

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 coadministration 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 24 h 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 suc-

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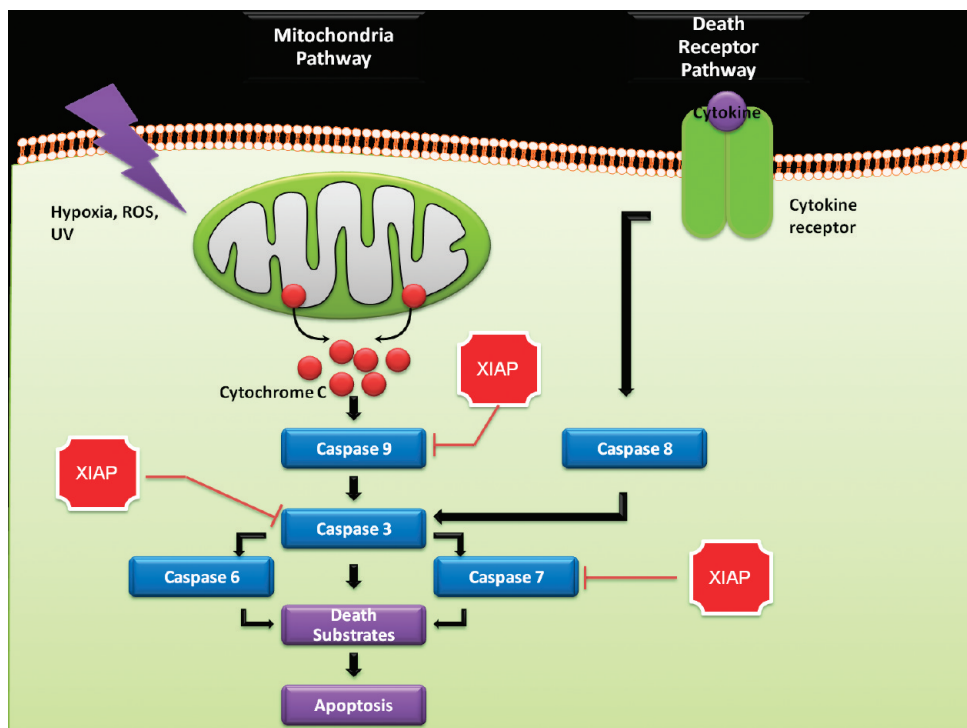


Figure 1. Scheme of apoptotic pathways and the role of XIAP.

cessful revascularization thereafter.^{7,8} Therefore, expression of an antiapoptotic gene to prevent β -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 coexpression 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 end point 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 Figure 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 antiapoptotic 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

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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. Emamaullee 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 48 h post transduction, almost 90% of the cells were detached from the flask. Then, the cells were collected, centrifuged and freeze and thawed 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 48 h post-transduction, AD-293 cells were fixed and incubated with Anti-Hexon antibody, followed by a secondary horse radish peroxidase (HRP)-conjugated antibody. Then the cells were stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate and positively stained cells were counted under a microscope, and the titer was determined.

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 24 h, INS-1E cells were transduced with Adv-hXIAP of indicated MOI for 3 h 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 medium 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 was incubated with diluted Adv-hXIAP in 300 μ L of medium in a 96-well plate for 12 h, followed by washing and further culturing in 1 mL of medium 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 μ L of 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

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run in triplicate. 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 \times 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 \times 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 an 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, that, when cleaved by caspases, will release luciferin to quantitatively determine caspase concentration. Briefly, 48 h following transduction and cytokine cocktail treatment for additional 3 h, 100 μL of Caspase-Glo reagent was added to 100 μL of culture supernatants in 96-well plates and incubated at room temperature for 1 h. 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 $\text{MOI} = 2$, INS-1E cells were further incubated with cytokine cocktail, including 5 ng/mL IL-1 β , 25 ng/mL TNF α , and 25 ng/mL IFN γ , for the indicated time. At the end of the cytokine treatment, INS-1E cells were further cultured with medium containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) for 2 h in 37°C . Then the medium was removed and 200 μL of

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 3 h, INS-1E cells were incubated with cytokine cocktail for 48 h. 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 3 h and incubation with the cytokine cocktail for an additional 48 h, 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 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 medium was carefully removed with a pipet, INS-1E cells and human islets were sequentially incubated in the medium containing 2.5 mM (basal) and 22 mM glucose (stimulated) at 37°C for 1 h. Supernatants were collected and analyzed for insulin release by Insulin Ultra-sensitive EIA kit (Alpco Diagnostics, Salem, NH). Insulin secretion was expressed as $\mu\text{U/mL}$, and the ratio of insulin levels at 22 mM to 2.5 mM 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 nontreated 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

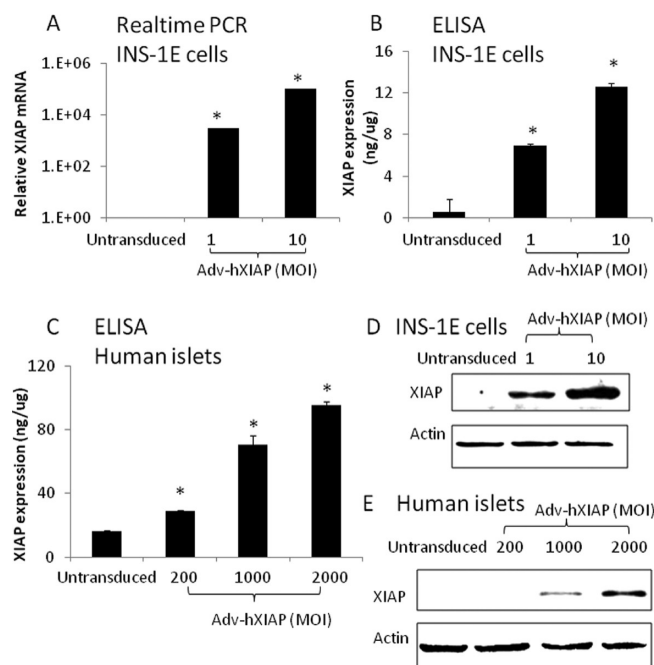


Figure 2. XIAP gene expression in 1×10^6 INS-1E cells per well and 1000 islet equivalent (IE) per well 2 days after Adv-hXIAP transduction. Realtime PCR (A), ELISA (B) and Western blot (D) illustrated that XIAP expression in INS-1E cells was upregulated by transduction with Adv-hXIAP. ELISA (C) and Western 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 \pm SD of $n = 3$.

glucose level ≥ 600 mg/dL after streptozotocin treatment and long time after transplantation, all mice were subjected to daily intraperitoneal administration of 10 U/kg insulin to maintain mouse survival. To minimize the interference to blood glucose measurement by insulin injection, all the measurements were done at least 5 h 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 before.²⁰ Sections of 5–7 μ m 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 counterstained hematoxylin (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.

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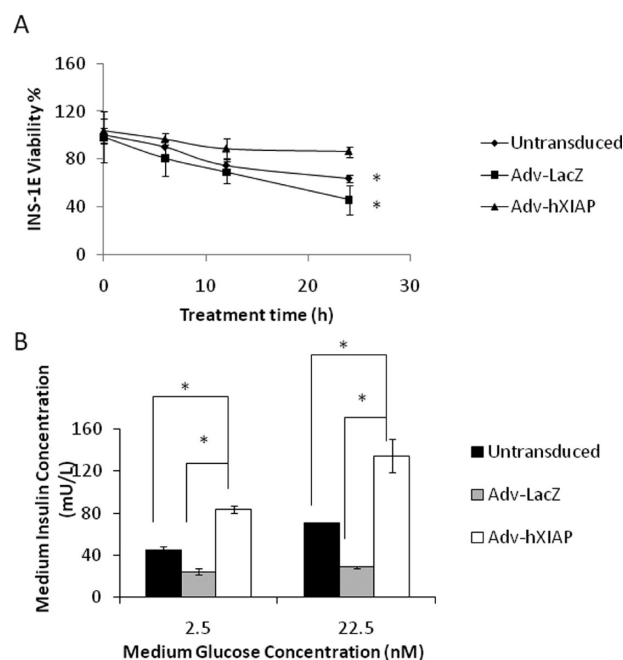


Figure 3. Beneficial effect of XIAP overexpression on INS-1E cell viability and function. (A) Higher viability of Adv-hXIAP transduced 1×10^6 INS-1E cells per well was observed in the presence of inflammatory cytokine cocktail, compared with untransduced cells and Adv-LacZ transduced cells. (B) More insulin production by INS-1E cells transduced with Adv-hXIAP was observed under cytokine treatment. Data are presented as the mean \pm SD of $n = 3$. * $p < 0.05$ under t test compared with Adv-XIAP group.

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-fold and 100000-fold after transduction of INS-1E cells with Adv-hXIAP at MOI of 1 and 10, respectively (Figure 2A). Similarly, XIAP protein concentration increased by 13-fold (from 0.54 ng/ μ g to 6.94 ng/ μ g) and 28-fold (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 (Figure 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 (Figure 2C). These results were in good agreement with our Western blot analysis (Figure 2D and 2E), where XIAP band density increased with increasing MOI, suggesting increase in XIAP protein expression after transduction.

XIAP Improves INS-1E Cell Viability and Function against Inflammatory Cytokines. Following transduction, INS-1E cells were further incubated with inflammatory cytokine cocktail for 48 h. INS-1E cell viability decreased after cytokine treatment, as determined by the MTT assay (Figure 3A). However, transduction of INS-1E cells with

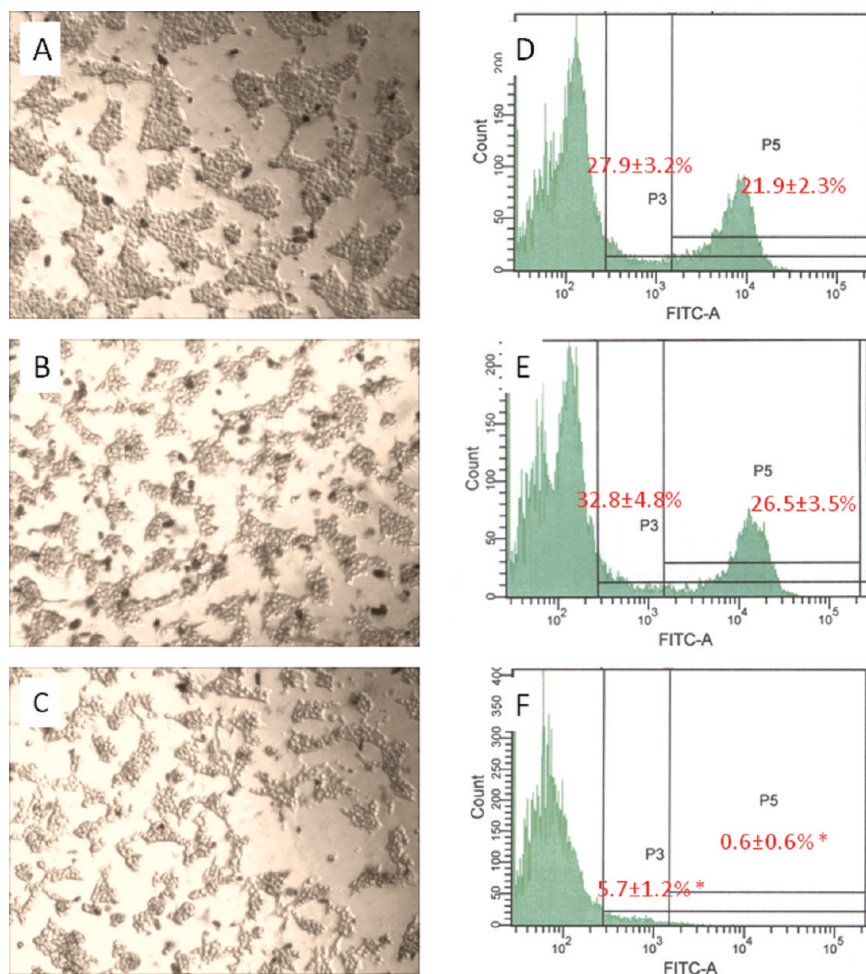


Figure 4. Antiapoptotic effect of XIAP overexpression on INS-1E cells. Left panel, dark stain of apoptotic INS-1E cells under cytokine treatment, as determined by DeadEnd Fluorometric TUNEL System. Right panel, apoptotic cell number counted by flow cytometry following fluorescent labeling by APO-DIRECT. 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 \pm SD of $n = 3$. * $p < 0.05$ under one-way ANOVA compared with untransduced group.

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%) (Figure 3A), suggesting the protective effect of XIAP. INS-1E cell function was determined by insulin stimulation test. Following transduction and cytokine treatment for 12 h, 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 (Figure 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 48 h 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 (Figure 4C), compared with untransduced INS-1E cells (Figure 4A), and Adv-LacZ transduced INS-1E cells (Figure 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 Figure 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

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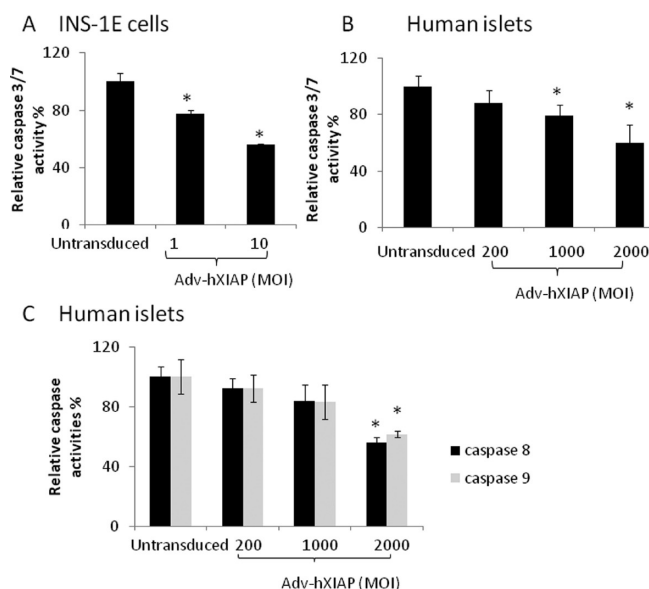


Figure 5. Effect of XIAP gene expression on caspase activities of 1×10^6 INS-1E cells/well and human islets after transduction with Adv-hXIAP and cytokine (5 ng/mL IL-1 β , 25 ng/mL TNF α , and 25 ng/mL IFN γ) treatment for an additional 3 h. XIAP expression significantly inhibited activities of caspase 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 \pm SD of $n = 3$. * $p < 0.05$ under one-way ANOVA compared with untransduced group.

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 (5 ng/mL IL-1 β , 25 ng/mL TNF α , and 25 ng/mL IFN γ for 3 h) (Figure 5). Caspase 3/7 activity decreased up to 40% post transduction of both INS-1E cells and human islets with Adv-hXIAP (Figure 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 (Figure 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 cotransduced human islets. Following transplantation, blood glucose level decreased to around 200 mg/dL for all

transplanted mice (Figure 6), suggesting the immediate reversal of diabetic hyperglycemia by islet transplantation. However, the blood glucose level of the mice transplanted with untransduced islets, Adv-LacZ transduced islets, and Adv-hXIAP plus embelin cotransduced islets gradually increased after the first week post-transplantation, and was maintained to the diabetic level (~ 600 mg/dL) since 2 weeks post-transplantation (Figure 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 until 6 weeks post-transplantation (Figure 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 10 U/kg insulin from the second week post-transplantation. To minimize the interference of insulin injections to blood glucose measurement, all the measurement were made 5 h 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 was also isolated and used as the negative control (Figure 7A). The positive staining of insulin was more evident in kidney section bearing Adv-hXIAP transduced islets (Figure 7B), while only rare insulin staining was observed in the kidney section bearing Adv-LacZ transduced islets (Figure 7C), suggesting the maintenance of insulin secretion. The serum of mice was also collected to measure serum c-peptide level by ELISA. Results showed that c-peptide level in the mice transplanted with Adv-hXIAP transduced islets was significantly higher than in the other group, indicating improved islet function in Adv-hXIAP transduced human islets post-transplantation (Figure 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

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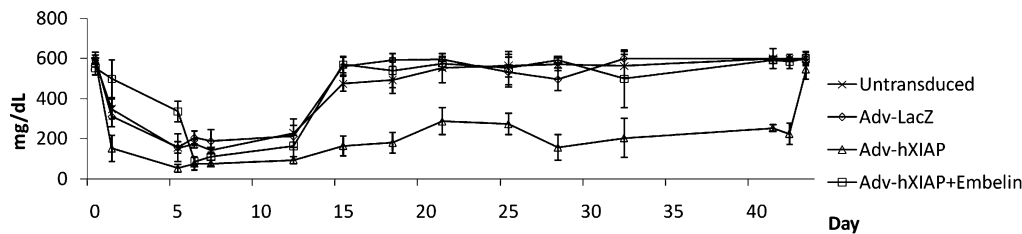


Figure 6. Effect of Adv-hXIAP transduction on the outcome of islet transplantation. (A) Blood glucose level of streptozotocin-induced diabetic NOD-SCID mice transplanted with 1000 Adv-hXIAP transduced islet equivalent (IE) under the kidney capsule. The mice transplanted with Adv-LacZ transduced and nontransduced 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 \pm SD of $n = 5$.

Table 1. Animal Weight Change after Transplantation of Adv-hXIAP Transduced Human Islets under Kidney Capsule of Diabetic NOD-SCID Mice^a

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

^a $p < 0.05$ under one-way ANOVA test. Data are presented as the mean \pm SD of $n = 5$.

treatment when concentration is below 200 nM (Figure 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 (Figure 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 50 nM embelin for 24 h, compared with 88% viability with just cytokine cocktail (Figure 8D), though 50 nM 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 (Figure 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 (Figure 6), suggesting the loss of islet viability and function by XIAP inhibition.

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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.

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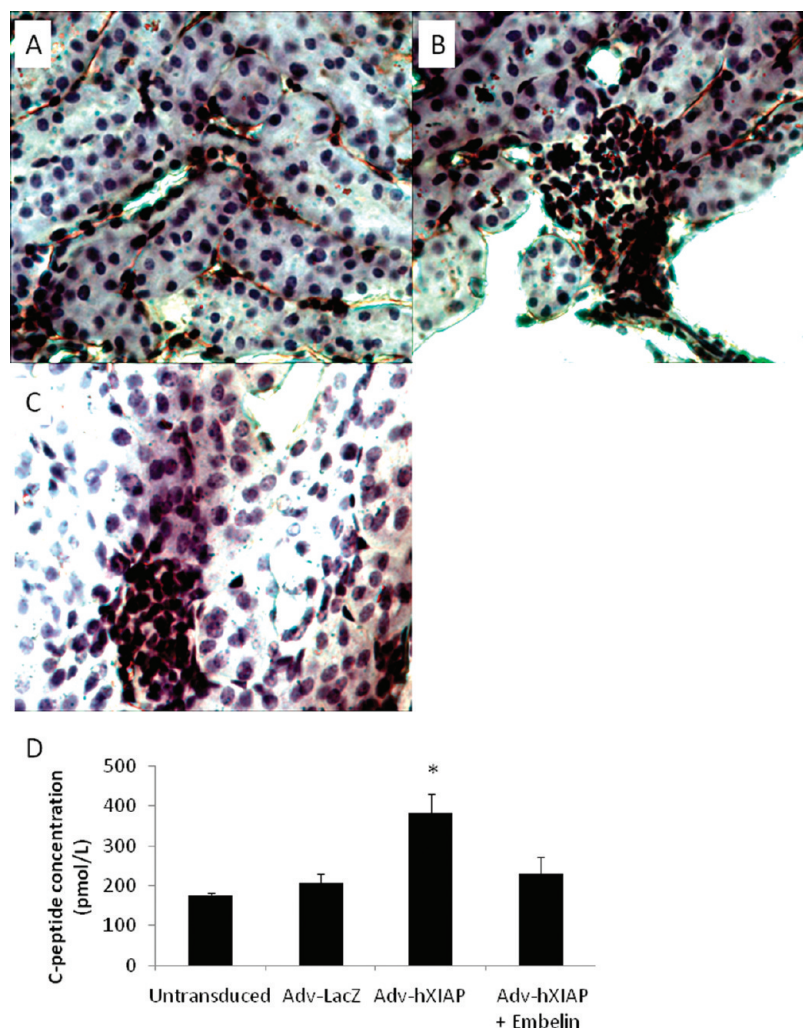


Figure 7. Immunohistochemical staining of kidney section at 42 days after transplantation. Kidney bearing no islet graft (A), Adv-hXIAP transduced islet graft (B), and Adv-LacZ transduced islet graft (C). Insulin was stained in brown color. Nucleus was stained in purple by hematoxylin. Increasing C-peptide (D) level in Adv-hXIAP group was observed in the serum collected at 42 days after transplantation. Data are presented as the mean \pm SD of $n = 5$. * $p < 0.05$ under one-way ANOVA compared with untransduced group.

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

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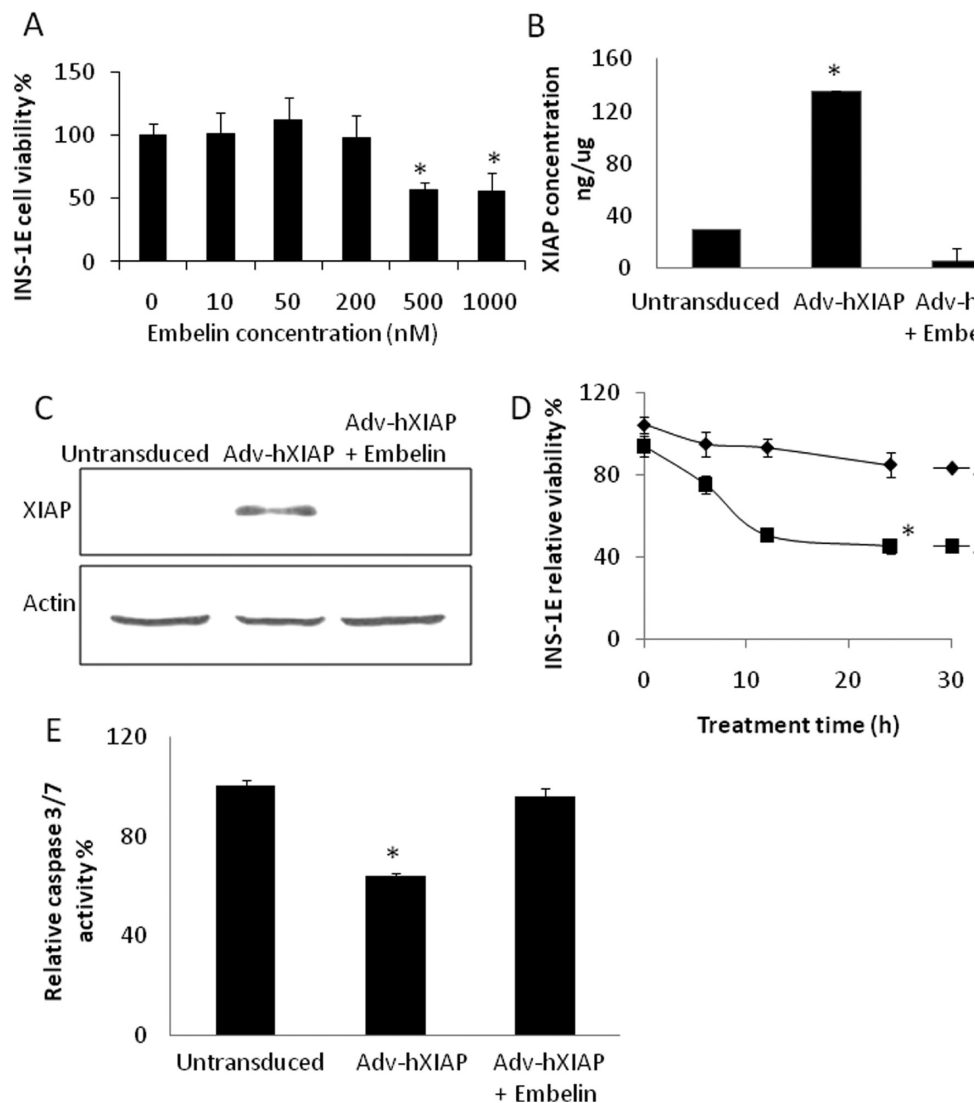


Figure 8. Beneficial effect of XIAP overexpression on INS-1E cells can be reversed by XIAP inhibitor embelin. Cytotoxicity study of embelin on 1×10^6 INS-1E cells/well for 24 h (A). Embelin can inhibit XIAP expression as illustrated by ELISA (B) and Western blot (C). Embelin reversed the cytoprotective effect of XIAP overexpression on 1×10^6 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 \pm SD of $n = 3$. * $p < 0.05$ under one-way ANOVA compared with zero embelin concentration (A) or untransduced group (B, D, E).

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 environ-

ment, will express a 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 (Figure 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

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in both INS-1E cells and human islets (Figure 5). The protective effect of Adv-hXIAP transduction on human islets 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 caspase 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 cytokine 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) domains. 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 were 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 a 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 are represented 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 coculturing with its inhibitor embelin, the blood glucose level of the diabetic NOD-SCID mice was decreased (Figure 6). This result suggested that the 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 10 U/kg insulin from the second week post-transplantation because it was necessary to maintain the survival of the mice 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 5 h 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

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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 (Figure 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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