production was due to the specific silencing of iNOS mRNA and not due to the off-target effects.

Effect of iNOS Gene Silencing on Human Islet Cell Death. We have shown that siRNA against iNOS mRNA could reduce the apoptosis of rat β-cells. In addition, siRNA can be delivered into human islets and reduce iNOS mRNA level. Therefore, we continued to test whether iNOS gene silencing can also prevent the cytokine induced apoptosis of human islets. TUNEL assay was used to detect DNA fragmentation. As shown in Figure 6A, siRNA against human iNOS reduced the percentage of apoptotic cells to 28.5% compared to the control siRNA group (36.2%). Although the prevention of apoptosis in human islets was not as high as shown in rat β cells (Figures 3B and 6A), this is not a surprise to get different results from INS-1E cell and human

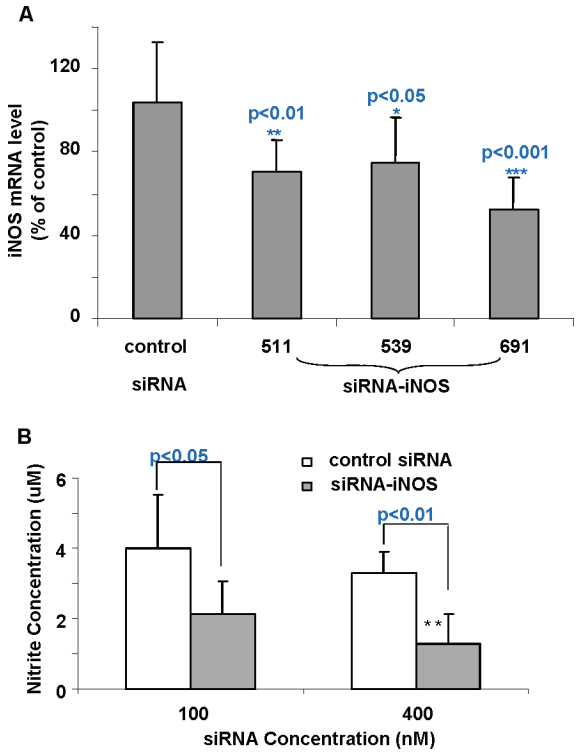

Figure 5. Effect of siRNA sequence on iNOS expression (A) and NO production (B) in human islets. Human islets (2000IE) were transfected for 12 h with 400 nM iNOS-siRNA complexed with 13.3 μg/mL of Lipofectamine 2000. Then human islets were treated for additional 12 h with a cytokine cocktail of IL-1β (50 pg/mL), TNF-α (5 ng/mL), and IFN-γ (50 ng/mL). (A) The iNOS mRNA were measured by real time RT-PCR and expressed as the percentage of control. (B) Nitrite concentration of the culture supernatants was determined by Griess assay. Human islets treated with control siRNA were used as control. Results are represented as the mean $\pm$ SD of triplicate. Significance established at <0.05 (*), <0.01 (**), and <0.001 (***).

islets, which is a cluster of 200–1000 cells. Increased caspase-3 activity is often used as a marker for apoptotic signaling cascade.[20] Therefore, we tested the effects of iNOS gene silencing on caspase-3 activities of human islets (Figure 6B). Compared with the control siRNA treated group, siRNA-iNOS reduced human islets caspase-3 activity by around 25%.

Human Islet Function after iNOS Gene Silencing. The effect of transfection process on *in vitro* islet function was determined by static incubation of islets with different concentration of glucose. There was increase in insulin secretion as a function of glucose concentration for islets transfected with lipid/siRNA-iNOS complexes, with stimulation index of 1.40. In contrast, there was little increase in insulin secretion with increase in glucose concentration

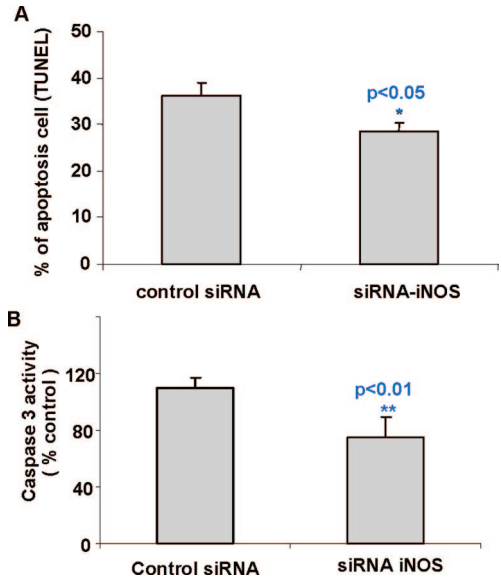

Figure 6. Effect of iNOS gene silencing human islets cell death. Following transfection with 400 nM siRNA-iNOS-691 as well as the control siRNA, human islets (2000IE) were incubated with the cytokine cocktail of IL-1β (50 pg/mL), TNF-α (5 ng/mL), and IFN-γ (50 ng/mL) for 48 h. (A) The TUNEL assay was used to analyze cell apoptosis. (B) Human islets were collected and lysed with 50 μL of lysis buffer. Ten microliters of cell lysate from each group sample were used for caspase-3 activity assay using Ac-DEVD-pNA substrate. Results are expressed as the percentage of control and represented as the mean $\pm$ SD of triplicates. Significance established at <0.05 (*) and <0.01 (**).

for islets transfected with lipid/siRNA-control (Figure 7). The results suggest that iNOS gene silencing partly protected human islet from inflammatory cytokine mediated destruction.

Discussion

Human islet transplantation has great potential for treating insulin-dependent diabetes, allowing stable glucose homeostasis without exogenous insulin regimens and thus avoiding several diabetic glucose complications. Despite some recent improvements in transplantation technology, a large number of transplanted islets do not function properly, which results in the need for multiple islet infusions for each patient. Moreover, the success of islet transplantation is currently dependent on chronic treatment of the patient with strong immunosuppressant drugs, which are often associated with serious side effects. Due to the limited supply of cadaver donors, it is essential to improve the survival of transplanted islets. Therefore, we and others have been working on genetic modification of islets to improve the outcome of islet transplantation.[21–26]

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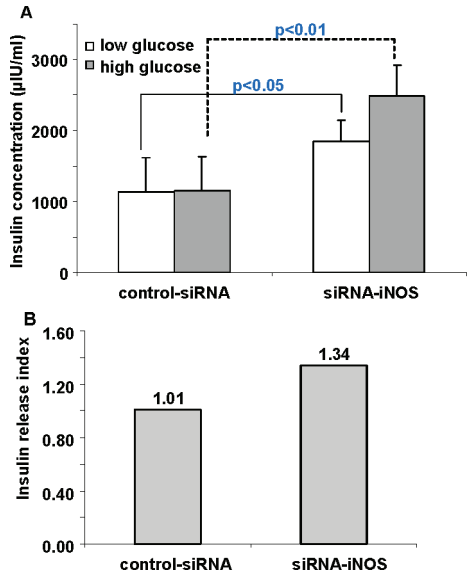

Figure 7. Human islet function after iNOS gene silencing. At 12 h post-transfection with Lipofectamine 2000/siRNA-iNOS or Lipofectamine 2000/siRNA−control complexes, islets were incubated with inflammatory cytokines cocktail of IL-1β (50 pg/mL), TNF-α (5 ng/mL), and IFN-γ (50 ng/mL) for 48 h. Then, islets were challenged with two different concentration of glucose (60 and 360 mg/dL). Insulin stimulation index (SI) was determined. Results are the average of three replicates: (A) insulin secretion as a function of glucose concentration, (B) stimulation index. The *p* value of <0.05 was considered statistically significant.

The overexpression of therapeutics genes has been demonstrated to improve the survival and function of islets post-transplantation.[23,27] Our previous work showed improved islet transplantation by genetic modification of human islets with human vascular endothelial growth factor (hVEGF) and human interleukin-1 receptor antagonist (hIL-1Ra).[23] The expression of hVEGF and hIL-1Ra synergistically suppress islet dysfunction and NO production in human islets. After transplantation of these transfected human islets into diabetic mice, reduced blood glucose levels and increased blood insulin and c-peptide level was observed.[23]

In contrast to the overexpression of therapeutic genes, *ex vivo* silencing of harmful genes offers an alternative approach to induce human islets to modify their internal signal transduction pathways to prevent the damage inflicted upon them by proinflammatory responses. Therefore, we recently determined the effect of chemically synthesized siRNAs against iNOS and NF-κB on iNOS gene expression, NO production, and apoptosis in insulin producing β-cell line, INS-1E, and demonstrated that silencing of iNOS is more effective than that of NF-κB.[13] Since human islets are clusters of 200–1000 cells, it is difficult to achieve high transfection efficiency. Therefore, in the present study, we compared the transfection efficiency of lipid/siRNA complexed in rat β cells and intact human islets. We first determined the transfection efficiency on INS-1E cells, which showed 95.9% cells were incorporated with fluorescein-labeled siRNA (Figure 1B). This result is consistent with a previous report by Hagerkvist et al.,[11] who demonstrated a transfection efficiency of 96.2% in another murine β cells line, β-TC-6 cells.

For human islets, as shown in Figure 4B, the transfection efficiency was 21.5% and 28.3% at siRNA concentrations of 100 and 400 nM in human islets, which are lower than that reported by Bradley et al.[10] (46.3% at siRNA concentration of 400 nM), but higher than that reported by Hagerkvist et al.[11] (10.9% at siRNA concentration of 100nM). This discrepancy may be due to the fact that these authors used mouse islets, while we used human islets. In addition, the difference in transfection protocol may also contribute to the varied transfection efficiency achieved. Nevertheless, this is an interesting finding suggesting that siRNA may penetrate beyond the periphery into a larger percentage of an intact islet cell mass than previously thought. Unlike intact islets, transfection efficiency was $>90\%$ in dispersed islets, which is close to that observed in cultured β-cells. However, we prefer to use intact islets, which are a cluster of 200–1000 cells and represent the real situation.[11] Nevertheless, these results shed light on the reasons that cause the different transfection efficiency between intact human islets and β-cell lines.

It is widely believed that the treatment of proinflammatory cytokine results in β-cell death possibly due to apoptosis and necrosis.[1,28] This cytotoxic effect of cytokines may be mediated by inducing iNOS and subsequently producing radical NO as well as of other free radicals, such as

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peroxynitrite ($OONO^-$) and superoxide (O_2^-).[29–31] As reported previously,[29] nearly 50% of the genes related to cytokine-induced β cell death are NO-dependent, which shows the essential role of the iNOS pathway in cytokine-mediated cell toxicity. Necrosis and apoptosis are found shortly after rat islet transplantation. Nonspecific inflammation at the graft site may contribute to the initial islet damage which results in massive loss of transplanted tissues and primary nongraft function.[32] iNOS gene expression on islets increased significantly after transplantation. The maximal iNOS gene expression was observed 1 day after transplantation and then declined progressively.[5] Increased iNOS gene expression led to an early inflammatory process in islet transplantation, suggesting iNOS is an important mediator of graft inflammation and islet damage in early islet transplantation.[5] Therefore, we hypothesized that the silencing of iNOS gene might reduce iNOS mediated inflammatory response and prevent islet cell death.

To determine iNOS gene silencing in human islets, we redesigned three siRNA sequences targeting different regions of human iNOS and used for transfection in human islets. As shown in Figure 5A, around 50% iNOS gene expression has been silenced by siRNA complexed with Lipofectamine 2000, which is similar to that reported by Bradley et al.,[10] who demonstrated 55% reduction of Ins-2 gene expression by isolated rodent islets. This finding is attractive and demonstrates the possibility of specifically reducing the target mRNA in human islets with siRNA. Various extents of iNOS gene silencing were achieved in the present study with different siRNA duplexes targeting different regions of iNOS mRNA, with the highest gene silencing for the siRNA-iNOS-691 group compared to the control siRNA group (Figure 5A). There was also significant decrease in NO production when islets were transfected with lipid/siRNA-iNOS-691 complexes (Figure 5B). As reported previously, the gene silencing effect of siRNA varied considerably depends on target sequences[33] and the secondary structure of target region that is essential for siRNA binding.[34] Therefore, a more potent siRNA could be found by designing and screening more siRNA sequences target different regions of mRNA.

Caspases play a key role in apoptosis. In mammals, there are 14 caspases; among them, Caspase-3 is the major effector caspase involved in apoptosis. It is activated by upstream proteases including caspase-6, caspase-8, and cytotoxic T-cell-derived granzyme B.[35] The critical role of caspase 3 in cytokine induced β cell apoptosis has been reported.[36–38] Both caspase 3 inhibitor (Z-DEVD-FMK)[39] and the X-linked inhibitor of apoptosis protein (XIAP)[26] have been shown to prevent apoptosis of transplanted human islets. We observed that siRNA targeting iNOS decreased proinflammatory cytokine induced caspase-3 activity in human islets (Figure 6B). The reduced caspase-3 activity probably contributes to the prevention of human islets from apoptosis.

One of the later steps in apoptosis is DNA fragmentation, which results from the activation of endonucleases during the apoptotic process. The free 3′-OH strand breaks resulting from DNA degradation can be measured by a TUNEL assay using the APO-DIRECT, which is a single-step staining method for labeling DNA breaks to detect apoptotic cells by flow cytometry analysis. Consistent with the results of caspase-3 activity, TUNEL assay demonstrated the prevention of β cell apoptosis on human islet as well as INS-1E cells. As shown in Figure 3B, siRNA-iNOS treatment significantly reduced cytokine-induced INS-1E cell apoptosis to 12.3%, compared to the control siRNA treated cells with 42.3% apoptotic cells. In human islets, the protective effect

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is less than that observed in INS-1E cells. The treatment of siRNA-iNOS decreased the percentage of cell apoptosis from 36.2% to 28.5% (Figure 6B). The difference in the protective effects can be explained by much lower transfection efficiency achieved in intact human islets compared to that achieved in INS-1E cells (21–28% vs 95%; Figures 1B and 4B). Besides, due to the heterogeneous feature of human islets, we cannot exclude the existence of other mechanisms involved in the apoptosis of human islets.[1]

To determine the effect of transfection process on the in vitro islet function, islets were transfected with lipid/siRNA-iNOS and lipid/siRNA-control complexes and then incubated with a cocktail of inflammatory cytokines. The islet function was determined by static incubation of islets with different concentration of glucose. There was increase in insulin release as a function of glucose concentration for the islets transfected with lipid/siRNA-iNOS complexes. In contrast, there was little increase in insulin secretion with increase in glucose concentration for the islets transfected with lipid/siRNA-control (Figure 7). The results suggest that iNOS gene silencing partly protected human islets from proinflammatory cytokine-mediated destruction. The stimulation index is somewhat lower than what we usually achieve, possibly due to the poor quality of human islets.

Our results demonstrate that silencing of iNOS gene expression results in reduction of NO production and reduced cell death in rodent β-cell line as well as in human islets. Due to the pivotal role of iNOS in cytokine signal cascade, it is not unexpected that we could prevent proinflammatory cytokine induced β cell death by silencing iNOS gene expression. iNOS gene silencing also partly protected human islets from inflammatory cytokine mediated destruction of glucose stimulated insulin secretion. The present study is the first to demonstrate the prevention of proinflammatory cytokine induced cell death in human islets with siRNA target iNOS gene.

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