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ORIGINAL ARTICLE

Effect of glucagon-like peptide-1 gene expression on graft function in mouse islet transplantation

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Summary

This study investigated the effect of local glucagon-like peptide-1 (GLP-1) production within mouse islets on cytoprotection *in vitro* and *in vivo* by gene transfer of GLP-1. Transduction of recombinant adenovirus vector expressing GLP-1 (rAd-GLP-1) induced a significant increase in bioactive GLP-1 in the mouse islet culture, whereas transduction with adenovirus vector expressing β-galactosidase (rAd-LacZ), as a control, had no effect on GLP-1 secretion. Islets transduced with rAd-GLP-1 were protected from H_2O_2-induced cell damage *in vitro*. In addition, glucose-stimulated insulin secretion was significantly increased in rAd-GLP-1-transduced islets. When transplanted under the kidney capsule of diabetic syngeneic mice, islet grafts retrieved 4 or 7 days after transplantation revealed that the rAd-GLP-1-transduced group had significantly more Ki67-positive cells as compared with the rAd-LacZ-transduced group. Regarding blood glucose control, diabetic mice transplanted with a marginal mass of rAd-GLP-1-transduced islets became normoglycemic more rapidly and 78% of the recipients were normoglycemic at 35 days post-transplant, whereas only 48% of the mice transplanted with rAd-LacZ-transduced islets were normoglycemic ($P < 0.05$). In conclusion, delivery of the GLP-1 gene to islets enhanced islet cell survival during the early post-transplant period, and preserved islet mass and functions over time in the transplants.

Introduction

Pancreatic islet transplantation into insulin-deficient diabetic patients is a potential way to restore the functional β-cell mass. Currently, allogeneic islet transplantation from a single donor usually fails to achieve long-term insulin independence in a diabetic recipient because of the early loss of transplanted islets [1,2]. Immediately after transplantation, the majority of islets fail to engraft and rapidly undergo cell death from hypoxic and inflammatory insults [3–5]. One strategy to improve the survival rates of transplanted islets is to engineer β-cells that are resistant to apoptotic cell death from these insults during the early post-transplant period. Protective effects on transplanted islet cells have been observed by the transduction of genes for the expression of anti-apoptosis molecules or angiogenic growth factors [6–11].

Glucagon-like peptide-1 (GLP-1) is a 30-amino-acid peptide hormone secreted from the L-cells of the intestinal epithelium in response to a meal. It enhances glucose-stimulated postprandial insulin release, and inhibits inadequate glucagon secretion and gastrointestinal motility [12,13]. GLP-1 and its long-lasting analog exendin-4 can significantly improve glycemic control in patients with

type 2 diabetes [14]. In addition to its insulinotropic effects on glucose metabolism by stimulating β-cells, GLP-1 acts as a growth factor for β-cells, increasing the β-cell mass via the proliferation of islet cells, neogenesis and differentiation of pancreatic ductal cells into insulin-producing cells, while inhibiting β-cell apoptosis [15–21].

Given its ability to stimulate β-cell growth and to inhibit apoptosis, GLP-1 or its analogs could be of potential benefit to islet transplantation by maintaining or enhancing β-cell mass over time, and has been of particular interest in the field of islet transplantation [22–25]. In recent clinical trials, adding exendin-4 combined with etanercept to recipients on an immunosuppression regimen improved islet graft function and facilitated the achievement of insulin-independence with fewer islets [24,25]. Although an overall positive effect of GLP-1/analogs on marginal mass islet transplantation was observed, the beneficial effect on β-cell mass could not be assessed in those clinical studies, and GLP-1/analog treatment might only temporarily mask actual deteriorating islet graft mass through its insulinotropic effects.

The expected β-cell 'trophic' effect of GLP-1/analogs could be enhanced by higher drug levels at islet grafts. The GLP-1 dose that promoted β-cell growth in rodents was 50–100 μg/kg body weight (BW), while the tolerable dose in humans is <2 μg/kg BW [26–28]. In islet culture studies, 10 nM exenatide led to cell proliferation or decreased apoptosis after exposure for days to weeks [29,30], while the peak exenatide levels are around 40 pM after peripheral injection in humans [31]. In addition, the tolerated dose of exenatide in islet allograft recipients with type 1 diabetes was lower than in patients with type 2 diabetes, due to increased side effects of nausea and vomiting and of hypoglycemia [22,23]. Consequently, it seems difficult to utilize the β-cell growth-promoting actions of GLP-1/analogs when they are administered by a systemic route [32].

Therefore, this study investigated the effect of local GLP-1 production directly within the mouse islet graft via adenovirus-mediated gene transfer of GLP-1 on the cytoprotection of islets *in vitro* and *in vivo*.

Materials and methods

Islet isolation

Male inbred Balb/c mice, 9–10 weeks of age, were purchased from Koatech (Pyeongtaek, Korea). Pancreatic islets were isolated from 12-week-old mice by digesting pancreatic tissues with 1 mg/ml collagenase P (Roche, Mannheim, Germany) followed by Ficoll (Biochrom AG, Berlin, Germany) gradient purification. The isolated islets were maintained in Medium 199 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 incubator. Individual islets were handpicked under an inverted microscope and quantified by dithizone staining in duplicate using a standard islet diameter of 150 μm as one islet equivalent (IEQ).

In vitro test of GLP-1 expression and secretion using rAd-GLP-1

Recombinant adenovirus vectors expressing GLP-1 (rAd-GLP-1) or β-galactosidase (rAd-LacZ), as a control, were constructed and produced, as described previously [33]. The isolated mouse islets were washed with fresh Medium 199 and infected with rAd-GLP-1 or rAd-LacZ at a multiplicity of infection (MOI) of 100. To check the expression of GLP-1 mRNA, islets infected with rAd-GLP-1 or rAd-LacZ were harvested 24 h after infection, and reverse transcriptase-polymerase chain reaction (RT-PCR) was performed, as described previously [33]. To measure GLP-1 secretion, the supernatant was collected 24 h after infection, and the amount of GLP-1 was analyzed using an Active GLP-1 enzyme-linked immunosorbent assay (ELISA) kit (Millipore, Tullagreen, Ireland).

Measuring cell viability and apoptosis

To evaluate the effect of GLP-1 expression on islet cell viability and apoptosis, mouse islets infected with rAd-GLP-1 or rAd-LacZ for 4 h were treated with 200 μmol/l H_2O_2 for 30 min. Islet cell viability was assessed using acridine orange/propidium iodide (AO/PI) (Sigma-Aldrich, St. Louis, MO, USA) fluorescence staining, as described previously [34]. Mitochondrial membrane potentials ($\Delta\psi m$) were determined by staining with the cationic fluorescent indicator JC-1 (Molecular Probes, Eugene, OR, USA) [34,35]. Islet cell apoptosis was detected using a Caspase-3/CPP32 assay kit (BioVision, Mountain View, CA, USA).

Glucose stimulated insulin secretion

To determine the effect of GLP-1 expression on insulin secretion in response to glucose, a static glucose stimulation test was performed with mouse islets. Twenty islets were incubated in RPMI-1640 medium containing 1.67 mmol/l glucose for a 1-h preincubation at 37 °C. The islets were then incubated sequentially for 1 h each in 2 ml of RPMI-1640 medium containing 1.67 mmol/l glucose (basal), 16.7 mmol/l glucose (stimulatory), and 1.67 mmol/l glucose (recovery). Medium was collected for insulin assays and stored at −20 °C. Insulin concentrations were measured using an insulin ELISA kit (Shibayagi, Gunma, Japan). The glucose stimulation index (GSI) was calculated as follows: GSI = (2 × stimulatory insulin)/(basal insulin + recovery insulin).

Islet transplantation

Mice were fed standard rodent chow in a barrier animal facility under a 12-h light/dark cycle. All animal experiments were in compliance with the guidelines of the Institutional Animal Care Committee of Hallym University. At 12 weeks of age, diabetes was induced by a single intraperitoneal injection of 200 mg/kg streptozotocin (STZ, Sigma) 3–5 days before transplantation. Mice were considered diabetic when their blood glucose levels were ≥300 mg/dl for two consecutive days. After 24 h of transduction with rAd-GLP-1 or rAd-LacZ, islets were transplanted as pellets under the left kidney capsule of diabetic syngeneic mice. We implanted 180 IEQ as a marginal number to cure diabetes. After transplantation, nonfasting blood glucose levels were measured 3 days per week for 35 days. Reversal of diabetes was defined as the consistent reversal of hyperglycemia to <200 mg/dl. At 35 days post-transplant, a left nephrectomy was performed in the cured mice to confirm a return to hyperglycemia. To assess the effect of GLP-1 expression on cell proliferation in early islet grafts, 300 rAd-GLP-1- or rAd-LacZ-infected IEQ were transplanted into diabetic recipients and a left nephrectomy was performed 4 or 7 days after transplantation.

Immunohistochemistry of islet grafts

The graft-bearing kidneys retrieved 4 or 7 days after transplantation were fixed in 10% formaldehyde, processed, and embedded in paraffin. The 4-um-thick sections were stained with Ki67 antibody (1:100 dilution; Bethyl, Montgomery, TX, USA) and streptavidin–biotin complexes to detect cell proliferation, and were counterstained with hematoxylin. The fraction of Ki67-positive cells in the islet grafts was quantified under a light microscope using a direct point-counting method, as described previously [11,36]. Briefly, a grid with 121 intersections was placed onto each tissue section under a light microscope (×400) and the number of intersections overlapping Ki67-positive cells was counted. In each graft, ≥10 tissue sections stained with Ki67 from all parts of the islet grafts were evaluated. The sections were also stained with insulin antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and streptavidin–biotin complexes.

Statistical analysis

All values are expressed as the mean ± SEM. Statistical significance was calculated using a Student's *t*-test or one-way analysis of variance (ANOVA). The Kaplan–Meier log-rank test was used to determine significance in the marginal islet mass transplantation experiments. A P-value < 0.05 was deemed statistically significant. All statistical analyses were performed using the program MedCalc (Mariakerke, Belgium).

Result

Expression of GLP-1 in rAd-GLP-1-transduced mouse islets

To check the expression of GLP-1 resulting from the rAd-GLP-1 vector, we infected mouse islets with rAd-GLP-1 or rAd-LacZ and examined the expression of GLP-1 mRNA by RT-PCR. GLP-1 mRNA was clearly expressed 24 h after rAd-GLP-1 infection, whereas GLP-1 mRNA was not detected in rAd-LacZ-infected islet cells. We examined the GLP-1 secretion by ELISA in rAd-GLP-1- or rAd-LacZ-transduced islet cells 24 h after infection. rAd-GLP-1 transduction induced a significant increase in bioactive GLP-1 in the culture medium of mouse islets, whereas transduction with rAd-LacZ, had no effect on GLP-1 secretion (Fig. 1).

Effect of rAd-GLP-1 transduction on the survival and function of mouse islets

To determine whether the GLP-1 expression in islets has a cytoprotective effect against cell stress, rAd-GLP-1 infected, rAd-LacZ infected, or noninfected islets were exposed to 200 μmol/l H_2O_2 for 30 min. On AO/PI staining, rAd-GLP-1-transduced islets showed significantly decreased H_2O_2-induced cell death (red PI-positive cells) as compared with rAd-LacZ-transduced or noninfected islets (Fig. 2a). On JC-1 staining, red aggregates were consistently found in the control islet cells, whereas the green fluorescence was predominant in the H_2O_2-treated rAd-LacZ-transduced or nontransduced islets, indicating mito-

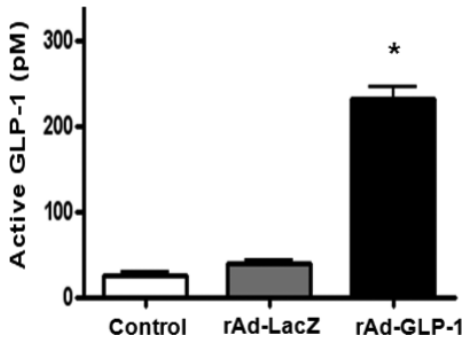

Figure 1 Glucagon-like peptide-1 (GLP-1) expression in mouse islets 24 h after transduction with rAd-LacZ or rAd-GLP-1. GLP-1 was measured in the culture media using an ELISA. *P < 0.05 vs. control or rAd-LacZ-transduced islets.

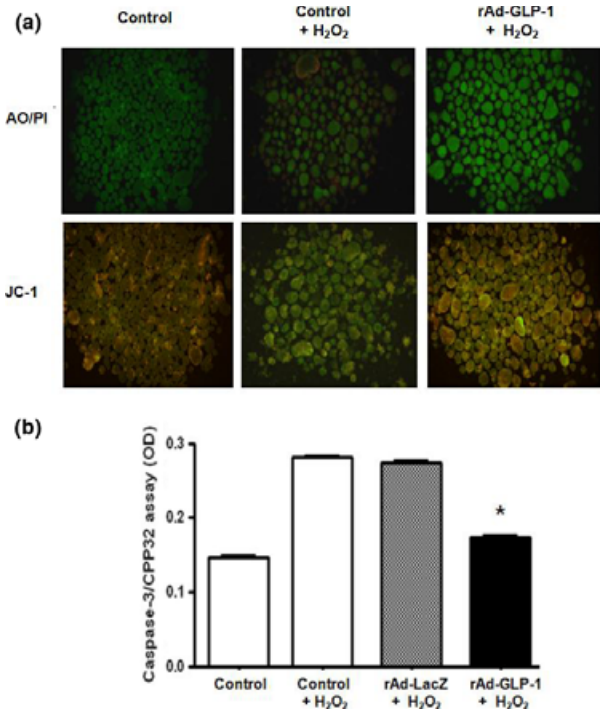

Figure 2 Effect of Glucagon-like peptide-1 (GLP-1) expression on H_2O_2-induced toxicity in mouse islet cells. (a) With AO/PI staining, H_2O_2 treatment (200 μmol/l for 30 min) decreased islet cell viability, as shown by the red PI-positive dead cells. The number of red cells was reduced in rAd-GLP-1-transduced islets. With JC-1 staining, Δψm was decreased in H_2O_2-treated islet cells, as shown by the predominant homogenous green fluorescence. Transduction with rAd-GLP-1 attenuated this decrease in Δψm, as reflected by some red aggregates in the cells. Representative images from three experiments are shown. (b) In the caspase-3/CPP32 assay, caspase-3 activity was lower in rAd-GLP-1-transduced islets after H_2O_2 treatment as compared with rAd-LacZ-transduced or nontransduced islets. *$P < 0.05$.

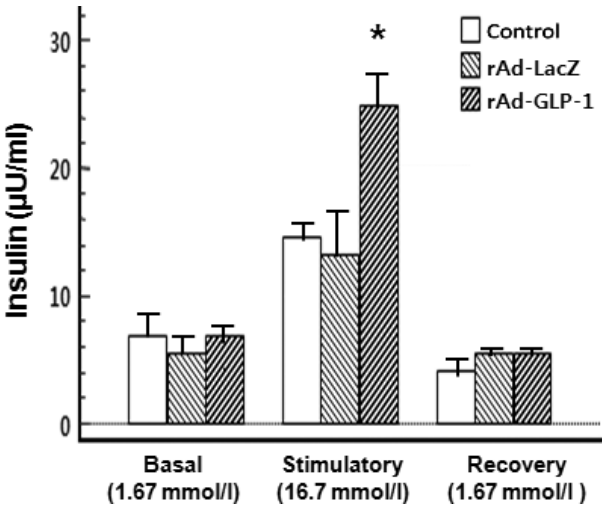

Figure 3 Effect of Glucagon-like peptide-1 (GLP-1) expression on glucose-stimulated insulin secretion in mouse islets. The rAd-GLP-1-transduced islets showed greater insulin secretion in response to glucose stimulation than did rAd-LacZ-transduced or nontransduced islets. *$P < 0.05$.

chondrial membrane depolarization and reduced Δψm. The transduction with rAd-GLP-1 attenuated this decrease in Δψm, as reflected by some red aggregates in the islet cells after H_2O_2 treatment. The caspase-3 activity was also significantly lower in rAd-GLP-1-transduced islets after H_2O_2 treatment, as compared with rAd-LacZ-transduced or nontransduced islets (Fig. 2b). The rAd-GLP-1-transduced islets secreted more insulin in response to glucose stimulation than the rAd-LacZ-transduced or nontransduced islets, with higher GSI (Fig. 3; 4.0 vs. 2.1 or 2.3, $P < 0.05$).

Effect of rAd-GLP-1 transduction on cell proliferation in early islet grafts

To determine whether the local production of GLP-1 in islets is beneficial for islet cell survival in early islet grafts, rAd-GLP-1 transduced or rAd-LacZ transduced islets were transplanted under the kidney capsule of chemically induced diabetic syngeneic mice. The islet grafts retrieved 4 or 7 days after transplantation were stained with insulin antibody or Ki67 antibody, which stains proliferating cells (Fig. 4a). In both the day 4 and the day 7 grafts, the rAd-GLP-1-transduced group had significantly more Ki67-positive cells as compared with the rAd-LacZ transduced group (Fig. 4b).

Effect of rAd-GLP-1 transduction on islet graft survival and function

To examine whether GLP-1 expression not only benefits islet cell proliferation in early grafts but also improves islet graft survival and function over time, 180 rAd-GLP-1-transduced or rAd-LacZ-transduced IEQ were transplanted under the kidney capsule of diabetic syngeneic mice. The transplantation outcome was evaluated for 35 days post-transplant (Fig. 5a). Diabetic mice transplanted with rAd-GLP-1-transduced islets became normoglycemic more rapidly than mice transplanted with rAd-LacZ-transduced islets (Fig. 5b). After 35 days, the grafts containing rAd-LacZ-transduced islets reversed hyperglycemia in only 48% of the diabetic mice, whereas the grafts containing rAd-GLP-1-transduced islets reversed hyperglycemia in 78% of the recipients. Based on the Kaplan–Meier analysis, the trend in the reversal of hyperglycemia by islet grafts differed between groups ($P < 0.05$).

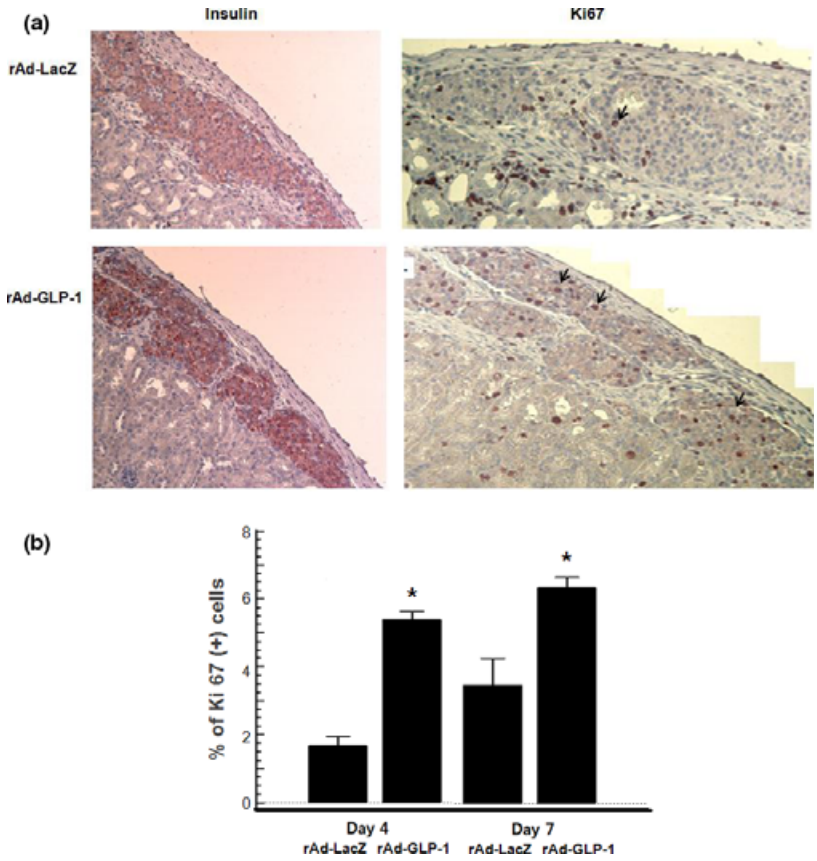

Figure 4 Insulin and Ki67 staining of day 4 or day 7 islet grafts. (a) In the day 7 grafts, Ki67-positive cells were scarce in the rAd-LacZ-transduced group, while the number of Ki67-positive cells increased in the rAd-GLP-1-transduced group. (b) The fraction of Ki67-positive cells in the islet grafts at day 4 or 7, obtained using a direct point-counting method. $*P < 0.05$ vs. rAd-LacZ-transduced islet grafts.

Discussion

The majority of GLP-1 gene therapy studies for treatment of diabetes have targeted tissues such as muscle and liver after systemic administration of GLP-1 transgene [37]. Using *ex vivo* strategy, Wideman *et al.* have shown that the expression of prohormone convertase (PC) 1/3 in α-cells increased GLP-1 secretion and improved transplantation outcome in a mouse model [32]. In this study, we evaluated the effect of the local GLP-1 production directly within mouse islets via *ex vivo* delivery of GLP-1 transgene on islet survival and function *in vitro* and *in vivo*. We used a recombinant adenovirus vector, which has relatively high transduction efficiency in islet cells. The rAd-GLP-1 contained the CMV promoter/enhancer, and albumin leader sequence, to facilitate secretion, followed by GLP-1 cDNA [33]. First, we demonstrated that the rAd-GLP-1 construct efficiently secreted GLP-1 *in vitro* in mouse islets. Next, we observed that islets transduced with rAd-GLP-1 were protected from H_2O_2-induced cell damage *in vitro*, confirming the anti-apoptotic action of local GLP-1 expression. In addition, glucose-stimulated insulin secretion was significantly higher in rAd-GLP-1-transduced islets than in rAd-LacZ-transduced or nontransduced islets. This insulinotropic effect of local GLP-1 expression in rAd-GLP-1-treated islets might be due to its autocrine or paracrine effect on β-cells.

When we assessed that the local production of GLP-1 in islets benefits islet cell survival in early islet grafts, the islet grafts retrieved 4 or 7 days after transplantation revealed that the rAd-GLP-1-transduced islets contained significantly more Ki67-positive cells than the rAd-LacZ-transduced group. These results suggest that the local GLP-1 secreted by the islet grafts was sufficient for GLP-1 to act as a growth factor for β-cells, leading to expansion of the β-cell mass via the proliferation. This finding is in concord with a previous study which showed that expression of GLP-1 in mouse β-cells *in vivo* via intraperitoneal injection of adeno-associated virus vector containing a GLP-1 transgene driven by the mouse insulin-II promoter significantly increased β-cell proliferation [38].

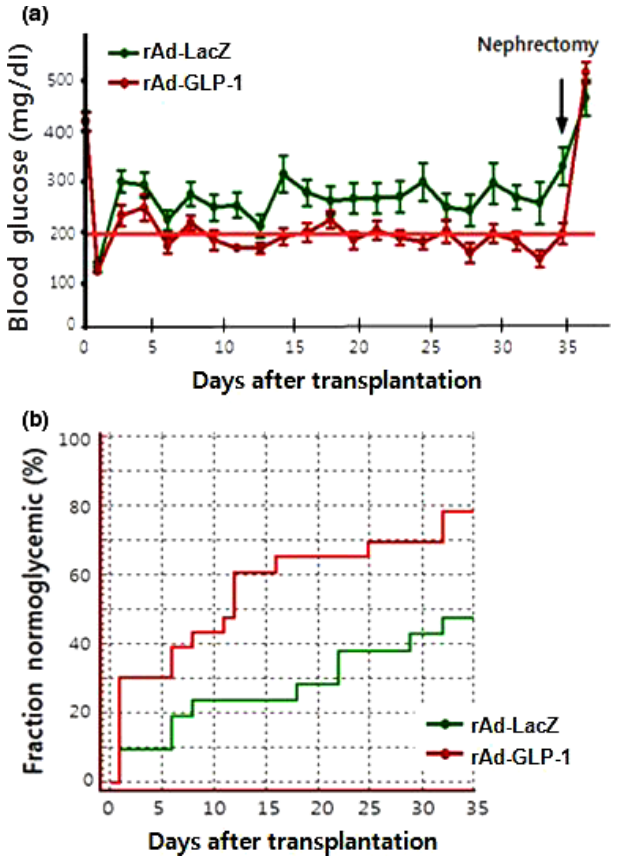

Figure 5 Blood glucose control with 180 IEQ grafts. Red denotes the rAd-GLP-1-transduced group (n = 23). Green denotes rAd-LacZ transduced group (n = 21). (a) Blood glucose levels of diabetic mice transplanted with rAd-GLP-1 transduced or rAd-LacZ transduced islets for 35 days post-transplant. (b) The fraction of normoglycemic mice at different time points for 35 days post-transplant is shown. The groups differed in the Kaplan–Meier analysis ($P < 0.05$).

Next, we examined whether GLP-1 expression not only benefits islet cell proliferation in early grafts, but also improves islet graft survival and function over time by transplanting a marginal mass of rAd-GLP-1-transduced islets into diabetic syngeneic mice. Diabetic mice transplanted with rAd-GLP-1-transduced islets became normoglycemic more rapidly than mice transplanted with rAd-LacZ-transduced islets. Although we did not measure circulating GLP-1 levels, it was shown that the GLP-1 expression in mouse islet β-cells of whole pancreas by systemic gene delivery did not change circulating GLP-1 levels [38]. This suggests that intra-islet graft production of GLP-1 can be a localized process while minimizing systemic delivery, if any. As GLP-1 has strong insulinotropic action in addition to stimulating β-cell proliferation, the achievement of rapid normoglycemia with rAd-GLP-1-transduced islet grafts may be due to both of these effects. However, the improved islet graft function continued for 5 weeks after transplantation in rAd-GLP-1-transduced islet grafts with significantly higher hyperglycemia reversal rates as compared with the rAd-LacZ-transduced islet grafts (78% vs. 48%). As adenovirus vectors typically have a short duration of transgene expression [39], the GLP-1 secretion in the rAd-GLP-1-transduced islet grafts is assumed to decline in less than a couple of weeks. Therefore, the cause of the better graft function observed with rAd-GLP-1-transduced islets over time might be that rAd-GLP-1 delivery favors islet survival through increased proliferation and possibly decreased apoptosis during the early vulnerable post-transplant period, which may lead to sustained β-cell mass and function. In an islet transplantation setting, the short duration of GLP-1 expression may be merited by preventing the over-regeneration of β-cells or possible tumorigenesis. In addition, local and transient production of GLP-1 directly within the islet could minimize systemic delivery and thereby unwanted side effects, such as vomiting and nausea; type 1 diabetic patients with functional transplanted islets showed more frequent side effects and a significant risk of hypoglycemia with systemic GLP-1/analogs therapy [22,23].

There are several potential uses for GLP-1/analogs in clinical islet transplantation that exploit its insulinotropic, anti-apoptotic, and β-cell growth promoting actions. First, GLP-1/analogs could be used as an alternative to insulin for patients who require additional therapy to optimize glycemic control after transplantation [22–25]. As type 1 diabetic patients with functional transplanted islets showed a significant risk of hypoglycemia with systemic GLP-1/analog therapy in clinical trials, this strategy needs to be optimized and its benefits over insulin therapy should be established through further studies. The blood levels of GLP-1/analogs achieved after peripheral injection might not be sufficient to induce β-cell-preserving effects. Second, GLP-1 may be a useful additive to isolation solutions or culture medium while harvesting islets to maintain cell viability. Recent studies have shown that short-term cultures of both rodent and human islets in the presence of GLP-1/analogs in the culture medium resulted in a lower rate of islet cell apoptosis and higher rate of islet recovery [40,41]. Finally, our data show that the local expression of GLP-1 in islet grafts during the early post-transplant period maintained or enhanced β-cell mass and function over time in a rodent transplantation model. Considering the potential implications of our data on clinical islet transplantation, we are now investigating the effect of GLP-1 plasmid delivery to islets using nonviral vectors, which are safer than adenovirus vector, although they have very low transfection efficiency.

In conclusion, delivery of the GLP-1 gene to islets enhances islet cell survival during the early post-transplant

period, and preserves islet mass and function over time in transplants. These results suggest that local GLP-1 expression in islets may be a useful strategy for *ex vivo* gene therapy in clinical islet transplantation to improve transplant outcomes.

Authorship

HYC: performed research and wrote the paper. JGK: collected and analyzed data. CSK: collected and analyzed data. SJL: performed research. ML: performed research. DK: contributed important reagents and performed research. HSJ: contributed important reagents and performed research. SHI: designed research, performed research and wrote the paper.

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