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RGD peptide-modified adenovirus expressing hepatocyte growth factor and X-linked inhibitor of apoptosis improves islet transplantation

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Abstract

Background—Islet transplantation has the potential for treating type I diabetes; however, its widespread clinical application is limited by the massive apoptotic cell death and poor revascularization of transplanted islet grafts.

Methods—We constructed a surface-modified adenoviral vector with RGD (Arg-Gly-Asp) sequences encoding human X-linked inhibitor of apoptosis and hepatocyte growth factor (RGD-Adv-hHGF-hXIAP). *In vitro* transgene expression in human islets was determined by enzyme-linked immunosorbent assay. RGD-Adv-hHGF-hXIAP-transduced human islets were transplanted under the kidney capsule of streptozotocin-induced diabetic NOD/SCID mice. The blood glucose levels of mice were measured weekly. The kidneys bearing islets were isolated at the end of the experiment and subjected to immunofluorescence staining.

Results—The transduction efficiency on human islets was significantly improved using RGD-modified adenovirus. HGF and XIAP gene expressions were dose-dependent after viral transduction. When exposed to a cocktail of inflammatory cytokines, RGD-Adv-hHGF-hXIAP-transduced human islets showed decreased caspase 3 activity and reduced apoptotic cell death. Prolonged normoglycemic control could be achieved by transplanting RGD-Adv-hHGF-hXIAP-transduced human islets. Immunofluorescence staining of kidney sections bearing RGD-Adv-hHGF-hXIAP-transduced islets was positive for insulin and von Willebrand factor (vWF) at 200 days after transplantation.

Conclusions—These results indicated that *ex vivo* transduction of islets with RGD-Adv-hHGF-hXIAP decreased apoptotic islet cell death and improved islet revascularization, and eventually might improve the outcome of human islet transplantation.

Keywords

adenovirus; apoptosis; islets; RGD; revascularization

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Introduction

Type 1 diabetes (T1D) is the result of autoimmune destruction of insulin-producing pancreatic β -cells and results in lifelong dependence on insulin injections. Islet transplantation has the potential to treat T1D. However, the widespread clinical application of transplantation is limited because of the lack of sufficient number of human islets from donors and the loss of islet viability after transplantation. Up to 70% of insulin-producing β -cells of transplanted islets is lost at the first 24 h post-transplantation [1]. Therefore, restoring β -cell function against inflammation after transplantation and protecting them from the immune reaction of the recipient are major challenges [2].

Islets are challenged by inflammatory cytokines, hypoxic environment, and reactive oxygen species (ROS) at the transplantation site [3–5]. Islet loss occurs mostly in the first 2 weeks after transplantation and decreases significantly thereafter because of successful revascularization [6]. Therefore, expression of an anti-apoptotic gene to prevent β -cell loss and expression of a growth factor gene to promote islet revascularization in the early stage of islet transplantation may be an effective strategy for improving islet survival and function after transplantation [2]. We previously demonstrated that adenovirus (Adv) mediated transgene expression of caspase 3 small hairpin RNA and that interleukin (IL)-1Ra in human islets prevented apoptotic islet death after transplantation [7,8]. In a recent study, we identified X-linked inhibitor of apoptosis (XIAP) as a better anti-apoptotic gene to improve islet transplantation because XIAP inhibited a wider range of executor caspases [9]. Besides apoptotic cell death, failure of revascularization is another reason for compromised islet transplantation in early stage. It has been reported that Adv-mediated hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) gene delivery to islets to enhance revascularization and prolong graft function [8,10,11]. We chose HGF in the present study because of its well-characterized angiogenic effect, as well as its potential for preventing apoptotic islet death and promoting the proliferation of pancreatic β -cells [12].

Because of the low level of the coxsackievirus and adenovirus receptors on the cell surface, human islets are usually hard to transduce with traditional Adv [13,14]. To solve this problem, we genetically modified the viral genome by incorporating the RGD (Arg-Gly-Asp) peptide sequence into the Adv fiber knob. We expected that the RGD modification could improve the transduction efficiency of Adv on human islets by providing an alternative viral entry pathway into human islets [15,16].

In the present study, we constructed an RGD-modified adenovirus, RGD-Adv-hHGF-hXIAP and determined its potential to improve the outcome of human islet transplantation by preventing apoptotic islet death and promoting functional revascularization.

Materials and methods

Culturing islets

Human islet preparations were received from the Integrated Islet Distribution Program funded by the National Institute of Diabetes and Digestive and Kidney Diseases and with support from the Juvenile Diabetes Research Foundation International. Upon arrival, islet

preparations were subjected to quality control assessment for viability by using calcein AM/propidium iodide staining and for purity by using dithizone staining. Dithizone binds zinc ions present in the β -cells of islets and therefore stains the islets red. Other exocrine tissue also present in the preparations does not bind dithizone, and is therefore not stained [17]. Islet preparations with purity and viability >90% were cultured in CMRL-1066 medium (Sigma Aldrich, St Louis, MO, USA) containing 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA) in an incubator at 37 °C.

Construction of RGD-modified Adv

RGD-Adv-GFP was a kind gift from Dr Hiroyuki Mizuguchi, Osaka University, Japan. Adv-GFP was constructed and passaged in our laboratory. To generate RGD-modified Adv, the genome encoding the fiber in adenovirus sero-type 5 (Ad5) (from 28592–30470) was cloned into the *SacII-KpnI* site of pBluescript II SK(+) (Stratagene, La Jolla, CA, USA), creating pSK5543, and the genome encoding the fiber in Ad5 (from 32123–32836) was cloned into the *EcoRI-HindIII* site of pSP72 (Promega, Madison, WI, USA), creating pSP72(713). For ease of incorporating the RGD epitope between the HI-loop (LNVTTQETGDTTPSAYSMSFSWD) of the fiber knob, immediately downstream of the following, the 11th threonine, new *BamHI* and *MroI* sites were added by polymerase chain reaction (PCR)-mediated site-directed mutagenesis. For PCR-mediated site-directed mutagenesis, the primer set used was: 5'-GAA ACA GGA GAC ACA *GGA TCCGCG TCC GGA* ACT CCA AGT GCA TAC-3' as the sense primer and 5'-GTA TGC ACT TGG AGT *TCC GGA CGC GGA TCCT* TGT GTC TCC TGT TTC-3' as the antisense primer. After PCR amplification, the resulting mutated PCR product, containing *BamHI* and *MroI* sites (underlined) to make pSP72 [713-BM], was verified using an ABI PRISM 337 automatic DNA sequencer (Applied Biosystems, Foster City, CA, USA). To construct a vector encoding the RGD epitope between the HI-loop of the fiber knob, two complementary oligonucleotides were synthesized and annealed to form a DNA duplex. This DNA duplex was designed to contain a *BamHI* overhang 5' end and an *MroI* overhang 3' end, so that the fragment could be inserted into the *BamHI* and *MroI* sites of the pSP72[713-BM] vector. The RGD epitope consisted of a nine-amino acid sequence, **CDCRGDCFC**. Sequences of the oligonucleotides were 5'-GA TCC *TGT GAC TGC CGC GGA GAC TGT TTC TGC T*-3' and 5'-CC GGA *GCA GAA TCA GTC TCC GCG GCA GTA ACA G*-3'. These oligonucleotides encoded the RGD epitope (boldface and italicized). The resulting plasmid was then digested with *NcoI* and *MfeI* and cloned into pSK5543 to generate an Adv fiber shuttle vector, pSK[5543-RGD]. To generate the RGD-modified Adv, the newly-constructed Adv fiber shuttle vector pSK [5543 + RGD] was digested with *SacII* and *XmnI*, and the viral vector dl324 was digested with *SpeI* for homologous DNA recombination in *Escherichia coli* BJ5183.

Generating RGD-Adv-hHGF-hXIAP

To generate an Adv encoding HGF and XIAP at the E1 and E3 regions, respectively, we first constructed an E3 shuttle vector expressing XIAP. The XIAP gene was cloned from pSport6-XIAP by using 5'-CG GGATCC ATG ACT TTT AAC AGT TTT GAA GGA TCT AAA-3' and 5'-CG GGATCC TTA AGA CAT AAA AAT TTT TTG CTT GAA AGT-3' primers and subcloned into the Adv E3 shuttle vector, pSP72-E3 [18], generating a pSP72-

XIAP. The newly-constructed pSP72-XIAP shuttle vector was linearized with *XmnI* digestion and then co-transformed with a replication-incompetent Adv, dl324-RGD, into *E. coli* BJ5183. It was then digested with *SpeI* for homologous recombination, yielding Adv-encoding plasmid, dl324-XIAP (E3)-RGD. Structures of the resulting recombinant vectors were then confirmed by restriction enzyme digestion and PCR analysis.

In addition, to construct an Adv E1 shuttle vector expressing HGF, HGF gene was excised from pCDNA3.1-HGF by using *Bam*HI and *Xba*I and subcloned into the replication-incompetent E1 shuttle vector, pCA14 (Microbix, Ontario, Canada). Then, the Adv E1 shuttle vector was linearized with *XmnI* digestion and co-transformed into *E. coli* BJ5183 with the *Bst*BI-digested dl324-XIAP-RGD for homologous recombination, generating dl324-HGF-XIAP-RGD. Recombinant Adv encoding plasmid was digested with *PacI* and transfected into 293 cells to generate the replication-incompetent Adv, RGD-Adv-hHGF-hXIAP (Figure 1). The propagation, purification, titration and quality analysis of all Adv used were performed as described previously [19].

Transduction efficiency

Human islets were first transduced with Adv-GFP and RGD-Adv-GFP at equal multiplicity of infection (MOI) to determine the effect of RGD modification on the transduction efficiency of Adv. To determine the optimal MOI for RGD-Adv-hHGF-hXIAP, 500 hand-picked human islets were transduced with serial dilutions of RGD-Adv-hHGF-hXIAP in 24-well plates for 12 h and incubated for an additional 48 h. Medium were collected. Human islets were washed with phosphate-buffered saline (PBS) and dispersed into a single-cell suspension by incubation with 0.25% trypsin/ethylene-diaminetetraacetic acid (EDTA) (Gibco, Gaithersburg, MD, USA) at 37 °C for 5 min followed by passage through a narrow-gauge pipette. Total protein was extracted with RIPA buffer (Sigma Aldrich) and stored at -80 °C. HGF concentration in the cultured medium and XIAP concentration in the total protein extract of human islets was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA).

Caspase detection

Caspase-Glo 3 assay kits were used to analyse caspase 3 activity as described previously [7]. Briefly, 48 h after transduction, a single-cell islet suspension was generated by 0.25% trypsin/EDTA digestion. Then, 100 µl of Caspase-Glo was added to 100 µl of the single-cell suspension of 10⁴ cells in 96-well plates and incubated at room temperature for 1 h. The contents were then transferred to culture tubes, and luminescence was determined using a luminometer (Berthold, Bad Wildbad, Germany).

Western blot

Five hundred human islets were transduced with RGD-Adv-hHGF-hXIAP at 500 MOI in 24-well plates for 12 h and incubated for an additional 48 h. A single-cell islet suspension was generated by 0.25% trypsin/EDTA digestion. The total protein samples were extracted with RIPA buffer supplemented with a protease inhibitor cocktail (Sigma Aldrich), mixed with 6 × Laemmli sodium dodecyl sulfate buffer (Boston BioProducts, Ashland, MA, USA) and then boiled for 5 min to denature the protein. Next, a 20-µg sample was loaded onto a 4–

15% Tris-HCl precast polyacrylamide gel (Bio-Rad, Hercules, CA, USA) for electrophoresis and subsequently transferred to an immobilon polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). 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 incubated with goat polyclonal immunoglobulin G to XIAP, BAX, BCL-2, p-AKT, AKT and GADPH (Abcam, Cambridge, MA, USA) primary antibodies (dilution 1:500) overnight at 4 °C. The membrane was then incubated with horseradish peroxidase-conjugated rabbit polyclonal antibody to goat (dilution 1:10000) (Abcam) for 1 h at room temperature. Target proteins were detected by enhanced chemiluminescence detection kit (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Apoptosis study

A cytokine cocktail of recombinant tumor necrosis factor (TNF) α (5 ng/ml) and IL-1 β (5 ng/ml) was used to mimic the *in vivo* challenge to the INS-1E cells and human islets by the inflammatory cytokines. INS-1E cells were transduced with Adv for 3 h, stimulated with cytokine cocktail for an additional 48 h and characterized by the DeadEnd™ Colorimetric TUNEL system (Promega, Madison, WI, USA), in which fragmented DNA from apoptotic cells was labeled with biotinylated nucleotide and detected using hydrogen peroxide and diaminobenzidine. Human islets were transduced with Adv for 12 h and incubated with cytokine cocktail for an additional 3 days. Then, islet cells were digested with 0.25% trypsin/EDTA into a single-cell suspension, stained with the Annexin V-FITC Apoptosis Detection Kit (Abcam) and analyzed with flow cytometry. Annexin V binds to phosphatidylserine on the cell surface, which is a feature found in apoptotic cells. Fluorescent intensity was analyzed using CellQuest software (BD Bioscience, Franklin Lakes, NJ, USA). Nontransduced cells without cytokine treatment served as a negative control. Three sets of independent transduction experiments were carried out for each assay.

Insulin release

The level of insulin secreted from human islets was determined by a dynamic islet perfusion assay. Briefly, after being stimulated with a cytokine cocktail of IL-1 β (5 ng/ml) and TNF α (5 ng/ml) for 3 days, 50 islets from each group were handpicked and loaded onto a Swinnex 13 chamber (Millipore, Burlington, MA, USA) and perfused with Krebs-Ringer bicarbonate buffer containing basal glucose (2.5 mM). The flow rate was maintained at 2 ml/min with a peristaltic pump (Thermo Fisher, Waltham, MA, USA) and the temperature was maintained at 37 °C with a solution heater (Warner Instruments, Hamden, CT, USA). Islets were first perfused with basal glucose (2.5 mM) for 45 min and stimulated with glucose (22.5 mM) for 15 min and finally perfused with basal glucose (2.5 mM) until insulin release reversed to the basal level. Samples were collected through a fraction collector (Waters, Milford, MA, USA) and analyzed for insulin content by ELISA (Calbiotech, Spring Valley, CA, USA).

Islet transplantation

Animal experiments were performed in accordance with NIH and Institutional Animal Care and Use Committee guidelines using an approved protocol. To induce diabetes, streptozotocin (STZ) (70 mg/kg) was administered to NOD/SCID mice by intraperitoneal injection for 2 consecutive days. Animals were classified as diabetic after two consecutive

measurements of blood glucose 400 mg/dl with a glucometer. Upon receiving human islets from the Integrated Islet Distribution Program distribution centers, they were immediately transduced with RGD-Adv-hHGF-hXIAP at a MOI of 500 for 12 h. Then islets were washed three times to remove free virus. Then, 500 islets were carefully hand-picked and transplanted under the kidney capsule of each streptozotocin-induced diabetic NOD/SCID mouse. Three shots of insulin (5 U/kg) were given to each mouse on the first 3 days after transplantation to relieve the hyperglycemic stress to the newly-transplanted islets. The nonfasted glucose levels of all the mice were measured from the snipped tail of each animal at 14.00 h during the first week after surgery and then weekly. At the end of study, the mice were anaesthetized to collect blood to measure serum insulin and c-peptide levels by ELISA. The graft-bearing kidneys were then removed from some animals to confirm the function of islet grafts by the return of blood glucose levels to 400 mg/dl.

Intraperitoneal glucose tolerance test

Thirty days after islet transplantation, glucose tolerance was determined in overnight-fasted mice as described by Garcia-Ocana *et al.* [11]. Briefly, the mice were subjected to intraperitoneal injection of glucose at 2 g/kg of body weight. Blood samples were obtained from the snipped tail at 15, 30, 60, 90 and 120 min after injection and analyzed for glucose levels with a glucometer.

Immunofluorescence staining

In an independent study, 500 hundred handpicked islets were transplanted under the kidney capsule of STZ-induced diabetic NOD/SCID mice. Six mice from each group (mice receiving untransduced human islets and mice receiving RGD-Adv-hHGF-hXIAP-transduced human islets at a MOI of 500) were sacrificed 30 days after transplantation. Another six from each group was sacrificed at 200 days after islet transplantation. The kidneys bearing islets were isolated, washed with PBS, fixed in 4% paraformaldehyde overnight, and embedded in optimal cutting temperature compound. Frozen sections of 10- μ m thickness were cut. To detect insulin-positive human islets, the slides were stained with guinea pig anti-insulin primary antibody (dilution 1:200) at 4 °C overnight and Alexa Fluor 568-conjugated goat anti-guinea pig secondary antibody (dilution 1:500) at room temperature for 1 h. To detect revascularization, the slides were stained with rabbit anti-von Willebrand factor (vWF) primary antibody (dilution 1:500) at 4 °C overnight and Dylight 488-conjugated goat anti-rabbit secondary antibody (dilution 1:500) at room temperature for 1 h. Slides were counter-stained with 4',6-diamidino-2-phenylindole.

Statistical analysis

Statistical significance of the difference between the two groups was determined by an unpaired Student's *t*-test and between several groups by one-way analysis of variance.

Results

Construction of RGD-Adv-hHGF-hXIAP

To improve the transduction efficiency of Adv on human islets, we chose to introduce an RGD peptide, which is known to bind with high affinity to several types of integrins present

on the surface of mammalian cells, into the HI loop of the fiber knob. An RGD-modified Adv was generated by sequentially incorporating an RGD epitope-expressing cassette, hXIAP cDNA and hHGF cDNA, into the Ad5 genome through homologous DNA recombination (Figure 1). Structures of the resultant recombinant vectors were then confirmed by restriction enzyme digestion and PCR analysis. A proper amount of recombinant plasmid was digested with *PacI* and transfected into 293 cells, thereby generating the replication-incompetent Adv, RGD-Adv-hHGF-hXIAP.

Transduction efficiency of RGD-Adv-hHGF-hXIAP on human islets

The transduction efficiency of traditional Adv-GFP and RGD-Adv-GFP was compared. The results showed that the RGD modification increased the expression of GFP in human islets, suggesting an improved transduction efficiency (Figure 2A). The transduction efficiency of RGD-Adv-hHGF-hXIAP and Adv-hHGF-hXIAP on human islets was also compared by transducing human islets with serial dilutions of these Adv vectors. The results showed that HGF and XIAP expressions were up-regulated in a dose-dependent manner. HGF and XIAP expression in RGD-Adv-hHGF-hXIAP-transduced human islets was significantly higher, indicating an improved transduction efficiency (Figure 2B). The gene expression profile of RGD-Adv-hHGF-hXIAP-transduced human islets over 2 weeks indicated the transient manner of Adv-mediated gene expression. The amount of HGF and XIAP produced by RGD-Adv-hHGF-hXIAP-transduced human islets peaked 2 days after transduction and decreased gradually over the next 14 days (Figure 2C). However, the levels of HGF and XIAP were still much higher compared to the endogenous expression of these two therapeutic genes (Figure 2C).

Pro-apoptotic gene is inhibited and anti-apoptotic gene is elevated

Both HGF and XIAP have anti-apoptotic effects. HGF inhibited the apoptotic pathway via activating AKT kinase, whereas XIAP is well known for its inhibition of caspase activities [20,21]. The results showed that the activity of caspase 3 was significantly decreased in RGD-Adv-hHGF-hXIAP-transduced human islets over 14 days after transduction (Figure 3A). Western blot results showed the up-regulation of p-AKT at 2 days after Adv transduction (Figure 3B). These results suggested an anti-apoptotic effect of RGD-Adv-hHGF-hXIAP on human islets. We also observed elevated anti-apoptotic protein BCL-2 and suppressed pro-apoptotic protein BAX (Figure 3B), which might be a result of XIAP overexpression, as suggested by previous studies [22,23].

Protection of RGD-Adv-hHGF-hXIAP on human islets

The TUNEL assay was used to determine the apoptotic cell death under cytokine treatment. We first tested the anti-apoptotic effect of RGD-Adv-hHGF-hXIAP on insulin-producing rat insulinoma (INS-1E) cells because it is relatively easier to stain and detect the dead cell in monolayer. Numerous apoptotic cells were observed in untransduced INS-1E cells after cytokine treatment, whereas fewer apoptotic cells were observed in RGD-Adv-hHGF-hXIAP-transduced INS-1E cells (Figure 4). Similarly, the fraction of apoptotic cells was significantly reduced in RGD-Adv-hHGF-hXIAP-transduced islets compared to untransduced islets (Figure 4). However, it should be noted that because flow cytometry did not differentiate insulin-producing β -cells from other cell types in human islets, these results

only suggested that the whole islet can be protected from cytokine-induced cell death. Another glucose perfusion experiment is needed to determine the viability and function of insulin-producing β -cells of human islets.

Human islets were first transduced with RGD-Adv-hHGF-hXIAP and then stimulated with a cytokine cocktail of IL-1 β and TNF α . The results showed that insulin secretion by human islets was significantly impaired after being treated with the cytokine cocktail (Figure 5). However, the insulin secretion of RGD-Adv-hHGF-hXIAP-transduced human islets was not significantly impaired under cytokine treatment (Figure 5), suggesting an improved viability and function of the insulin-producing β -cells of RGD-Adv-hHGF-hXIAP-transduced human islets.

RGD-Adv-hHGF-hXIAP transduction prolonged islet survival after transplantation

The blood glucose levels of the diabetic mice were decreased to below 300 mg/ml shortly after islet transplantation (Figure 6). However, the mice transplanted with RGD-Adv-hHGF-hXIAP-transduced islets showed a better transplantation outcome in terms of mean blood glucose level, duration of normoglycemia, and insulin-independent ratio (Figure 6). These results demonstrated the improved viability and function of human islets genetically modified with RGD-Adv-hHGF-hXIAP.

An intraperitoneal glucose tolerance test was performed 30 days after islet transplantation to determine whether *ex vivo* transduction of islets with RGD-Adv-hHGF-hXIAP helped islets engraft and respond to elevated blood glucose in real time. Before glucose injection, all mice displayed similar baseline blood glucose levels after overnight fasting. Blood glucose levels increased immediately after glucose (2 g/kg) injection, peaked at 15 min in all groups, and then decreased over time (Figure 7). There was a faster and better response to the glucose boost in those mice receiving RGD-Adv-hHGF-hXIAP-transduced human islets relative to the mice receiving untransduced islets (Figure 7). These results demonstrated that RGD-Adv-hHGF-hXIAP-transduced human islets had a better engraftment outcome and led to superior glucose control and tolerance compared to untransduced islets.

RGD-Adv-hHGF-hXIAP transduction improved islet revascularization after transplantation

Immunofluorescence staining showed the successful engraftment of insulin-positive islets under the kidney capsule 30 days after islet transplantation. Untransduced islets and RGD-Adv-hHGF-hXIAP-transduced islets both showed proper engraftment and function at this time (Figure 8). At 200 days after transplantation, the clear insulin staining in the kidney bearing RGD-Adv-hHGF-hXIAP-transduced islets indicated morphological integrity and appropriate function of transplanted islets (Figures 9C and 9D). In addition, the potent staining of vWF in proximity to these islets indicated the functional revascularization of islet grafts under the kidney capsule (Figure 9D). However, in the kidney bearing untransduced islets, only scarce vWF staining was seen and weak insulin staining indicated poor morphology and function of transplanted islets (Figures 9A and 9B).

Discussion

T1D diabetes is an autoimmune disease resulting from the destruction of insulin-producing pancreatic β -cells, which necessitates a lifelong daily glucose monitoring and injection of insulin. Islet transplantation, which is still an experimental treatment for diabetes, could be a permanent cure for type I diabetes if transplanted islets could actively maintain normal blood glucose under all conditions and escape graft rejection as a result of inflammatory and immune reactions. However, the clinical application of islet transplantation to treat diabetes is greatly hindered by the massive loss of islet grafts immediately after transplantation [1,24]. Immunosuppressive regimens are capable of preventing islet failure from months to years, although the agents used in these treatments may induce significant side effects, resulting in a progressive decline of graft function [25]. Moreover, because of the extensive post-transplantation challenges such as inflammatory cytokines, ROS and hypoxia [2], a patient needs at least 10 000 islet equivalents per kilogram of body weight (extracted from two or more donor pancreases) for an optimal transplantation outcome, making the current shortage of islet supply even worse [1,26].

Dinareello *et al.* [27] summarized that IL-1 and TNF are the two most important effectors in autoimmune diseases. Donath *et al.* [28] concluded that anti-inflammatory therapeutic approaches to block β -cell apoptosis could be an important strategy for treating type 1 and 2 diabetes. We previously reported that IL-1Ra expression in human islets effectively blocked the IL-1 β -mediated apoptotic islet death [8,29] but did not stop the apoptosis caused by other inflammatory cytokines, such as TNF α and interferon- γ . We also reported that XIAP expression in human islets repressed apoptotic islet death by inhibiting caspase activation [30], although its effect is limited because of a low transduction efficiency. In the present study, we genetically engineered Adv with an RGD peptide to improve transduction efficiency and co-expressed HGF and XIAP, both of which have an anti-apoptotic effect but through different pathways [21,31]. The results obtained demonstrated that the levels of HGF and XIAP were significantly up-regulated and gradually decreased 14 days after Adv transduction (Figure 2C), which is in accordance with the transient property of Adv-mediated gene expression. Consequently, a high HGF level activated the PI3K-Akt pathway and a high XIAP level suppressed caspase 3 activity in the first 14 days after Adv transduction (Figure 3). In an inflammatory condition mimicked by a cytokine cocktail of IL-1 β and TNF α , the TUNEL assay and islet perfusion study indicated that overexpression of HGF and XIAP gene before cytokine treatment effectively protected islet viability and function from apoptotic cell death (Figures 4B and 5). The *in vivo* viability and function were further confirmed by immune fluorescence analysis (Figure 8).

Revascularization is another crucial factor for successful islet transplantation. Islets are a cluster of heterogeneous cell types with extensive intra-islet vessels. These vessels become disrupted during islet isolation, leading to a collapse of vasculature, an accumulation of endothelial fragments and a compromised perfusion in the core of the islets [32]. Therefore, extensive and functional revascularization is required to promote the survival of islet grafts post-transplantation [33]. VEGF is a popular angiogenic factor, although its effect is so potent that caution should be exercised to avoid aberrant angiogenesis [34]. Recently, HGF has been seen as a more favourable growth factor because it can prevent apoptotic islet death

and promote the proliferation of pancreatic β -cells [12]. In the present study, we demonstrated that HGF expression of human islets can be up-regulated over 14 days after Adv transduction (Figure 2C). Consequently, extensive revascularization was observed in the mice transplanted with RGD-Adv-hHGF-hXIAP-transduced human islets in the immune fluorescence study (Figure 9D).

Islet destruction after transplantation can be greatly reduced by gene therapy [35]. Because the islet comprises a compact cluster of approximately 1000 nondividing cells, it is difficult to transfect intact islets by the available nonviral approaches, such as cationic liposomes and polymer-based systems, which are also toxic at high doses [36]. By contrast, replication deficient (E1 or E3 deleted) Adv vectors are known to efficiently transduce islets. In addition, adenoviral vectors can be produced in high titers and there is no risk of insertional mutagenesis because they do not integrate into host genome. However, Adv transduction of human islets leads to increasing immune responses because some viral genomes co-express with therapeutic genes and elicit an increased immune response and allograft rejection [37,38]. This problem may be exaggerated when a relatively higher MOI is required to achieve optimal transduction efficiency on solid organs such as human islets [13,14]. We incorporated an RGD peptide sequence into the Adv fiber knob to interact with $\alpha_v\beta$ integrins aiming to facilitate Adv transduction into human islets [39]. The results obtained indicated that the transduction efficiency on human islets could be significantly improved using RGD-modified Adv (Figures 2A and 2B). Transduction efficiency could be further increased when the MOI was increased to 1000 and 5000 (data not shown). However, we decided not to transduce islets with an MOI higher than 500 to avoid any toxic side effects. The insulin perfusion study demonstrated that there was no decrease in insulin production from RGD-Adv-hHGF-hXIAP-transduced islets (Figure 5). RGD modification of Adv may be of significant importance for the development of new viral vectors, which can be genetically redirected to specialized cells as gene delivery vehicles. A similar RGD sequence placed in the virus hexon coat protein increased gene transfer to cells that were normally refractory to Adv transduction [40]. Similarly, adding a cationic polylysine sequence (pK7) to the fiber knob COOH terminus, which facilitates viral binding to negatively-charged cell surface molecules such as heparan sulfates, increased transduction to macrophages, endothelial cells, smooth muscle cells, fibroblasts and T cells [41,42]. In the present study, we also evaluated a bipartite Adv vector for multiple gene delivery to human islets for promoting revascularization and inhibiting inflammatory cytokine-mediated destruction. Because transfection with the bipartite plasmid encoding hHGF and hXIAP (dI324-hHGF-hXIAP-RGD) (Figure 1) produced very little hHGF or hXIAP (data not shown), viral vector appears to be the only choice for gene therapy on human islets. Bipartite Adv vectors are usually constructed with one therapeutic gene and another marker gene, especially GFP [43,44]. Using a bipartite vector not only simplifies the amplification and purification process of Adv vectors, but also decreases the use of total Adv backbone compared to the use of two single Adv vectors. This procedure is expected to minimize the immunogenicity and toxicity of Adv vectors.

Islet isolation and preparation is another state-of-the-art in clinical islet transplantation. Unlike other species from which islets can be isolated with little contamination, human islet preparations are usually composed of a majority (50–60%) of β -cells and many other cell

types, including duct cells, acinar cells, α cells and lymph node cells [45,46]. Regardless of their origin, all cells within an islet preparation may contribute to the immune response induced after transplantation. Therefore, the purity and viability of islets used for transplantation has a significant impact on the outcome of islet transplantation. Dithizone staining is carried out to discriminate between endocrine and nonendocrine cells aiming to increase the purity of the islet preparation to 70–95% [46,47]. Using hand-picked islets helped to further improve islet purity by discarding other tissues such as acinar cells and lymph nodes [48]. However, further purification of the islet preparations remains a challenge because of a remarkable loss of insulin-producing cells.

To summarize, the present study demonstrated that RGD modification is a useful tool to improve the transduction efficiency of Adv. *Ex vivo* transduction of islets with RGD-Adv-hHGF-hXIAP decreased apoptotic islet death and improved islet revascularization, and eventually improved the outcome of human islet transplantation.

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References

1. Ryan EA, Paty BW, Senior PA, et al. Five-year follow-up after clinical islet transplantation. *Diabetes*. 2005; 54:2060–2069. [PubMed: 15983207]
2. Narang AS, Mahato RI. Biological and biomaterial approaches for improved islet transplantation. *Pharmacol Rev*. 2006; 58:194–243. [PubMed: 16714486]
3. Mandel TE, Koulmanda M. Effect of ischemia and temperature on fetal mouse pancreas. Insulin production in vitro, and function after isotransplantation. *Diabetes*. 1984; 33:376–382. [PubMed: 6423431]
4. Sarvetnick N, Shizuru J, Liggitt D, et al. Loss of pancreatic islet tolerance induced by beta-cell expression of interferon-gamma. *Nature*. 1990; 346:844–847. [PubMed: 2118234]
5. Kessler L, Jesser C, Lombard Y, et al. Cytotoxicity of peritoneal murine macrophages against encapsulated pancreatic rat islets: in vivo and in vitro studies. *J Leukoc Biol*. 1996; 60:729–736. [PubMed: 8975875]
6. Pawelec K, Juszczak MT, Kumar A, et al. Time course of islet loss after intraportal transplantation. *Ann NY Acad Sci*. 2008; 1150:230–233. [PubMed: 19120301]
7. Cheng G, Zhu L, Mahato RI. Caspase-3 gene silencing for inhibiting apoptosis in insulinoma cells and human islets. *Mol Pharm*. 2008; 5:1093–1102. [PubMed: 18828606]
8. Panakanti R, Mahato RI. Bipartite adenoviral vector encoding hHGF and hIL-1Ra for improved human islet transplantation. *Pharm Res*. 2009; 26:587–596. [PubMed: 19002565]
9. Wu H, Panakanti R, Li F, et al. XIAP Gene expression protects beta-cells and human islets from apoptotic cell death. *Mol Pharm*. 2010; 7:1655–1666. [PubMed: 20677802]
10. Narang AS, Sabek O, Gaber AO, et al. Co-expression of vascular endothelial growth factor and interleukin-1 receptor antagonist improves human islet survival and function. *Pharm Res*. 2006; 23:1970–1982. [PubMed: 16906455]
11. Garcia-Ocana A, Takane KK, Reddy VT, et al. Adenovirus-mediated hepatocyte growth factor expression in mouse islets improves pancreatic islet transplant performance and reduces beta cell death. *J Biol Chem*. 2003; 278:343–351. [PubMed: 12403787]
12. Garcia-Ocana A, Vasavada RC, Cebrian A, et al. Transgenic overexpression of hepatocyte growth factor in the beta-cell markedly improves islet function and islet transplant outcomes in mice. *Diabetes*. 2001; 50:2752–2762. [PubMed: 11723058]

13. Kay MA, Glorioso JC, Naldini L. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med.* 2001; 7:33–40. [PubMed: 11135613]
14. Contreras JL, Wu H, Smyth CA, et al. Double genetic modification of adenovirus fiber with RGD polylysine motifs significantly enhances gene transfer to isolated human pancreatic islets. *Transplantation.* 2003; 76:252–261. [PubMed: 12865820]
15. Dmitriev I, Krasnykh V, Miller CR, et al. An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J Virol.* 1998; 72:9706–9713. [PubMed: 9811704]
16. Kurozumi K, Nakamura K, Tamiya T, et al. BDNF gene-modified mesenchymal stem cells promote functional recovery and reduce infarct size in the rat middle cerebral artery occlusion model. *Mol Ther.* 2004; 9:189–197. [PubMed: 14759803]
17. Ricordi C, Gray DW, Hering BJ, et al. Islet isolation assessment in man and large animals. *Acta Diabetol.* 1990; 27:185–195.
18. Yun CO, Kim E, Koo T, et al. ADP-overexpressing adenovirus elicits enhanced cytopathic effect by induction of apoptosis. *Cancer Gene Ther.* 2005; 12:61–71. [PubMed: 15375379]
19. Chartier C, Degryse E, Gantzer M, et al. Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *J Virol.* 1996; 70:4805–4810. [PubMed: 8676512]
20. Xiao GH, Jeffers M, Bellacosa A, et al. Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways. *Proc Natl Acad Sci USA.* 2001; 98:247–252. [PubMed: 11134526]
21. Huang Y, Park YC, Rich RL, et al. Structural basis of caspase inhibition by XIAP: differential roles of the linker vs. the BIR domain. *Cell.* 2001; 104:781–790. [PubMed: 11257231]
22. Deveraux QL, Leo E, Stennicke HR, et al. Cleavage of human inhibitor of apoptosis protein XIAP results in fragments with distinct specificities for caspases. *EMBO J.* 1999; 18:5242–5251. [PubMed: 10508158]
23. Bratton SB, Lewis J, Butterworth M, et al. XIAP inhibition of caspase-3 preserves its association with the Apaf-1 apoptosome and prevents CD95- and Bax-induced apoptosis. *Cell Death Differ.* 2002; 9:881–892. [PubMed: 12181739]
24. Hering BJ, Kandaswamy R, Ansie JD, et al. Single-donor, marginal-dose islet transplantation in patients with type 1 diabetes. *JAMA.* 2005; 293:830–835. [PubMed: 15713772]
25. Polastri L, Galbiati F, Bertuzzi F, et al. Secretory defects induced by immunosuppressive agents on human pancreatic beta-cells. *Acta Diabetol.* 2002; 39:229–233. [PubMed: 12486498]
26. Scharp DW, Lacy PE, Santiago JV, et al. Results of our first nine intraportal islet allografts in type 1, insulin-dependent diabetic patients. *Transplantation.* 1991; 51:76–85. [PubMed: 1987709]
27. Dinarello CA. Inflammatory cytokines: interleukin-1 and tumor necrosis factor as effector molecules in autoimmune diseases. *Curr Opin Immunol.* 1991; 3:941–948. [PubMed: 1665333]
28. Donath MY, Storling J, Maedler K, et al. Inflammatory mediators and islet beta-cell failure: a link between type 1 and type 2 diabetes. *J Mol Med (Berl).* 2003; 81:455–470. [PubMed: 12879149]
29. Panakanti R, Mahato RI. Bipartite vector encoding hVEGF and hIL-1Ra for ex vivo transduction into human islets. *Mol Pharm.* 2009; 6:274–284. [PubMed: 19067524]
30. Wu H, Panakanti R, Li F, et al. XIAP gene expression protects beta-cells and human islets from apoptotic cell death. *Mol Pharm.* 2010; 7:1655–1666. [PubMed: 20677802]
31. Ozaki M, Haga S, Zhang HQ, et al. Inhibition of hypoxia/reoxygenation-induced oxidative stress in HGF-stimulated antiapoptotic signaling: role of PI3-K and Akt kinase upon rac1. *Cell Death Differ.* 2003; 10:508–515. [PubMed: 12728249]
32. Cheng K, Fraga D, Zhang C, et al. Adenovirus-based vascular endothelial growth factor gene delivery to human pancreatic islets. *Gene Ther.* 2004; 11:1105–1116. [PubMed: 15238996]
33. Menger MD, Beger C, Vajkoczy P. Restitution of intra-islet portal system in pancreatic islet isografts. *Transplant Proc.* 1994; 26:688. [PubMed: 7513464]
34. Ozawa CR, Banfi A, Glazer NL, et al. Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis. *J Clin Invest.* 2004; 113:516–527. [PubMed: 14966561]

35. Mahato RI. Gene expression and silencing for improved islet transplantation. *J Control Release*. 2009; 140:262–267. [PubMed: 19376168]
36. Mahato RI, Rolland A, Tomlinson E. Cationic lipid-based gene delivery systems: pharmaceutical perspectives. *Pharm Res*. 1997; 14:853–859. [PubMed: 9244140]
37. Kay MA, Holterman AX, Meuse L, et al. Long-term hepatic adenovirus-mediated gene expression in mice following CTLA4Ig administration. *Nat Genet*. 1995; 11:191–197. [PubMed: 7550348]
38. Yang Y, Wilson JM. Clearance of adenovirus-infected hepatocytes by MHC class I-restricted CD4+ CTLs in vivo. *J Immunol*. 1995; 155:2564–2570. [PubMed: 7650386]
39. Wickham TJ, Mathias P, Cheres DA, et al. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell*. 1993; 73:309–319. [PubMed: 8477447]
40. Vigne E, Mahfouz I, Dedieu JF, et al. RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection. *J Virol*. 1999; 73:5156–5161. [PubMed: 10233980]
41. Hidaka C, Milano E, Leopold PL, et al. CAR-dependent and CAR-independent pathways of adenovirus vector-mediated gene transfer and expression in human fibroblasts. *J Clin Invest*. 1999; 103:579–587. [PubMed: 10021467]
42. Wickham TJ, Tzeng E, Shears LL II, et al. Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. *J Virol*. 1997; 71:8221–8229. [PubMed: 9343173]
43. Mistry SJ, Bank A, Atweh GF. Targeting stathmin in prostate cancer. *Mol Cancer Ther*. 2005; 4:1821–1829. [PubMed: 16373697]
44. Silvertown JD, Geddes BJ, Summerlee AJ. Adenovirus-mediated expression of human prorelaxin promotes the invasive potential of canine mammary cancer cells. *Endocrinology*. 2003; 144:3683–3691. [PubMed: 12865351]
45. Sever CE, Demetris AJ, Zeng J, et al. Composition of human islet cell preparations for transplantation. *Acta Diabetol*. 1992; 28:233–238. [PubMed: 1576361]
46. Street CN, Lakey JR, Shapiro AM, et al. Islet graft assessment in the Edmonton Protocol: implications for predicting long-term clinical outcome. *Diabetes*. 2004; 53:3107–3114. [PubMed: 15561940]
47. Deng S, Vatamaniuk M, Huang X, et al. Structural and functional abnormalities in the islets isolated from type 2 diabetic subjects. *Diabetes*. 2004; 53:624–632. [PubMed: 14988246]
48. Szot GL, Koudria P, Bluestone JA. Transplantation of pancreatic islets into the kidney capsule of diabetic mice. *J Vis Exp*. 2007; 9:404. [PubMed: 18989445]

Figure 1. Construction of the RGD-modified Adv genome encoding HGF and XIAP in E1 and E3 deletion. RGD epitope expressing cassette, hXIAP cDNA and hHGF cDNA were sequentially incorporated into the Ad5 genome by homologous DNA recombination.

Figure 2.

Transduction efficiency of RGD-Adv-hHGF-hXIAP into human islets. (A) GFP expression in human islets transduced with Adv-GFP and RGD-Adv-GFP. (B) Dose-dependent expression of HGF and XIAP in RGD-Adv-hHGF-hXIAP and Adv-hHGF-hXIAP-transduced human islets. (C) Time-dependent expression of HGF and XIAP in RGD-Adv-hHGF-hXIAP-transduced human islets at a MOI of 500. Data are presented as the mean $\pm$ SD ($n = 6$). * $p < 0.05$ as determined by an unpaired Student's t -test.

Figure 3. HGF and XIAP expression led to inhibition of the pro-apoptotic gene and elevation of the anti-apoptotic gene. (A) Caspase 3 activity in RGD-Adv-hHGF-hXIAP-transduced human islets was measured up to 14 days after transduction by the Caspase 3 Glo kit. (B) Expression of proapoptotic marker BAX and anti-apoptotic markers Bcl-2 and p-Akt was determined by western blot. GADPH was used as a control. MOI: 0, 5, 10, 50, 100 and 500 (from left to right).

Figure 4.

Overexpression of HGF and XIAP protected INS-1E cells and human islets from inflammatory cytokine-induced apoptotic cell death. After transduction with RGD-Adv-hHGF-hXIAP, INS-1E cells and human islets were incubated with a cytokine cocktail of IL- β (5 ng/ml) and TNF α (5 ng/ml) for 48 h and 3 days, respectively. (A) Apoptotic INS-1E cells were stained dark brown with the DeadEndTM Colorimetric TUNEL System. (B) Islets were dispersed with trypsin/EDTA into a single-cell suspension. Apoptotic cells were stained with annexin V-FITC and counted by flow cytometry. P3 indicates the percentage of apoptotic cells. All experiments were performed in triplicate and at least twice with similar results being obtained. A representative image is shown. Results are presented as the mean $\pm$ SD. * p < 0.05 as determined by Student's t -test.

Figure 5.

Overexpression of HGF and XIAP improved insulin secretion of islets against inflammatory cytokines. Briefly, after transduction with RGD-Adv-hHGF-hXIAP and further stimulation with a cytokine cocktail of IL- β (5 ng/ml) and TNF α (5 ng/ml) for 3 days, 50 islets from each group were sequentially perfused with basal glucose (2.5 mM) for 45 min and stimulated with glucose (22.5 mM) for 15 min and finally perfused with basal glucose (2.5 mM) until insulin release reversed to the basal level. The flow rate was maintained at 2 ml/min. Samples were collected through a fraction collector and analyzed for insulin content by ELISA. All experiments were performed in triplicate. Results are presented as the mean $\pm$ SD.

Figure 6. Effect of RGD-Adv-hHGF-hXIAP transduction on the outcome of islet transplantation. (A, B) The blood glucose level of every single mouse after receiving 500 untransduced (A) or RGD-Adv-hHGF-hXIAP-transduced islets (B). (C) The mean blood glucose level of mice after islet transplantation. (D) Reversed-diabetes ratio of the NOD/SCID mice after islet transplantation. Blood glucose 200 mg/dl (dashed line) was identified as reversed-diabetes (dashed line in A, B). White squares indicate mice receiving 500 untransduced islets; black triangles indicate mice receiving 500 RGD-Adv-hHGF-hXIAP-transduced islets. Data are presented as the mean $\pm$ SD ($n = 10$). * $p < 0.05$ as determined by Student's t -test.

Intraperitoneal Glucose Tolerance Test

Figure 7.

Intraperitoneal glucose tolerance test at 30 days after islet transplantation. The results obtained from nontreated diabetic mice and nondiabetic mice are shown as negative and positive controls, respectively. Data are presented as the mean $\pm$ SD ($n = 6$). * $p < 0.05$ as determined by Student's t -test compared to mice receiving untransduced human islets.

Figure 8. Immunofluorescence staining of the kidney section bearing RGD-Adv-hHGF-hXIAP-transduced human islets at 30 days after islet transplantation. (A, B) Insulin was stained in red to indicate the functional human islets of mice receiving untransduced human islets (A) and RGD-Adv-hHGF-hXIAP-transduced human islets (B).

Figure 9. Immunofluorescence staining of the kidney section bearing RGD-Adv-hHGF-hXIAP-transduced human islets at 200 days after islet transplantation. Insulin was stained in red and vWF factor was stained in green to indicate the neo-revascularization of transplanted human islets. (A, B) Kidney section bearing untransduced human islets (A) and a higher magnification of transplanted islets (B). (C, D) Kidney section bearing RGD-Adv-hHGF-hXIAP-transduced human islets (C) and a higher magnification of transplanted islets (D).