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Protection of Human Pancreatic Islets Using a Lentiviral Vector Expressing Two Genes: cFLIP and GFP

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Pancreatic islet transplantation can provide insulin independence to diabetic patients. However, apoptosis of islets often leads to early graft failure. Genetic engineering with protective gene(s) can improve the viability of these cells. Here we show successful transduction of human islets with a feline immunodeficiency virus (FIV) vector expressing both a cytoprotective (cFLIP) gene and the green fluorescent protein (GFP). Despite using low virus titers to maximize safety, transduced islets expressed both genes, resulting in improved β-cell metabolic activity and viability. Although only ~10% of total islet cells were transduced, the significant viability advantages suggest a "barrier" effect in which protecting the periphery of the islet shields the core. These results provide the first demonstration that a lentiviral vector can express two genes in islets. Furthermore, the engineered islets are resistant to a variety of apoptotic stimuli, suggesting the potential of this approach in enhancing the viability of transplanted cells.

Key words: Diabetes; Pancreatic islet transplantation; Feline immunodeficiency virus (FIV); Gene therapy; cFLIP

INTRODUCTION

Type 1 diabetes is a T-cell-mediated autoimmune disease resulting in the destruction of insulin-producing β-cells of the islets of Langerhans. Despite insulin therapy, many patients cannot achieve glycemic control and develop clinical complications that dramatically affect quality of life and life expectancy (24). Dramatically improved islet isolation techniques and immunosuppressive regimens have made islet transplantation a therapeutic option for these patients (34). Islet transplantation can restore endocrine function and physiological control of glucose metabolism, leading to the reversal of both vascular (15) and cardiovascular complications (16). A major drawback to this approach, however, is the shortage of organs available for transplantation exacerbated by the fact that insulin independence is generally observed only after sequential transplantation of two or more islet grafts. The need for a large number of islets is related to early events including anoikis, hypoxia, and activation of the implant microenvironment, all of which lead to inflammatory stimuli, islet cell death, and/or functional impairment (29). These phenomena ultimately activate proapoptotic pathways and contributes substantially to the reduction of functional islet mass (7,42). To confer cytoprotection, islets may be genetically engineered in vitro prior to transplantation, an approach that has led to a reduction in the number of islets required for successful transplantation in experimental models (8,18).

Vectors based on the feline immunodeficiency virus (FIV) have been shown to stably transduce islets without bearing the immunogenicity concerns associated with adenoviral vectors (11,14,30), which have been shown to induce release of inflammatory mediators from islets (46). Also, being derived from a nonhuman pathogen, FIV vectors afford several potential safety advantages for use in human subjects (10). FIV vectors have shown impressive efficacy in the limited number of preclinical disease models in which they have been used thus far (1,6,43). Pertinent to this study, our laboratory has shown that FIV transduces islets effectively and stably with no untoward effect on the insulin secretion capacity of the β-cells (12,14).

The delivery of more than one gene may be highly advantageous to protect islets in the transplant setting.

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Simultaneous transduction of pancreatic islets with two genes has been accomplished using adenovirus (4) as well as adeno-associated virus (23). Until now, only one gene has been expressed in islets transduced with lentiviral vectors. The present study was designed to determine whether an FIV vector could deliver two genes to isolated human pancreatic islets.

Infections were carried out at a modest multiplicity of infection (MOI < 10) so that the efficiency of transduction could be accurately gauged, and so that we could determine whether transduction of predominantly the outer cell layer of the islets might confer protection to the inner islet mass via a "barrier" effect. Additionally, there is significant concern about deleterious effects caused by gene transfer vector insertional mutagenesis (3). Limiting the number of "hits" per cell dramatically decreases this risk and leaves the inner islet cells largely untouched.

We chose to utilize the antiapoptotic gene cFLIP (cellular Fas-associated death domain-like IL-1-converting enzyme inhibitory protein), an inactive homologue of caspase-8 that binds to the death effector domains of both Fas-associated death domain and caspase-8 (19,21). Both cFLIP and synthetic caspase inhibitors have been shown to protect insulin-producing cells (9,13,15). The downregulation of cFLIP in islets exposed to high glucose as part of the ensuing apoptotic cascade implicates this gene in the death pathways of transplanted islets (26). Furthermore, because Fas, a transmembrane cell surface receptor, is thought to be involved in the death of islets by inflammatory cytokines (32), we tested the ability of the transduced cFLIP to protect islets from anti-Fas death induction.

For the first time, we show successful transduction of islets with a lentiviral vector expressing two genes. Despite using low MOIs of recombinant viral vector we demonstrate significant protective effects of cFLIP on the whole islet mass, probably due to bystander effects. The fact that cFLIP expression results in improved β-cell viability following exposure to proinflammatory cytokines, anti-Fas, and doxorubicin has important implications for the protection of transplanted islets.

MATERIALS AND METHODS

Isolation of Human Islets of Langerhans

Human pancreata were obtained from multiorgan cadaveric donors and processed at the Human Cell Processing Facility of the Diabetes Research Institute. Islets were obtained by a mechanically enhanced enzymatic digestion (Liberase®; Roche-Boeringher-Mannheim) using a modification of the automated method (37) followed by purification on discontinuous gradients (36). Islet purity was assessed by dithizone (Sigma, St. Louis, MO) staining, and islets were counted and scored for size. An algorithm was used for the calculation of 150-μm-diameter islet equivalent number (IEQ) (35). After isolation, islets were cultured overnight at 37°C, 5% CO_2, in CMRL medium (Mediatech Inc., Herndon, VA) with 2% human albumin (Talecris Biotherapeutics, Research Triangle Park, NC), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 2 mM L-glutamine, and 25 mmol/ml HEPES buffer.

Construction of FIV Expression Vector With Two Functional Genes

Second-generation FIV-based FELIX vectors have been previously described (11) and served as backbones for the vector used. Murine cFLIP was cloned into Rous sarcoma virus pRSV-huGFP [pRC/RSV (Invitrogen, Carlsbad, CA) based huGFP2-1 expression vector] upstream of the RSV-huGFP cassette. The resulting cFLIP-RSV-GFP product was then cloned out and inserted in the FIV transfer vector pTiger downstream of the internal CMV promoter. Thus, in pFIV-cFLIP-GFP, the CMV promoter drives cFLIP while the RSV promoter drives GFP (Fig. 1A).

FIV Production

Transfections for FIV production were performed using FuGENE 6 (Roche, Indianapolis, IN) as described previously (11). In all cases, 31 μg of structural protein vector, 19 μg transfer vector, and 12 μg envelope vector were transfected per 15-cm plate. The day prior to transfection, 12.5×10^6 293T cells were plated per 15-cm plate. Cells were washed and media changed at 30 h posttransfection. At 42–44 h posttransfection plates were moved to 32°C. At 48 h posttransfection, virus was collected and cleared by filtration through a 0.45-μm filter. Virus was concentrated ~15-fold by centrifugation in a Beckman JA-25.50 rotor at 20000 RPM for 1.5 h and resuspended in TNE.

Islet Transduction

Islets were transduced at a MOI of 5×10^2–5×10^3 infectious units per islet equivalent (IEQ) in the presence of 5 μg/ml Polybrene (Sigma, St. Louis, MO) using spinoculation (11). The algorithm used for the calculation of 150-μm-diameter islet equivalent has been previously described (35). Islets were placed in six-well plates and incubated in complete CMRL with virus and polybrene and spun using 2000 rpm at 22°C for 1 h. After the spinoculation plates were placed overnight at 22°C (5% CO_2) in fresh complete culture media prior to incubation at 37°C (5% CO_2).

Transgene Expression: GFP

Seventy-two hours posttransduction, islets were mounted with Vectashield mounting medium with DAPI

(4′,6 diamidino-2-phenylindole) (Vector Laboratories) for nuclear counterstaining and examined for GFP expression at the Diabetes Research Institute Imaging Core using a Zeiss Axioplat unit with a fluorescent light and appropriate filters and photographed using a LSM 500 software laser scanning confocal system attached to a Zeiss Axiovent 100M (Carl Zeiss, Thornwood, NY). To determine transduction efficiency, islets were dissociated in 0.05% trypsin-EDTA, washed, and resuspended in phosphate-buffered saline containing 0.5% bovine serum albumin, and then assessed (minimum 1×10^5 cells/condition/experiment) by flow cytometry (FacsCalibur; BD Pharmingen, San Diego CA) for GFP expression.

cFLIP Expression

cFLIP expression was assessed by Western blot analysis where purified transduced and nontransduced human islets were solubilized by sonication in 20 mM Tris, pH 7.4, containing 2% SDS. Protein (15 μg) was separated by electrophoresis on 10% polyacrylamide gels (Cambrex, Bioscience, Rockland, ME) under denaturing and reducing conditions. Proteins were transferred to nitrocellulose membranes and membranes were used for immunoblotting. Membranes were incubated with 0.5 μg/ml rabbit anti-human cFLIP (R&D Systems, Minneapolis, MN) and detected after incubation with goat-anti-rabbit IgG peroxidase conjugate (Sigma, St. Louis, MO) by chemiluminescence (Pierce, Rockford, IL). Recombinant human cFLIP (R&D Systems) was included as a positive control. Membranes were stripped and reprobed with rabbit anti-actin (1:5000 dilution; Sigma) and detected as described above.

RNA Preparation and Reverse Transcription

Total RNA of islets was purified by using RNeasy Mini Kit (Qiagen, Valencia, CA). Subsequently, the first-strand cDNA was synthesized by using random primers with a Superscript First-Strand Synthesis System for RT-PCR (Invitrogen).

Real-Time PCR Assay

Real-time PCR reaction was carried out in the AB 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Relative quantification of cFLIP was performed using TaqMan cFLAR Gene Expression Assay using Taqman 18S Expression Assay as the normalization control. Cycle threshold used for this assay was normalized to the cycle number at which the fluorescence signal crosses the threshold. RQ values are the relative quantification values for the study.

Caspase 8 Levels

To measure caspase 8 levels the membranes were incubated with 1.0 μg/ml rabbit anti-human caspase 8 antibody (R&D Systems). After incubation and development with the secondary antibody the membranes were stripped and reprobed for actin. Chemiluminescent images were captured with a digital imaging system (Fluor Chem 8000, Alpha Innotech, San Leandro, CA) and the integrated pixel intensities measured using the Fluor Chem software. The total caspase-8 (or procaspase-8) comprises a band at 55–57 kDa whereas the active caspase-8 comprises a band around 18 kDa.

Functional Assessment of Transduced Islets

Islets were analyzed for their response to a dynamic stimulation assay by perifusing them in a chromatography column (Bio-gel Fine 45–90 nm; Bio-Rad, Hercules, CA) with a buffer containing 125 mM NaCl, 5.9 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM HEPES, 0.1% bovine serum albumin, and 3 mM glucose at 37°C. Islets were perifused in the same buffer for 1 h (preincubation period). Following the preincubation period islets were perifused 5 min with the same buffer (3 mM glucose) and then sequentially exposed to 11 mM glucose (10 min), 3 mM glucose (15 min), 25 mM KCl (5 min), and 3 mM glucose (5 min). Fractions of the perfusate were collected every 12 min during the preincubation phase, and every minute during stimulation. The collected fractions were then assayed for human insulin concentrations by ELISA (Mercodia, Salem, NH).

Glucose-Stimulated Insulin Secretion (GSIS)

GSIS is expressed as secreted insulin upon glucose challenge and consists of measuring the insulin released by 50–100 islet equivalents (IEQ) after a 1-h incubation in high glucose (20 mM) and subsequently over the insulin released during low glucose incubation (2.8 mM) and expressing a ratio between high and low. Insulin was measured by ELISA (Mercodia, Salem, NH).

Apoptosis Induction

Cytokine induction (IL-1β, TNF-α, and IFN-γ). Seventy-two hours after transduction, control and transduced islets were cultured overnight at 1,000 IEQ/well and apoptosis was induced with a cytokine cocktail containing IL-1β (50 U/ml), TNF-α (1×10^3 U/ml), and IFN-γ (1×10^3 U/ml) (R&D Systems). Alternatively, islets were treated in the identical fashion and then exposed to 10 μM actinomycin D (G Biosciences, St. Louis, MO) for 30 min, followed by a 4-h incubation with anti-human CD95, 50 ng/ml (anti-Fas) (BD Biosciences, San Diego, CA).

Doxorubicin Induction. Seventy-two hours after transduction, control and transduced islets were cultured overnight at 1,000 IEQ/well and apoptosis was induced with 2 μM doxorubicin (Gene Technology, St. Louis,

MO). After incubation, islets were dissociated into single cell suspension in 0.05% trypsin-EDTA. The proportion of apoptotic cells was measured by FACS using the Annexin V-7AAD Apoptosis Detection Kit (BD Pharmingen, San Diego CA). Cells positive for Annexin V alone (early apoptosis) and cells positive for 7AAD and Annexin V (end-stage apoptosis and death) were counted and expressed as percentage of apoptotic cells for each experimental condition.

Measurement of Apoptosis. Quantitative analysis of β-cell viability was performed on dissociated human islet cells using a method for the assessment of fractional β-cell viability developed at the Diabetes Research Institute that allows differentiation between β-cell and non-β-cell subsets and defines subsets of viable and apoptotic cells as described in detail by Ichii et al. (20). Briefly, after the percentages of dead cells(7AAD+) were evaluated, the percentages of viable (nonapoptotic) β-cells were calculated by %Newport green positive × %TMRE positive in β-cells.

RESULTS

FIV Transduction of Islets Results in Expression of Both cFLIP and GFP

Islets were transduced with a self-inactivating lentiviral vector in which cFLIP is driven off an internal cytomegalovirus (CMV) promoter and huGFP expression is controlled by a downstream RSV promoter (FIV-cFLIP-GFP) (Fig. 1A). In this vector, expression of the upstream gene, cFLIP, is considerably stronger than that of the downstream GFP gene (data not shown); however, cells positive for GFP are almost invariably positive for the upstream gene. Confocal microscopy shows GFP expression in 100% of transduced islets, predominantly in the exterior cells (Fig. 1B). Transduction efficiency of individual cells within an islet was determined by flow cytometry of GFP fluorescence on dissociated islets, and ranged between 8% and 12%. Similar efficiencies at these MOIs have been reported previously, as has proof that a substantial number of the transduced cells are insulin-secreting β-cells (12).

The level of cFLIP expression was measured by Western blot analysis (Fig. 1C). The 55-kDa band corresponding to full-length cFLIP was detected in both the transduced and the nontransduced islets. In the transduced islets, the intensity of the cFLIP band was approximately twofold higher than in the control islets. Presence of cFLIP message was established by RT-PCR (Fig. 1D). The results normalized to 18S RNA and expressed as fold increase over control show that cFLIP expression increases three- to fourfold in transduced islets induced with cytokines when compared to mock transduced islets. These results demonstrate that two transgenes are simultaneously expressed in pancreatic islets using a lentiviral vector.

Increased cFLIP Expression Results in Decreased Levels of Caspase-8

To test the effect of cFLIP at the intracellular level, islet apoptosis was induced with cytokines and anti-Fas and then caspase-8 levels were analyzed. Previously, Cottet et al. (9) showed, in a murine cell line, that cytokine-induced apoptosis was abolished by transfection with cFLIP due to its direct interaction with and inhibition of caspase-8. To examine the effect of cFLIP transduction in human islets in relation to caspase-8 levels, apoptosis was induced using a cytokine cocktail alone (IL-1β, TNF-α, and IFN-γ) or by the identical treatment followed by actinomycin-D and anti-Fas. Western Blot analysis shows that the levels of caspase-8 are significantly lower in islets that express cFLIP whether or not apoptosis is induced (Fig. 2A, B). Furthermore, levels of activated caspase-8 were also determined (Fig. 2C, D). Activation of caspase-8 involves a two-step proteolysis: the cleavage of caspase-8 to generate a 43- and a 12-kDa fragment, which is further processed to 10 kDa. The 43-kDa protein is then cleaved to yield a 26-kDa fragment and the active form of caspase-8 p18 (18kDa). The 55-kDa results are shown in Figure 2A and B, whereas those for the p18 form are shown in Figure 2C and D. In both cases caspase-8 levels are decreased in islets transduced with FIV-cFLIP-GFP when the islets are treated with cytokines or with cytokines and anti-FAS. These results show that the transduction with cFLIP results in decreased levels of caspase-8 as predicted, thereby implying the expression of functional cFLIP.

Islets Expressing FIV-cFLIP-GFP Exhibit a Metabolic Advantage Relative to Untransduced Islets

A key requirement for the applicability of genetic engineering of islets is that the regulated insulin secretion capability not be affected by the transduction or the expression of the transgene. In vitro islet function was tested measuring insulin release in human islets using two approaches. In the first, a perifusion profile of the islets reflected their response to low glucose, high glucose, and KCl challenge after infection with FIV-cFLIP-GFP. In the second (GSIS), the high glucose response was compared to the low glucose response and expressed as a ratio. Both approaches were based on comparisons of control islets to islets transduced with FIV-cFLIP-GFP.

The results shown in Figure 3A demonstrate that the islets transduced with cFLIP are at a metabolic advantage when compared to untransduced islets as shown by perifusion. To confirm the perifusion data, a glucose-

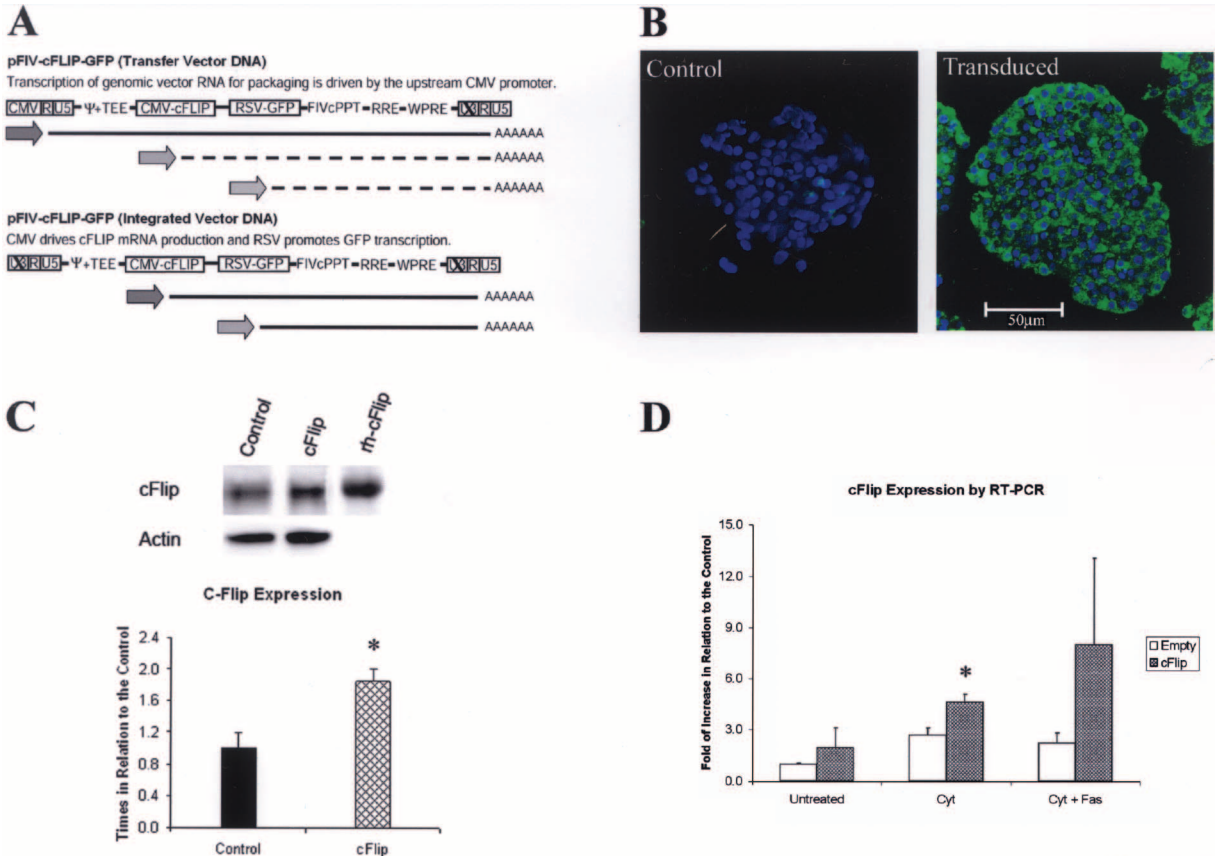

Figure 1. FIV vectors and gene expression. (A) pFIV-cFLIP-GFP is depicted in its plasmid DNA form used in FIV vector production as well as in its integrated proviral form. TEE denotes a small *cis*-acting element from FIV, which may enhance infectivity. The FIV cPPT and packaging signal are also marked. The vector contains a 3′ self-inactivating (SIN) LTR mutation, which renders both LTRs in the integrated form of the vector transcriptionally inert. The CMV promoter drives production of cFLIP, while the Rous sarcoma virus (RSV) promoter drives GFP expression. (B) Expression of GFP in human islets transduced with FIV-cFLIP-GFP 3 days posttransduction (left panel mock, right panel transduced). DAPI (blue) staining shows the nuclei using the appropriate fluorescent light showing GFP in green as seen using confocal microscopy. Scale bar: 50 μm. (C) Nontransduced (control, black column) and FIV-cFLIP-GFP-transduced islets (cFLIP, hatched column) were solubilized and 15 μg of protein was separated by electrophoresis on 10% polyacrylamide gels under denaturing and reduced conditions. Proteins were transferred to nitrocellulose membranes for immunodetection of cFLIP and actin. Western blot shows an immunoreactive band coincident with the recombinant human cFLIP (rh-cFLIP), which was included as a positive control. cFLIP band intensities were normalized for intensity of the actin band to account for differences in sample loading and results are representative of two experiments. There is a 1.8-fold increase in cFLIP immunoreactivity in the transduced versus control islets. (D) Expression of transgene-derived and endogenous cFLIP mRNA in transduced islets measured by real-time PCR. Three days after transduction control islets transduced with empty virus (white columns) and FIV-cFLIP-GFP-transduced islets (hatched columns) were cultured overnight either in control media (untreated), media containing cytokine cocktail (TNF-α, IFN-γ, and IL-1β) (cyt) or media containing the same cytokine cocktail plus anti-FAS (cyt + FAS). Relative quantification expressed as fold increase after normalization for 18S RNA is depicted from four independent experiments. A statistically significant ($*p = 0.040$) increase is seen in cFLIP message levels when transduced islets are induced with cytokines using paired Student's *t*-test.

stimulated insulin secretion (GSIS) assay was performed using islets treated with cytokines + FAS. The GSIS results were as follows. Control islets had a ratio of high glucose insulin secretion versus low glucose insulin secretion of 1.25, islets transduced with empty vector had a ratio of 1.32 ± 0.03, whereas islets transduced with cFLIP-GFP had a significantly higher ($p = 0.001$) GSIS ratio of 1.89 ± 0.18.

FIV-cFLIP-transduced islets were stimulated with cytokines and anti-FAS; β-cell metabolism was significantly protected compared to islets treated in the same manner that had not been transduced with cFLIP. These results confirm that the β-cell response to glucose challenge in vitro is neither adversely affected by the infection protocol nor by expression of two transgenes. Furthermore, the data suggest that expression of cFLIP in

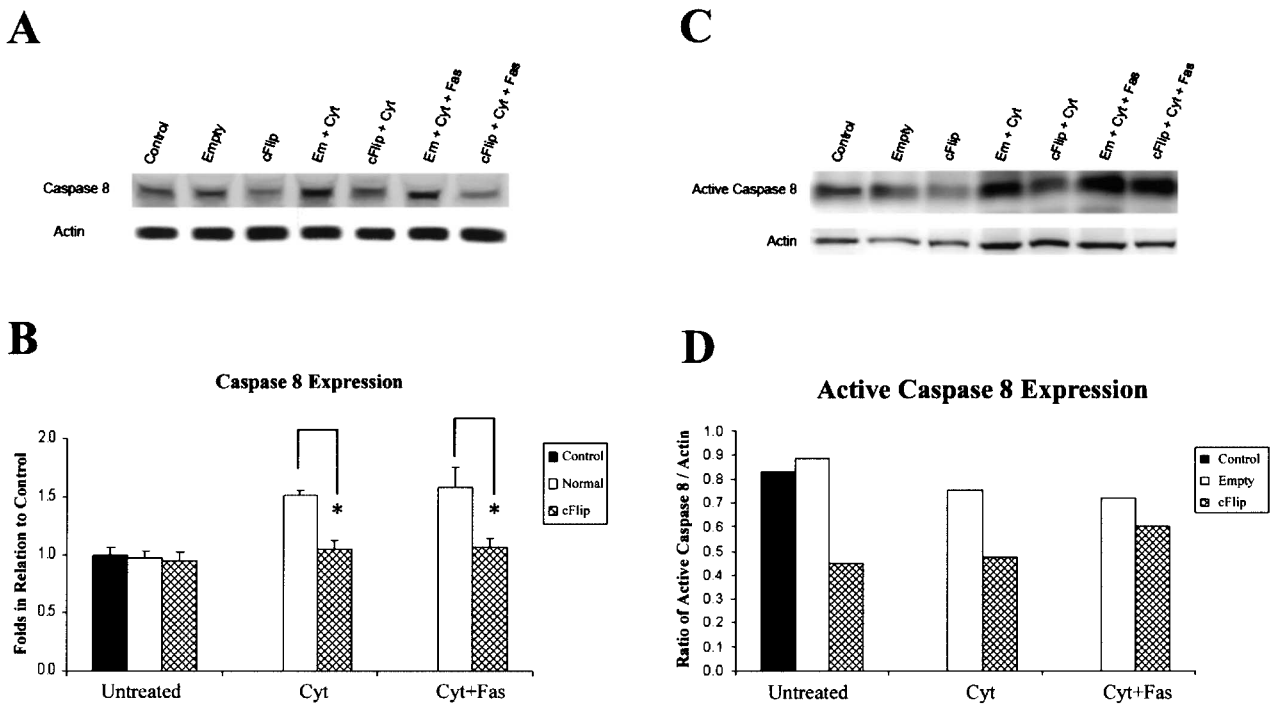

Figure 2. Caspase-8 and active caspase-8 expression. Three days after transduction control islets transduced with empty virus (white columns) and FIV-cFLIP-GFP-transduced islets (hatched columns) were cultured overnight either in control media (untreated), media containing cytokine cocktail (TNF-α, IFN-γ, and IL-1β) (cyt), or media containing the same cytokine cocktail plus anti-FAS treatment (cyt + FAS). Islets were solubilized and 15 μg of protein was separated by electrophoresis on 10% polyacrylamide gels under denaturing and reduced conditions. Proteins were transferred to nitrocellulose membranes for immunodetection of caspase-8, active caspase-8, and actin. Western blots show an immunoreactive band coincident with caspase-8 (A) or active caspase-8 (C); relative fold increase over control is shown in (B) and the ratio active caspase 8/actin in (D). A control untreated sample is included as a black column to confirm that the transduction with an empty vector does not alter caspase-8 levels. Data are shown with ±SEM from at least three independent experiments for caspase-8 and for one experiment for active caspase-8. *FIV-cFLIP-GFP-transduced islets have significantly less caspase-8 when treated with cytokines or cytokines + Fas [one-way ANOVA followed by Tukey (post hoc) $p < 0.02$].

the FIV-transduced islets results in improved in vitro function likely due to enhanced viability.

Expression of cFLIP Is Cytoprotective When Apoptosis Is Induced by Proinflammatory Cytokines and Fas Activation

Because the binding of cFLIP can prevent the activation of both caspase-3 and caspase-8 (21), we examined the antiapoptotic effect of cFLIP expression on pathways dependent on these caspases. Control and transduced islets were exposed to a cytokine cocktail (IL-1β, TNF-α, and IFN-γ) with or without Fas activation as previously described (20). It should be noted that each assay is done on different preparations of human islets and each has a different level of apoptosis prior to treatment. The data are therefore presented as a ratio of viable β-cells following proapoptotic treatment compared to the initial viability. cFLIP-transduced islets showed a significant increase in the percentage of viable (nonapoptotic) β-cells in islets infected with FIV-cFLIP-GFP compared with controls as determined by FACS analysis (Fig. 4, $*p = 0.01$ and $**p = 0.003$).

Expression of cFLIP Is Cytoprotective When Apoptosis Is Induced by Doxorubicin

Matta et al. (27) have shown that cFLIP protects against cell death induced by doxorubicin, a chemotherapeutic agent whose mechanism of action is mediated by the upregulation of TRAIL death receptor 5 (28). The effect of cFLIP protection from doxorubicin-induced apoptosis was examined in control islets and compared to islets expressing cFLIP. Doxorubicin (2 μM) was added to cultures of control and transduced islets overnight and the proportion of apoptotic cells under each condition was assessed by flow cytometry using Annexin V and 7AAD. Figure 4B shows that cFLIP-transduced islets are almost completely resistant to doxorubicin-induced apoptosis. This result suggests that the expression of cFLIP in these islets also protects against doxorubicin-induced death.

DISCUSSION

Transplantation of pancreatic islets offers the best hope for a curative treatment for type 1 diabetes; however, before this treatment can become widely available to patients suffering with type 1 diabetes, means must be developed to render islets resistant to the numerous noxious stimuli that may trigger cell death following transplantation. Recently, a study using gene array technology showed that human islets express a large variety of proinflammatory genes that can potentially lead to the initiation of apoptosis in islet cells upon transplantation (22). Death of β-cells in the transplant setting is a complex process suggesting that an integrated interventional approach, targeting multiple pathways that affect islet cell viability and function, will be necessary to maximize the success of islet transplantation. Blocking multiple proapoptotic pathways may prove essential in promoting islet engraftment and in improving clinical outcomes, suggesting that a successful gene therapy approach in this setting will require gene transfer and expression of multiple genes (5,31). Combined expression

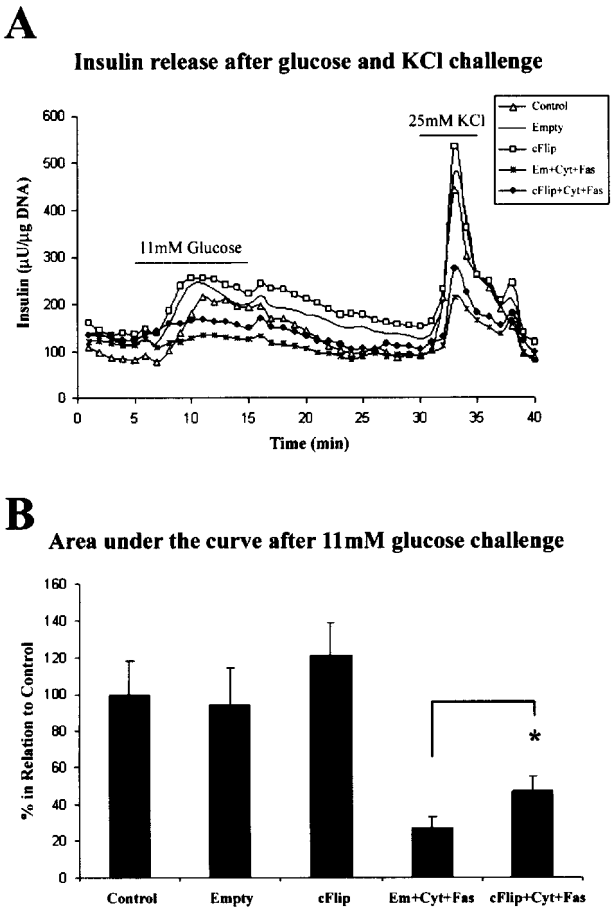

Figure 3. Assessment of islet function by perifusion. Islets were analyzed for their response to a dynamic stimulation assay in vitro and the results of the collected fractions are plotted as graphs representing the insulin release after the sequential challenges: 3 mM glucose (5 min), 11 mM glucose (10 min), 3 mM glucose (15 min), 25 mM KCl (5 min), and 3 mM glucose (5 min). The figure shows a representative graph of four independent experiments. The columns in (B) represent the area under the perifusion curve after 11 mM glucose challenge in relation to the control islets. Islets transduced with an empty vector are compared to islets transduced with the FIV-cFLIP-GFP vector and challenged by cytokines and anti-FAS. The islets with functional cFLIP have significantly better function than challenged islets without the cFLIP gene, as shown by a one-way AANOVA followed by Bonferroni (post hoc). $*p = 0.003$.

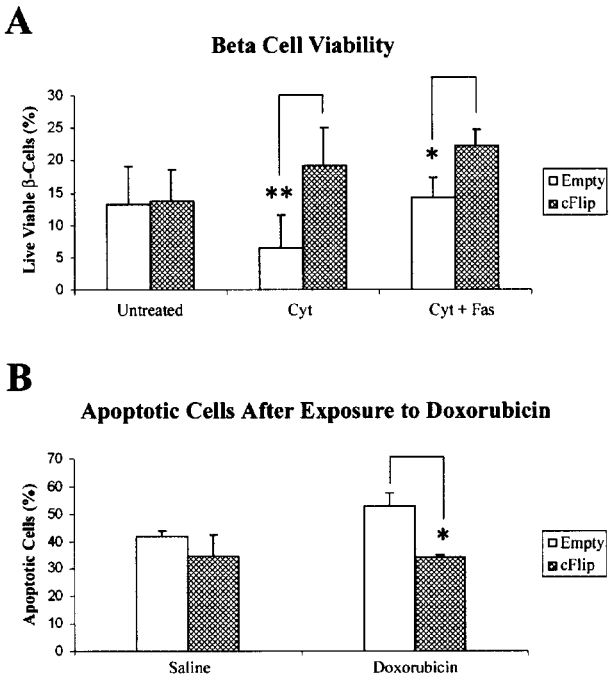

Figure 4. Effect of cFLIP expression on cytokine-induced and doxorubicin-induced apoptosis. (A)Three days after transduction control islets transduced with empty virus (white columns) and FIV-cFLIP-GFP-transduced islets (hatched columns) were cultured overnight either in control media (untreated), media containing cytokine cocktail (TNF-α, IFN-γ, and IL-1β) (cyt), or media containing the same cytokine cocktail plus anti-FAS treatment (cyt + FAS). The proportion of viable β-cells was assessed using the method developed by our group and previously described. Data from five experiments are shown as proportion of viable beta cells ($*p = 0.01$, $**p = 0.003$), indicating that overexpression of cFLIP protects β-cell viability from cytokines and anti-FAS induction. (B) Three days post-transduction, untransduced (control) and FIV-cFLIP-GFP-transduced islets were cultured overnight in culture media containing saline or 2 µM doxorubicin. The proportion of apoptotic cells was assessed in each condition by flow cytometry analysis using Annexin V and 7AAD (for details, please refer to Materials and Methods section). Data are shown as mean proportion of apoptotic cells ± SEM from at least three independent experiments. *Two-tailed, paired *t*-test: $p = 0.042$.

of two cytoprotective genes by the means of individual vectors has been shown to be effective in delaying the development of autoimmune diabetes in NOD mice (25). Prior to the present study, no lentiviral vector has been shown to deliver more than one gene to pancreatic islets, although bicistronic lentiviral vectors have been shown to be effective in targeting other cell types (33,41,45). In showing that FIV can successfully deliver two genes to islets, we have established its suitability as a vector for future experiments in assaying the efficacy of therapeutic gene combinations.

In the present study the reporter gene (GFP) was delivered downstream of the gene encoding for the antiapoptotic protein cFLIP. This allows transduction efficiency to be measured by flow cytometry. GFP expression was seen in all transduced islets but the transduction efficiency measured by flow cytometry on dispersed islet cells averaged only 10%, which is similar to previous results using similar MOIs (12). Islets are composed of spherical clusters and it has been shown that the transduced cells are seldom in the core no matter what vector is used (12,17). This and confocal microscopy data lead to the conclusion that the 10% of cells transduced appear to be on the exterior of the islet. It is interesting, therefore, that protecting the exterior cells of an islet from apoptotic stimuli would also confer protection to those cells within. It is likely that this protection has to do with the bystander effect caused by the proximity of cells within an islet.

While the expression of cFLIP in transduced islets as determined by Western blotting appeared modest, there is no question that it was sufficiently high to functionally protect the genetically engineered islets. Also cFLIP lowered the levels of proapoptotic caspase-8, proving that the amount of cFLIP expressed in these islets was sufficient to functionally affect islet viability. Our results indicate that cFLIP may block some of the key events in the progressive loss of islets due to inflammation and thus provide a potentially effective strategy for β-cell protection. Transduction with FIV proved efficient enough that a significant metabolic advantage was conferred to these islets in vitro even using a low MOI (approximately 0.5–5 per islet cell). Infection of whole islets favors the cells on the exterior of the islet mass to which virus has the easiest access. It is possible that these cells are also the most exposed to external apoptotic stimulus and that engineering them to express cFLIP converts them into an outer "barrier" that shields the untransduced cells of the inner islet from death. Future work will address the exact mechanism of the protection observed in these studies as well as the in vivo effects of this protection in transplant models. Furthermore, improved designs for bicistronic lentiviral vectors that are now being applied to FIV-based vectors are likely to further augment transduction efficiency and coexpression levels of both genes of interest. Although cFLIP improved islet viability when delivered at low MOI, some potentially cytoprotective genes may require greater or lesser levels of transduction depending on their mechanism of protection.

The construct used in our study encoded for cFLIP, which inhibits apoptosis both through death receptors (39) and through Fas activation (2,40). The capacity of cFLIP to inhibit the downstream mediators of TNF signaling is critical in preventing cytokine-induced apoptosis (44). In order to determine the cytoprotective effect of cFLIP, we measured islet apoptosis in the presence or absence of TNF-α and IL-1β, which are known to upregulate caspase-8 (38). As further proof of cFLIP protection the chemotherapeutic drug doxorubicin, which upregulates caspase-3 and against which cFLIP is known to protect, was also tested (27). This result would suggest that the death receptor 5 may be involved in the protection of islets via cFLIP.

Our data showed that islets transduced with FIV-cFLIP-GFP were protected from apoptosis induced by these proapoptotic stimuli. These results confirm the measurable biologic effect of the cFLIP gene in human islets. In addition, islets transduced with the lentiviral vector expressing both genes showed improved metabolic function relative to controls, as measured by insulin secretion in response to glucose challenge in vitro. These results demonstrate that the expression of the two transgenes and the transduction protocol itself did not adversely affect islet β-cell function. These data are consistent with our previous studies also showing the lack of islet cell impairment when using FIV lentiviral vectors (12). Furthermore, they show that the introduction of cFLIP protects islets from a variety of proapoptotic stimuli.

While we have demonstrated the capacity of FIV to deliver more than one gene to islets, the challenge remains to find a gene combination that is capable of protecting islets against the variety of immunologically mediated events following transplantation. It is clear that no single gene is likely to protect transplanted islets without immunosuppression; however, the capacity of FIV vectors to simultaneously transfer two protective genes may prove invaluable in the field of islet transplantation. Also, the significantly larger packaging capacity of FIV relative to adeno-associated virus (AAV) vectors allows for the coexpression and regulation of multiple genes in a way that isn't possible in AAV. Our immediate plans include replacing GFP with secreted immunomodulatory molecules, or alternately with direct inhibitors of the lytic proteases carried by T cells and NK cells. Our data suggest that FIV vectors may be used to deliver polycistronic constructs to human islet cells and that by choos-

ing the appropriate genes this delivery system may result in β-cells that are cytoprotected. This approach may provide an important molecular tool that will contribute to reversing diabetes with a reduced number of modified islets.

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