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XIAP Gene Expression Protects β -cells and Human Islets from Apoptotic Cell Death

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Abstract

Islet transplantation has the potential to treat type I diabetes, however, its clinical application is limited due to the massive apoptotic cell death and other post-transplantation challenges to islet grafts. Therefore, the objective of this study was to determine whether ex vivo transduction of rat insulin producing INS-1E cells and human islets with adenoviral vector encoding human X-linked inhibitor of apoptosis (Adv-hXIAP) can protect them from inflammatory cytokines and improve their viability and function. There was dose dependent XIAP gene expression. XIAP expression led to decrease in the activities of caspase 3/7, 8 and 9, resulting in reduced apoptotic cell death induced by a cocktail of inflammatory cytokines such as IL-1 β , TNF α , and IFN γ . Prolonged normoglycemic control could be achieved by transplantation of Adv-XIAP transduced human islets under the kidney capsule of streptozotocin induced diabetic NOD-SCID mice. Immunohistological staining of the islets bearing kidney sections at day 42 after transplantation was positive for insulin. Moreover, the protective effect of XIAP was reversed by co-administration of XIAP inhibitor embelin. These results indicate that ex vivo transduction of islets with Adv-XIAP will decrease cytokine induced apoptosis and improve the outcome of islet transplantation.

Keywords

Human islets; adenoviral vectors; XIAP; apoptosis; islet transplantation; diabetes

INTRODUCTION

Islet transplantation has the potential to treat type I diabetes. However, its widespread application in the clinic is limited due to the lack of sufficient number of human islets from donors and the loss of islet viability after transplantation. Insulin producing β -cells of transplanted islets lose up to 70% at about 24h post transplantation.¹⁻² Therefore, how to restore β -cell function against the inflammation after transplantation and protect them from the immune reaction of the recipient becomes a major barrier to overcome.

Success of islet transplantation greatly depends on the graft viability and function against post-transplantation challenges including inflammatory cytokines, hypoxic environment, and reactive oxygen species (ROS) at the transplantation site.³⁻⁶ Islet loss occurs mostly in the first two weeks after transplantation, and will decrease significantly due to successful revascularization thereafter.⁷⁻⁸ Therefore, expression of an antiapoptotic gene to prevent β -

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cell loss and expression of a growth factor gene to promote islet revascularization may be an effective strategy to improve islet survival and function post transplantation.⁹ Ex vivo transduction of islets with adenoviral vector encoding human interleukin-1 receptor antagonist (Adv-hIL-1Ra) has been reported to prevent IL-1 β induced apoptotic cell death of islets.¹⁰ In our group, Narang and colleagues demonstrated the synergistic effect of vascular endothelial growth factor (VEGF) and IL-1Ra co-expression in improving the islet viability and function.¹¹

IL-1 β is just one of the several inflammatory cytokines which induce apoptosis. Both extrinsic and intrinsic pathways will eventually upregulate caspase 3, which is the endpoint of the apoptotic pathway. Therefore, we further demonstrated that the viability and function of islets can be better improved by caspase-3 inhibition after transplantation. Caspase-3 gene silencing by Adv-caspase-3-shRNA could partially prevent the islet loss post-transplantation.¹² Moreover, we recently reported that inducible nitric oxide synthase (iNOS) gene silencing can also prevent inflammatory cytokine induced β -cell apoptosis.¹³

As shown in Fig. 1, extracellular stress can activate intracellular caspase cascades through cytokines-death receptor-caspase 8 pathway or hypoxia, reactive oxygen species, and UV-mitochondria-cytochrome C-caspase 9 pathway. Caspase 8 and caspase 9 then can activate the converging point, caspase 3. Caspase 3 itself is an executioner caspase, which can cleave the death substrates to induce apoptosis, it can also activate other executioner caspases, such as caspase 6 and caspase 7 to expand the apoptotic signal. X chromosome linked inhibitor of apoptosis (XIAP) is a potent anti-apoptotic factor inhibiting the activities of caspase 3, 7 and 9. The BIR2 domain of XIAP inhibits caspase 3 and caspase 7, while BIR3 domain inhibits caspase 9.¹⁴⁻¹⁶ Therefore, XIAP holds great potential to inhibit the apoptosis of human islets caused by both hypoxic environment and inflammatory cytokines in the transplantation sites. Emamaulle and colleagues demonstrated that XIAP overexpression minimizes the injury in pancreatic β -cells caused by hypoxia and reperfusion.¹⁷ Hui and colleagues demonstrated the reversal of the negative effects of immunosuppressive drugs by XIAP overexpression on human islets.¹⁸ XIAP has also been proven to improve the murine islet viability after isolation.¹⁹ However, the major reason for the cytoprotective effect of XIAP on pancreatic β -cells and human islets against cytokines was not determined in these studies, and the mechanism underlying the protective effect of XIAP was also not discussed. Promising long term data of in vivo normoglycemic control after islet transplantation is still needed. Therefore, in this study, we transduced rat insulinoma cells (INS-1E cells) and human islets with replication deficient Adv-hXIAP to determine whether XIAP overexpression protects β cells and islets from cytokine induced cell death and discussed about the underlying mechanism of its protective effect.

MATERIALS AND METHODS

Materials

The replication deficient ($\Delta E1/\Delta E3$) adenoviral vector encoding human XIAP (Adv-hXIAP) was purchased from Vector Biolabs (Philadelphia, PA). RPMI 1640 medium was purchased from Invitrogen (Carlsbad, CA). CMRL-1066 medium was purchased from Sigma Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was purchased from MediaTech Cellgro (Herndon, VA). Phosphate buffer solution (PBS) was purchased from GIBCO-BRL (Gaithersburg, MD). Human XIAP ELISA kits and cytokines such as IL-1 β , TNF- α , and IFN- γ were purchased from R&D Systems (Minneapolis, MN). Human Insulin ELISA kits were purchased from Alpco Diagnostics (Windham, NH). RNA extraction kit and Caspase-Glo 3/7 assay kit, DeadEnd Colorimetric TUNEL system, and APO-DIRECT TUNEL system were purchased from Promega (Madison, WI). SYBR Green real-time PCR master

mix and reverse transcription reagents were purchased from Applied Biosystems (Foster city, CA).

Virus Amplification and Titer Determination

Adv-hXIAP was transduced into 293 cells. At 48h post transduction, almost 90% of the cells were detached from the flask. Then, the cells were collected, centrifuged and frozen and thaw three times to release Adv-hXIAP viral particles. Then, the viral solution was used to determine the viral titer using an Adeno-X rapid titer kit from Clontech (Mountain View, CA). Briefly, different dilutions of Adv-hXIAP was taken and transduced to AD-293 cells, using untransduced cells as a control. After 48h post-transduction, AD-293 cells were fixed and incubated with Anti-Hexon antibody, followed by a secondary horse radish peroxidase (HRP)-conjugated antibody. Then the cells were stained with 3, 3' diaminobenzidine tetrahydrochloride (DAB) substrate and positively stained cells were counted under a microscope, and the titer was determined.

Cell and Islet Culture and Transduction

Rat insulinoma (INS-1E) cells, a gift from Professor Claes B. Wolheim (University Medical Center, Geneva, Switzerland) were seeded at a density of 10^6 cells per 24-well plate. After 24h, INS-1E cells were transduced with Adv-hXIAP of indicated MOI for 3h followed by washing and further culturing for 2 days. 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 receiving, the islets were cultured in CMRL-1066 medium containing 10 % fetal bovine serum (FBS) and 1 % Penicillin Streptomycin. INS-1E cells and human islets were transduced with Adv-hXIAP, while nontransduced human islets as well as the islets transduced with Adv-LacZ were used as controls. To determine the optimal multiplicity of infection (MOI) of Adv vectors in terms of plaque forming units (pfu) per islet equivalent (IE), 1000 IE were incubated with diluted Adv-hXIAP in 300µl media in a 96 well plate for 12 h, followed by washing and further culturing in 1ml of media in 24-well plates for 2 days.

Quantitative Real-time PCR

INS-1E cells were collected to determine XIAP mRNA level by quantitative Real-time PCR post-transduction. Briefly, INS-1E cells were collected and total RNA was isolated using RNeasy mini isolation kit from Promega (Madison, WI) 3 days post-transduction. RNA concentration was measured by UV spectrophotometer. Then 170 ng of extracted RNA was converted into cDNA using MultiScribe reverse transcriptase and random hexamers (Applied Biosystems, Inc., Foster City, CA) by incubation at 25°C for 10 min, followed by reverse transcription at 48°C for 30 min and enzyme inactivation at 95°C for 5 min. Then 2 µl cDNA was used as a template and analyzed by SYBR Green-I dye universal PCR master mix on a LightCycler 480 Instrument. Caspase 3 mRNA expression was also determined by the same method. β-actin was used as an internal control. All samples were run in triplicates. The primer sequences were as follows: human XIAP (forward) 5'-TGT TTC AGC ATC AAC ACT GGC ACG-3', (reverse) 5'-TGC ATG ACA ACT AAA GCA CCG GAC-3'; rat caspase 3 (forward) 5'-CAT GAC CCG TCC CTT GAA-3', (reverse) 5'-CCG ACT TCC TGT ATG CTT ACT CTA-3'; rat β actin (forward) 5'-AGT CAT GTA CGT AGC CAT-3', (reverse) 5'-CTC TCA GCT GTG GTG GTG-3'.

ELISA and Western Blot

At the indicated time post-transduction, INS-1E cells or human islets were collected to determine XIAP protein level by ELISA or Western Blot. Total protein was extracted by lysing INS-1E cells or human islets with RIPA buffer (Sigma) and stored in -80 °C for

ELISA and Western Blot study. For ELISA, XIAP levels were measured as per the manufacturer's protocol (R&D Systems, Minneapolis, MN). For Western Blot analysis, the samples were mixed with 6 × Laemmli sodium dodecyl sulfate (SDS) buffer (Boston BioProducts) and then boiled for 5 min to denature the protein. Then groups of samples were loaded to 4%-15% Tris-HCl Precast Polyacrylamide Gel (Bio-Rad, Hercules, CA) for electrophoresis and subsequently transferred to Immobilon Polyvinylidene Fluoride (PVDF) membrane (Millipore). After blocking with 3% bovine serum albumin in 1×*** PBST (PBS containing 0.05% Tween-20) for 1 h at room temperature, the membranes were further incubated with goat polyclonal IgG to XIAP and actin (Santa Cruz) primary antibodies (1:500) overnight at 4 °C. Membrane was then incubated with horseradish peroxidase (HRP)-conjugated rabbit polyclonal antibody to goat (1:10000) (Abcam) for 1 h at room temperature. Target proteins were detected by enhanced chemiluminescence (ECL) detection kit (GE Healthcare Life Sciences, Pittsburgh, PA).

Total protein concentration of INS-1E cells and islet extracts was also determined using the bicinchoninic acid (BCA) protein assay kit to normalize the results. Actin was used as a internal control for Western Blot.

Caspase Detection

Caspase-Glo 3/7, 8 and 9 assay kits were used to analyze caspase 3/7, 8 and 9 activities, respectively, as per the manufacturer's protocol (Promega). This assay kit provides a proilluminescent caspase substrate, DEVD, when cleaved by caspases, will release luciferin to quantitatively determine caspase concentration. Briefly, 48h following transduction and cytokine cocktail treatment for additional 3h, 100 µl Caspase-Glo reagent was added to 100 µl of culture supernatants in 96-well plates and incubated at room temperature for 1h. The contents were then transferred into culture tubes and luminescence was determined using a luminometer (Berthold, Germany).

Cell Viability

Cell viability was determined using MTT assay. Briefly, following transduction with adenoviral vectors at MOI=2, INS-1E cells were further incubated with cytokine cocktail, including 5ng/ml IL-1β, 25ng/ml TNFα, and 25ng/ml IFNγ, for indicated time. At the end of the cytokine treatment, INS-1E cells were further cultured with medium containing 0.5mg/ml 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) for 2h in 37°C. Then the medium was removed and 200ul DMSO was added into each well to dissolve the formazan crystals. The absorbance was measured in 550 nm using a microplate reader (Bio-Rad, Hercules, CA). The relative cell viability (%) was calculated.

TUNEL Assay

To determine the apoptotic cell death, following transduction for 3h, INS-1E cells were incubated with cytokine cocktail for 48h. Apoptotic cells were dark stained using DeadEnd Fluorometric TUNEL System from Promega (Madison, WI) as per the manual.

To quantitatively determine the effect of XIAP expression on cytokine induced INS-1E cell apoptosis, APO-DIRECT kit (Promega, Madison, WI) was used to detect apoptotic cells. Briefly, following transduction for 3h and incubation with the cytokine cocktail for additional 48h, INS-1E cells were made to single cell suspension by trypsin digestion 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 propidium iodide. Fluorescent intensity was measured by flow cytometry and analyzed using CELLQUEST software (BD Bioscience). Nontransduced cells without cytokine treatment were served as a

negative control. Three sets of independent transduction experiments were carried out for each assay.

Insulin Stimulation Test

In vitro function of INS-1E cells and human islets after transduction with Adv-hXIAP was determined by the static incubation method. Briefly, after transduction media was carefully removed with pipette, INS-1E cells and human islets were sequentially incubated in the media containing 2.5mM (basal) and 22mM glucose (stimulated) at 37°C for 1h. Supernatants were collected and analyzed for insulin release by Insulin Ultrasensitive EIA kit (Alpco Diagnostics, Salem, NH). Insulin secretion was expressed as $\mu\text{U/ml}$ and the ratio of insulin levels at 22mM to 2.5mM glucose was used to calculate the stimulation index.

Islet Transplantation Studies

Animal experiments were performed as per the NIH (<http://grants1.nih.gov/grants/olaw/references/phspol.htm>) and institutional animal care and use guidelines using the approved protocol. To induce diabetic animal model, streptozotocin (STZ) (40 mg/kg) was administered to NOD-SCID mice by intraperitoneal injection for 5 consecutive days. Animals were considered to be diabetic after two consecutive measurements of blood glucose ≥ 300 mg/dl using a One Touch Ultra Glucometer (LifeScan, Inc.). Before transplantation, human islets were grouped in four and transduced with blank vehicle, Adv-hXIAP, Adv-LacZ, and Adv-hXIAP with 50 ng/ml embelin at the dose of 1,000 MOI for 12 h and washed with PBS. About 1,000 transduced or non-treated islets were transplanted under the left kidney capsules of diabetic mice. The nonfasted glucose levels were measured from the snipped tail of each animal up to 42 days post-transplantation. Then the mice were anesthetized to collect blood to measure serum c-peptide level by ELISA; Alpco Diagnostics (Windham, NH), and the graft-bearing kidneys were removed from some animals to confirm the function of islet grafts by the return of blood glucose levels to ≤ 300 mg/dL for two consecutive days. Since most mice developed high blood glucose level ≥ 600 mg/dL after streptozotocin treatment and long time after transplantation, all mice were subjected to daily intraperitoneal administration of 10U/kg insulin to maintain mice survival. To minimize the interference to blood glucose measurement by insulin injection, all the measurement were done at least 5h after insulin injection.

To determine the effect of ex vivo Adv-hXIAP transduction prior to transplantation on insulin production of human islets post transplantation, transplanted mice were sacrificed at day 43 and kidneys bearing islets were isolated, washed with PBS, fixed in 4% paraformaldehyde overnight, and embedded in paraffin as described before²⁰. Sections of 5–7 μm thickness were cut and immune-stained with rabbit anti-insulin first antibody (Abcam) 1:500 overnight. Then immune-reactivity was detected using goat anti-rabbit IgG, H&L chain specific peroxidase conjugate (Abcam) and subsequent incubated with DAB substrate. Sildes were further counter-stained Haematoxylin (Sigma).

Statistical Analysis

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

RESULTS

XIAP gene expression in INS-1E cells and human islets

Real-time PCR results showed increase in XIAP mRNA level of INS-1E cells by 3000 folds and 100,000 folds after transduction of INS-1E cells with Adv-hXIAP at MOI of 1 and 10, respectively (Fig. 2A). Similarly, XIAP protein concentration increased by 13 folds (from

0.54 ng/ μ g to 6.94 ng/ μ g) and 28 folds (from 0.54 ng/ μ g to 12.59 ng/ μ g) for INS-1E cells after transduction with Adv-hXIAP at MOI of 1 and 10 (Fig. 2B). In human islets, dose-dependent XIAP gene expression was also observed after Adv-hXIAP transduction. XIAP protein level increased from the basal level of 16.17 ng/ μ g to 28.87, 75.58, and 95.55 ng/ μ g when 1000 islet equivalents were transduced with Adv-hXIAP at MOI of 200, 1000, and 2000, respectively (Fig. 2C). These results were in good agreement with our Western Blot analysis (Fig. 2D and 2E), where XIAP band density increased with increasing MOI, suggesting increase in XIAP protein expression after transduction.

XIAP improves INS-1E cells viability and function against inflammatory cytokines

Following transduction, INS-1E cells were further incubated with inflammatory cytokine cocktail for 48h. INS-1E cell viability decreased after cytokine treatment, as determined by the MTT assay (Fig. 3A). However, transduction of INS-1E cells with Adv-hXIAP showed only 14% decrease in cell viability, compared with untransduced INS-1E cells (37%) and INS-1E cells transduced with Adv-LacZ (55%) (Fig. 3A), suggesting the protective effect of XIAP. INS-1E cell function was determined by insulin stimulation test. Following transduction and cytokine treatment for 12h, INS-1E cells were sequentially incubated with 2.5 nM and 22.5 nM glucose medium and secreted insulin was determined by ELISA. The results showed that under cytokine treatment, Adv-hXIAP transduced INS-1E cells did produce more insulin than untransduced INS-1E cells and Adv-LacZ transduced INS-1E cells (Fig. 3B), suggesting the protective effect of XIAP on INS-1E cell function.

XIAP inhibits INS-1E cell apoptosis against inflammatory cytokines

One crucial step in apoptosis is DNA fragmentation, resulting from the activation of endonucleases during the apoptotic process.²¹ Therefore, the analysis of DNA fragmentation with TUNEL assay is a useful approach to gain information regarding cell apoptosis. After 48h of incubation with cytokine cocktail post-transduction, Adv-hXIAP transduced INS-1E cells showed significant decrease in the number of apoptotic cells, which were stained dark brown (Fig. 4C), compared with untransduced INS-1E cells (Fig. 4A), and Adv-LacZ transduced INS-1E cells (Fig. 4B). In a parallel experiment, all cells were fixed, stained with FITC, and run through flow cytometry to count the relative ratio of apoptotic cells. As shown in Fig. 4D-F, Adv-hXIAP transduced INS-1E cells showed an average of 5.7% of apoptosis, significantly lower than 27.9% and 32.8% of untransduced INS-1E cells and Adv-LacZ transduced INS-1E cells, respectively.

XIAP inhibits caspase activity in INS-1E cells and human islets

XIAP binds to caspase 3 and caspase 7 with its BIR2 domain and caspase 9 with its BIR3 domain,²² and is known to inhibit their activities.²³ Since caspase 3/7 was the converging point of apoptotic pathways, we determined the effect of XIAP gene expression on caspase 3/7 activity. XIAP overexpression led to dose dependent caspase inhibition under cytokine treatment (5ng/ml IL-1 β , 25ng/ml TNF α , and 25ng/ml IFN γ for 3h) (Fig. 5). Caspase 3/7 activity decreased up to 40% post transduction of both INS-1E cells and human islets with Adv-hXIAP (Fig. 5A and 5B). Similarly, XIAP expression also inhibited caspase 8 and caspase 9 activities in human islets, resulting in a total inhibition of 45% and 40%, respectively (Fig. 5C).

Prolonged normoglycemic control of diabetic mice by Adv-hXIAP transduced human islets

The effect of XIAP gene expression on the islet survival and function post-transplantation was determined in terms of blood glucose, animal weight gain, and insulin production, by comparing mice transplanted with Adv-hXIAP transduced human islets and mice transplanted with untransduced human islets, Adv-LacZ transduced human islets, and Adv-

hXIAP with embelin co-transduced human islets. Following transplantation, blood glucose level decreased to around 200 mg/dl for all transplanted mice (Fig. 6), suggesting the immediate reversal of diabetic hyperglycemia by islet transplantation. However, the blood glucose level of the mice transplanted with un-transduced islets, Adv-LacZ transduced islets, and Adv-hXIAP plus embelin co-transduced islets gradually increased after the first week post transplantation, and was maintained to the diabetic level (~600 mg/dl) since 2 weeks post transplantation (Fig. 6), suggesting the impaired normoglycemic control caused by the impaired islet viability and function. This result is also consistent with previous reports. 24-26 In contrast, the mice transplanted with Adv-hXIAP showed prolonged normoglycemic control till 6 weeks post transplantation (Fig. 6), probably due to the improvement in transplanted islet viability and function by XIAP overexpression. It should be noted that since most mice became highly diabetic after streptozotocin treatment and the experiment last for more than 6 weeks, all mice were subjected to daily intraperitoneal administration of 10U/kg insulin from the second week post transplantation. To minimize the interference of insulin injections to blood glucose measurement, all the measurement were made 5h after insulin injection.

As listed in Table 1, the mice transplanted with Adv-hXIAP transduced human islets showed significant increase in average body weight six weeks after transplantation, probably due to the prolonged normoglycemic control by XIAP overexpression. To further monitor the long-term islet function following transplantation, the kidney bearing Adv-LacZ transduced islets and Adv-hXIAP transduced islets were isolated at the end of experiment and subjected to immunohistochemical analysis. The untransplanted opposite kidney were also isolated and used as the negative control (Fig. 7A). The positive staining of insulin was more evident in kidney section bearing Adv-hXIAP transduced islets (Fig. 7B), while only rare insulin staining was observed in the kidney section bearing Adv-LacZ transduced islets (Fig. 7C), suggesting the maintenance of insulin secretion. The serum of mice was also collected to measure serum c-peptide level by ELISA. Result showed that c-peptide level in the mice transplanted with Adv-hXIAP transduced islets was significantly higher than the other group, indicating improved islet function in Adv-hXIAP transduced human islets post transplantation (Fig. 7D).

Protective effect of Adv-hXIAP transduction will be reversed by XIAP inhibitor embelin

Embelin cytotoxicity was first determined ex vivo by MTT. Embelin does not show significant toxicity on INS-1E cells in 24 h treatment when concentration is below 200 nM (Fig. 8A), and 50 nM was selected as a proper concentration of embelin to inhibit XIAP expression. XIAP expression in human islets was greatly inhibited after embelin treatment (Fig. 8B, 8C), even lower than the basal XIAP expression in normal islets, which was consistent with the literature.²⁷⁻²⁸ The cytoprotective effect of XIAP overexpression was impaired by XIAP inhibitor embelin. Adv-hXIAP transduced INS-1E cell viability was decreased to 44% after incubation with cytokine cocktail and 50nM embelin for 24h, compared with 88% viability with just cytokine cocktail (Fig. 8D), though 50nM embelin did not cause any significant toxicity to INS-1E cells in more than 3 days (data not shown). Caspase 3/7 activity was restored to the untransduced level after embelin treatment (Fig. 8E). This suggests the inhibitory effect of XIAP gene expression on caspase activity can be reversed by embelin. The prolonged normoglycemic control of diabetic mice by Adv-hXIAP transduced human islets can also be impaired by additional embelin incubation before transplantation, in which the duration of normoglycemia was significantly decreased from 6 weeks to 2 weeks (Fig. 6), suggesting the loss of islet viability and function by XIAP inhibition.

DISCUSSION

Human islet transplantation has great potential to provide type 1 diabetic patients with sustained and improved normoglycemic control and a period of insulin independence. However, most islet grafts get destroyed in the early days post-transplantation due to inflammatory cytokines, ROS and failure to revascularize induced by immune rejection, let alone the islet loss in islet isolation, transportation and transplantation processes.¹⁻² Although the use of immunosuppressant to the transplant recipient can partially relieve graft rejection by the host immune system, these drugs have significant toxicity including nephrotoxicity, ulcers, hyperglycemia, osteoporosis, and increased risk of infection and neoplasms.²⁹⁻³² Therefore, islets from two to four donors are generally necessary for successful islet transplantation.³³ Since the limited islet supply nowadays cannot meet the increasing islet need for transplantation, improving islet graft viability and function post transplantation is one of the major barriers to overcome for successful islet transplantation.

As we mentioned before, the first two weeks post islet transplantation is crucial for graft survival. This makes adenoviral vector a good candidate for ex vivo gene therapy because of its high transduction efficacy and stable expression ability up to eight weeks.³⁴⁻³⁵ In our group, we have been working on the adenovirus mediated gene therapy method to improve the islet viability and function against graft rejection post transplantation for years. Briefly, besides the growth factor gene delivery to reduce apoptotic β -cell death and promote β -cell proliferation post transplantation, we also demonstrated that proapoptotic gene silencing such as iNOS silencing and caspase 3 silencing by siRNA and shRNA respectively, could reduce the apoptotic β -cell death caused by inflammatory cytokines.^{11-13, 26}

Compared with other gene therapy methods, XIAP gene delivery holds its own merits. The major problem with growth factor gene therapy was that growth factors were secreted protein, whose effects were determined by microenvironment concentration. Therefore, in Adv-transduced human islet clusters, growth factor concentration in extracellular microenvironment could not be uniformly distributed and finely regulated. Accumulation of microenvironmental VEGF in the outer layer of human islets may finally lead to aberrant angiogenesis and islet loss.³⁶⁻³⁷

XIAP is strictly localized in the cytoplasm in a granular supranuclear pattern.³⁸ Therefore, after Adv-hXIAP transduction, the outer layer of human islets which will be more exposed to the inflammatory cytokines and hypoxic environment, will express higher level of XIAP and achieve an optimal defense mechanism against cytokine and hypoxia challenge in the early stage of graft survival. Transduction of INS-1E cells and human islets with Adv-hXIAP resulted in significant increased XIAP expression (Fig. 2). With adenovirus mediated XIAP overexpression method, we can easily reach the caspase inhibition effect in human islets without inducing acute immune response by ex vivo transduction. Adv-hXIAP ex vivo transduction also led to ~40% inhibition of caspase 3/7, caspase 8, and caspase 9 activities in both INS-1E cells and human islets (Fig. 5). The protective effect of Adv-hXIAP transduction on human islets was lasted for more than six weeks after transplantation. Another advantage of XIAP expression, though not discussed in this paper, is its ability to reverse the islet loss caused by hypoxia,³⁹⁻⁴⁰ which made it a good candidate for ex vivo transduction of islets prior to transplantation.

Probably as a result of caspases inhibition, we also observed the protective effect of XIAP expression on INS-1E cells against cytokine cocktail treatment. Compared with untransduced INS-1E cells, Adv-XIAP transduced INS-1E cells showed significant improved cell viability, decreased apoptotic cells and increased insulin production under cytokines treatment. One interesting point we observed in this experiment is that Adv-LacZ

transduced INS-1E cells showed decreased cell viability and decreased insulin production under cytokines treatment, despite the fact that Adv-LacZ at such low MOI (MOI=2) has no significant effect on INS-1E cell viability (data not shown), suggesting the cytotoxic or proapoptotic effect of inflammatory cytokines could be possibly exaggerated by viral transduction. But since XIAP expression effectively blocked the caspases activation, it reversed the cytotoxic or proapoptotic effect of cytokines.

XIAP, as an endogenous inhibitor of apoptosis, has been proven to bind caspase 3, caspase 7, and caspase 9 and inhibit their activities. XIAP contains 3 tandem baculovirus IAP repeat (BIR). The second BIR domain of XIAP (BIR2) inhibits caspases 3 and 7, while the third BIR domain (BIR3) inhibits caspase 9. 14-16 We demonstrated that the activities of caspase 3/7 and caspase 9 was inhibited in a dose dependent manner with increasing Adv-hXIAP transduction into INS-1E cells and human islets, suggesting XIAP as a potent therapeutic target to improve cell viability and function against inflammatory cytokine induced apoptosis in INS-1E cells and human islets. We also observed the inhibition effect on caspase 8 by Adv-hXIAP transduction, which seems to be contradictory to previous report that XIAP does not bind to caspase 8 and inhibit its activity.⁴¹ However, other IAP protein members, cIAP-1 and cIAP-2 have been reported to partially inhibit caspase 8 activity when assisted by TRAF.⁴² Further, the proapoptotic effect of IAP family inhibitor has been shown to get reversed by caspase 8 inhibitor on cancer cells,⁴³ suggesting the extensive interactions between caspase 8 and members of IAP protein family. Apoptosis analysis was also conducted using TUNEL assay and flow cytometry. Our data proved that cytokine induced apoptosis was greatly inhibited in INS-1E cells transduced with Adv-XIAP. However, for human islets which represents as clusters of cells varying in size and function, apoptosis analysis is very difficult to do in both TUNEL and flow cytometry. We will continue to find a proper solution in the future.

Our in vivo data showed the prolonged normoglycemia control of highly diabetic NOD-SCID mice and increasing insulin production after transplantation with Adv-hXIAP transduced human islets for six weeks, which was much longer than the previous reports,^{11, 17, 26} probably due to the protective effect of XIAP in the first two weeks after transplantation. However, when transplanted with human islets in which XIAP expression was almost silenced by co-culturing with its inhibitor embelin, the blood glucose level of the diabetic NOD-SCID mice was decreased.(Fig. 6) This result suggested XIAP level played a crucial role in determining islet viability after transplantation in highly diabetic patients. However, it should also be addressed that all mice were subjected to daily intraperitoneal administration of 10U/kg insulin from the second week post transplantation because it was necessary to keep the mice survival in all the other three groups except the one transplanted with Adv-hXIAP transduced human islets or these mice might not have survived for six weeks with such high blood glucose levels. To minimize the interference of insulin injection to blood glucose measurement to the lowest extent, all the measurement were made at least 5h after insulin injection.

Adv-hXIAP transduced INS-1E cells and human islets, when exposed to small molecular XIAP inhibitor embelin, had greatly decreased XIAP activity and therefore decreased cell viability and insulin production under inflammatory cytokines. Our data suggest that embelin inhibited XIAP activity by blocking protein synthesis, which is in good agreement with the previous report that embelin regulated apoptotic related gene expression,⁴⁴ not by structure based protein-protein interaction as described before.²⁷ Embelin could inhibit XIAP expression to an extremely low level which was even lower than the basal XIAP level in normal INS-1E cells and human islets, and hence reversed the protective effect of XIAP in INS-1E cells and human islets against cytokine, suggesting the important role of XIAP expression in graft viability and function post transplantation.

The therapeutic range of Adv-hXIAP transduction is very hard to determine. The MOI of Adv vector used for transduction must be carefully selected to achieve optimal transduction efficacy, with little toxicity. Our data showed that Adv-hXIAP could elevate XIAP expression in human islets in a dose dependent manner (Fig. 2) without significant toxicity, and this result was in good agreement with previous reports.11-12, 24, 26, 39

Taken together, these data confirm that XIAP overexpression in human islets enhances islet viability and function against inflammatory cytokines and in doing so helped to achieve prolonged normoglycemic control in diabetic mice, suggesting that clinical application of this protocol could immediately and greatly enhance the availability and long-term outcome of islet transplantation for type I diabetes.

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REFERENCES

1. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E. Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. *Diabetes*. 2002; 51(1):66–72. [PubMed: 11756324]
2. Ryan EA, Paty BW, Senior PA, Bigam D, Alfadhli E, Kneteman NM, Lakey JR, Shapiro AM. Five-year follow-up after clinical islet transplantation. *Diabetes*. 2005; 54(7):2060–9. [PubMed: 15983207]
3. Mandel TE, Koulmanda M. Effect of ischemia and temperature on fetal mouse pancreas. Insulin production in vitro, and function after isotransplantation. *Diabetes*. 1984; 33(4):376–82. [PubMed: 6423431]
4. Sarvetnick N, Shizuru J, Liggitt D, Martin L, McIntyre B, Gregory A, Parslow T, Stewart T. Loss of pancreatic islet tolerance induced by beta-cell expression of interferon-gamma. *Nature*. 1990; 346(6287):844–7. [PubMed: 2118234]
5. Kessler L, Jesser C, Lombard Y, Karsten V, Belcourt A, Pinget M, Poindron P. Cytotoxicity of peritoneal murine macrophages against encapsulated pancreatic rat islets: in vivo and in vitro studies. *J Leukoc Biol*. 1996; 60(6):729–36. [PubMed: 8975875]
6. Ritz-Laser B, Oberholzer J, Toso C, Brulhart MC, Zakrzewska K, Ris F, Bucher P, Morel P, Philippe J. Molecular detection of circulating beta-cells after islet transplantation. *Diabetes*. 2002; 51(3):557–61. [PubMed: 11872650]
7. Pavlakis M, Khwaja K. Transplantation for type 1 diabetes: whole organ pancreas and islet cells. *Curr Diab Rep*. 2006; 6(6):473–8. [PubMed: 17118232]
8. Pawelec K, Juszczak MT, Kumar A, Powis SH, Press M. Time course of islet loss after intraportal transplantation. *Ann N Y Acad Sci*. 2008; 1150:230–3. [PubMed: 19120301]
9. Narang AS, Mahato RI. Biological and biomaterial approaches for improved islet transplantation. *Pharmacol Rev*. 2006; 58(2):194–243. [PubMed: 16714486]
10. Giannoukakis N, Rudert WA, Ghivizzani SC, Gambotto A, Ricordi C, Trucco M, Robbins PD. Adenoviral gene transfer of the interleukin-1 receptor antagonist protein to human islets prevents IL-1beta-induced beta-cell impairment and activation of islet cell apoptosis in vitro. *Diabetes*. 1999; 48(9):1730–6. [PubMed: 10480601]
11. Narang AS, Sabek O, Gaber AO, Mahato RI. Co-expression of vascular endothelial growth factor and interleukin-1 receptor antagonist improves human islet survival and function. *Pharm Res*. 2006; 23(9):1970–82. [PubMed: 16906455]
12. Cheng G, Zhu L, Mahato RI. Caspase-3 gene silencing for inhibiting apoptosis in insulinoma cells and human islets. *Mol Pharm*. 2008; 5(6):1093–102. [PubMed: 18828606]
13. Li F, Mahato RI. iNOS gene silencing prevents inflammatory cytokine-induced beta-cell apoptosis. *Mol Pharm*. 2008; 5(3):407–17. [PubMed: 18217711]

14. Takahashi R, Deveraux Q, Tamm I, Welsh K, Assa-Munt N, Salvesen GS, Reed JC. A single BIR domain of XIAP sufficient for inhibiting caspases. *J Biol Chem.* 1998; 273(14):7787–90. [PubMed: 9525868]
15. Riedl SJ, Renatus M, Schwarzenbacher R, Zhou Q, Sun C, Fesik SW, Liddington RC, Salvesen GS. Structural basis for the inhibition of caspase-3 by XIAP. *Cell.* 2001; 104(5):791–800. [PubMed: 11257232]
16. Chai J, Shiozaki E, Srinivasula SM, Wu Q, Datta P, Alnemri ES, Shi Y. Structural basis of caspase-7 inhibition by XIAP. *Cell.* 2001; 104(5):769–80. [PubMed: 11257230]
17. Emamaullee J, Liston P, Korneluk RG, Shapiro AM, Elliott JF. XIAP overexpression in islet beta-cells enhances engraftment and minimizes hypoxia-reperfusion injury. *Am J Transplant.* 2005; 5(6):1297–305. [PubMed: 15888033]
18. Hui H, Khoury N, Zhao X, Balkir L, D'Amico E, Bullotta A, Nguyen ED, Gambotto A, Perfetti R. Adenovirus-mediated XIAP gene transfer reverses the negative effects of immunosuppressive drugs on insulin secretion and cell viability of isolated human islets. *Diabetes.* 2005; 54(2):424–33. [PubMed: 15677500]
19. Plesner A, Liston P, Tan R, Korneluk RG, Verchere CB. The X-linked inhibitor of apoptosis protein enhances survival of murine islet allografts. *Diabetes.* 2005; 54(9):2533–40. [PubMed: 16123340]
20. Narang AS, Cheng K, Henry J, Zhang C, Sabek O, Fraga D, Kotb M, Gaber AO, Mahato RI. Vascular endothelial growth factor gene delivery for revascularization in transplanted human islets. *Pharm Res.* 2004; 21(1):15–25. [PubMed: 14984253]
21. Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, Sikorska M. Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *Embo J.* 1993; 12(9):3679–84. [PubMed: 8253089]
22. Duckett CS, Li F, Wang Y, Tomaselli KJ, Thompson CB, Armstrong RC. Human IAP-like protein regulates programmed cell death downstream of Bcl-xL and cytochrome c. *Mol Cell Biol.* 1998; 18(1):608–15. [PubMed: 9418907]
23. Deveraux QL, Reed JC. IAP family proteins--suppressors of apoptosis. *Genes Dev.* 1999; 13(3):239–52. [PubMed: 9990849]
24. Panakanti R, Mahato RI. Bipartite vector encoding hVEGF and hIL-1Ra for ex vivo transduction into human islets. *Mol Pharm.* 2009; 6(1):274–84. [PubMed: 19067524]
25. Olerud J, Kanaykina N, Vasilovska S, King D, Sandberg M, Jansson L, Kozlova EN. Neural crest stem cells increase beta cell proliferation and improve islet function in co-transplanted murine pancreatic islets. *Diabetologia.* 2009
26. Panakanti R, Mahato RI. Bipartite adenoviral vector encoding hHGF and hIL-1Ra for improved human islet transplantation. *Pharm Res.* 2009; 26(3):587–96. [PubMed: 19002565]
27. Nikolovska-Coleska Z, Xu L, Hu Z, Tomita Y, Li P, Roller PP, Wang R, Fang X, Guo R, Zhang M, Lippman ME, Yang D, Wang S. Discovery of embelin as a cell-permeable, small-molecular weight inhibitor of XIAP through structure-based computational screening of a traditional herbal medicine three-dimensional structure database. *J Med Chem.* 2004; 47(10):2430–40. [PubMed: 15115387]
28. Danquah M, Li F, Duke CB 3rd, Miller DD, Mahato RI. Micellar delivery of bicalutamide and embelin for treating prostate cancer. *Pharm Res.* 2009; 26(9):2081–92. [PubMed: 19415464]
29. Corbett JA, Wang JL, Misko TP, Zhao W, Hickey WF, McDaniel ML. Nitric oxide mediates IL-1 beta-induced islet dysfunction and destruction: prevention by dexamethasone. *Autoimmunity.* 1993; 15(2):145–53. [PubMed: 7692996]
30. Burke JF Jr, Pirsch JD, Ramos EL, Salomon DR, Stablein DM, Van Buren DH, West JC. Long-term efficacy and safety of cyclosporine in renal-transplant recipients. *N Engl J Med.* 1994; 331(6):358–63. [PubMed: 8028616]
31. Dantal J, Hourmant M, Cantarovich D, Giral M, Blanche G, Dreno B, Souillou JP. Effect of long-term immunosuppression in kidney-graft recipients on cancer incidence: randomised comparison of two cyclosporin regimens. *Lancet.* 1998; 351(9103):623–8. [PubMed: 9500317]

32. Newstead CG. Assessment of risk of cancer after renal transplantation. *Lancet*. 1998; 351(9103): 610–1. [PubMed: 9500312]
33. Street CN, Lakey JR, Shapiro AM, Imes S, Rajotte RV, Ryan EA, Lyon JG, Kin T, Avila J, Tsujimura T, Korbitt GS. Islet graft assessment in the Edmonton Protocol: implications for predicting long-term clinical outcome. *Diabetes*. 2004; 53(12):3107–14. [PubMed: 15561940]
34. Nadeau I, Garnier A, Cote J, Massie B, Chavarie C, Kamen A. Improvement of recombinant protein production with the human adenovirus/293S expression system using fed-batch strategies. *Biotechnol Bioeng*. 1996; 51(6):613–23. [PubMed: 18629828]
35. Svensson EC, Marshall DJ, Woodard K, Lin H, Jiang F, Chu L, Leiden JM. Efficient and stable transduction of cardiomyocytes after intramyocardial injection or intracoronary perfusion with recombinant adeno-associated virus vectors. *Circulation*. 1999; 99(2):201–5. [PubMed: 9892583]
36. Ozawa CR, Banfi A, Glazer NL, Thurston G, Springer ML, Kraft PE, McDonald DM, Blau HM. Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis. *J Clin Invest*. 2004; 113(4):516–27. [PubMed: 14966561]
37. von Degenfeld G, Banfi A, Springer ML, Wagner RA, Jacobi J, Ozawa CR, Merchant MJ, Cooke JP, Blau HM. Microenvironmental VEGF distribution is critical for stable and functional vessel growth in ischemia. *Faseb J*. 2006; 20(14):2657–9. [PubMed: 17095533]
38. Vischioni B, van der Valk P, Span SW, Kruyt FA, Rodriguez JA, Giaccone G. Expression and localization of inhibitor of apoptosis proteins in normal human tissues. *Hum Pathol*. 2006; 37(1): 78–86. [PubMed: 16360419]
39. Emamaullee JA, Rajotte RV, Liston P, Korneluk RG, Lakey JR, Shapiro AM, Elliott JF. XIAP overexpression in human islets prevents early posttransplant apoptosis and reduces the islet mass needed to treat diabetes. *Diabetes*. 2005; 54(9):2541–8. [PubMed: 16123341]
40. Emamaullee JA, Shapiro AM, Rajotte RV, Korbitt G, Elliott JF. Neonatal porcine islets exhibit natural resistance to hypoxia-induced apoptosis. *Transplantation*. 2006; 82(7):945–52. [PubMed: 17038911]
41. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS, Reed JC. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *Embo J*. 1998; 17(8):2215–23. [PubMed: 9545235]
42. Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS Jr. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science*. 1998; 281(5383):1680–3. [PubMed: 9733516]
43. Vince JE, Wong WW, Khan N, Feltham R, Chau D, Ahmed AU, Benetatos CA, Chunduru SK, Condon SM, McKinlay M, Brink R, Leverkus M, Tergaonkar V, Schneider P, Callus BA, Koentgen F, Vaux DL, Silke J. IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell*. 2007; 131(4):682–93. [PubMed: 18022363]
44. Ahn KS, Sethi G, Aggarwal BB. Embelin, an inhibitor of X chromosome-linked inhibitor-of-apoptosis protein, blocks nuclear factor-kappaB (NF-kappaB) signaling pathway leading to suppression of NF-kappaB-regulated antiapoptotic and metastatic gene products. *Mol Pharmacol*. 2007; 71(1):209–19. [PubMed: 17028156]

Fig. 1.
Scheme of apoptotic pathways and the role of XIAP.

Fig. 2.

XIAP gene expression in 106 INS-1E cells per well and 1000 islet equivalent (IE) per well 2 days after Adv-hXIAP transduction. Realtime PCR (A), ELISA (B) and western blot (D) illustrated that XIAP expression in INS-1E cells was upregulated by transduction with Adv-hXIAP. ELISA (C) and western blot (E) illustrated that XIAP expression in human islets was upregulated by transduction with Adv-hXIAP. * $p < 0.05$ under one way ANOVA compared with untransduced group.

Data are presented as the mean + SD of $n = 3$.

Fig. 3.

Beneficial effect of XIAP overexpression on INS-1E cell viability and function. (A) Higher viability of Adv-hXIAP transduced 106 INS-1E cells per well was observed in the presence of inflammatory cytokine cocktail, compared with untransduced cells and Adv-LacZ transduced cells. (B) More insulin production by INS-1E cells transduced with Adv-hXIAP was observed under cytokine treatment. Data are presented as the mean + SD of $n = 3$.

* $p < 0.05$ under t test compared with Adv-XIAP group.

Fig. 4.

Anti-apoptotic effect of XIAP overexpression on INS-1E cells. Left panel, dark stain of apoptotic INS-1E cells under cytokine treatment, as determined by DeadEnd Fluorometric TUNEL System. Right panel, apoptotic cell number counted by flow cytometry following fluorescent labeling by APO-DIRECT. Untransduced INS-1E cells (a, d), Adv-LacZ transduced INS-1E cells (b, e) and Adv-hXIAP transduced INS-1E cells (c, f). The values show the percentage of positive cells. Data are presented as the mean + SD of n = 3.

*p<0.05 under one way ANOVA compared with untransduced group.

Fig. 5.

Effect of XIAP gene expression on caspase activities of 106 INS 1E cells/well and human islets after transduction with Adv hXIAP and cytokine (5ng/ml IL 1 β , 25ng/ml TNF α , and 25ng/ml IFN γ) treatment for additional 3 h. XIAP expression significantly inhibited activities of caspase 3/7, 8, and 9 in a dose dependent manner. Caspase 3/7 activity by in INS 1E cells (A) and human islets (B). caspase 8 and caspase 9 activity in human islets (C). Data are presented as the mean + SD of n = 3. *p<0.05 under one way ANOVA compared with untransduced group.

Fig. 6.

Effect of Adv-hXIAP transduction on the outcome of islet transplantation. (A) Blood glucose level of streptozotocin-induced diabetic NOD-SCID mice transplanted with 1000 Adv-hXIAP transduced islet equivalent (IE) under the kidney capsule. The mice transplanted with Adv-LacZ transduced and nontransduced islet equivalent (IE) were used as controls. Embelin was also used as negative control since it can reverse the effect of XIAP overexpression. The nonfasting glucose levels were measured in each animal up to 42 days post-transplantation. At day 43 after transplantation, the islet graft-bearing kidney was removed and mice were sacrificed. Data are presented as the mean + SD of $n = 5$.

Fig. 7.

Immunohistochemical staining of kidney section at 42 days after transplantation. Kidney bearing no islet graft (A), Adv-hXIAP transduced islet graft (B), and Adv-LacZ transduced islet graft (C). Insulin was stained in brown color. Nuclear was stained in purple by haematoxylin. Increasing C-peptide (D) level in Adv-hXIAP group was observed in the serum collected at 42 days after transplantation. Data are presented as the mean + SD of n = 5. * $p < 0.05$ under one way ANOVA compared with untransduced group.

Fig. 8.

Beneficial effect of XIAP overexpression on INS-1E cells can be reversed by XIAP inhibitor embelin. Cytotoxicity study of embelin on 106 INS-1E cells/well for 24 h (A). Embelin can inhibit XIAP expression as illustrated by ELISA (B) and western blot (C). Embelin reversed the cytoprotective effect of XIAP overexpression on 106 INS-1E cells/well against cytokine treatment (D). Embelin reversed the caspase inhibition effect of XIAP overexpression on INS-1E cells (E). Data are presented as the mean + SD of $n = 3$. * $p < 0.05$ under one way ANOVA compared with zero embelin concentration (A) or untransduced group (B, D, E).

Table 1

Animal weight change after transplantation of Adv-hXIAP transduced human islets under kidney capsule of diabetic NOD-SCID mice.

	Day 0	Day 7	Day 28	Day 42
Control	18.06±1.01	17.90±2.43	18.90±1.99	19.21±2.05
Adv-LacZ	17.93±1.33	17.20±2.35	18.87±2.28	18.93±2.40
Adv-hXIAP	18.39±0.69	17.83±1.85	20.49±2.36 *	22.33±2.43 *
Adv-hXIAP+Embelin	18.18±0.93	18.07±2.70	18.76±2.07	19.13±3.07

* p<0.05 under one way ANOVA test. Data are presented as the mean + SD of n = 5.