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Research Paper

Protection of rat islet viability following heme oxygenase-1 gene transfection via adenoviral vector *in vitro*☆

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Abstract

Objective: To investigate the effect of Heme oxygenase-1 (HO-1) gene transfection on the viability of cultured rat islets, and to explore the potential value of HO-1 gene in islet transplantation. **Methods**: Recombinant adenovirus vector containing human HO-1 gene (Ad-HO-1) or enhanced green fluorescent protein gene (Ad-EGFP) was generated by using AdEasy system respectively. The rat islets were transfected with Ad-HO-1, Ad-EGFP or blank vector and then cultured for 7 days. Transfection was confirmed by expression of EGFP and human HO-1 protein detected by fluorescence photographs and western blot, respectively. The insulin release upon different concentration of glucose stimulation was detected using insulin radioimmunoassay kit, and stimulation index (SI) was calculated. Glucose-stimulated insulin release was used to assess islet viability. **Results**: Adenovirus vector successfully transferred HO-1 gene to rat islet cells *in vitro*, and the insulin release upon high level of glucose stimulation and stimulation index (SI) of Ad-HO-1-infected islets were significantly higher than those of Ad-EGFP-infected islets and control islets ($P < 0.05$). **Conclusion**: Adenovirus-mediated HO-1 gene transfection is a feasible strategy to confer cytoprotection and therefore protect the viability of cultured rat islets.

Keywords: adenovirus vectors; heme oxygenase-1; pancreatic islet; gene transfection

INTRODUCTION

Beta-cell replacement by allotransplantation of pancreatic islets represents a promising approach to cure type 1 diabetes[1]. Many transplant protocols incorporate a period of short-term (24 to 72 hours) islet culture before transplantation to the recipients to be treated with immunodepleting agents[2, 3], and to provide time for *in vitro* assessment of islet quality. Short-term islet culture indeed has some benefits, such as purification of islet preparation, immunomodulation[4], and possible improvemot of allograft survival, but cultured islets are known to lose viability and functional responsiveness to glucose stimulation with the extension of culturing time[5]. Keeping the cultured islets viable is a key point for successful islet transplantation. An attractive strategy for preventing the loss of islets is to use gene therapy to transduce islets with cytoprotective gene that can make islets more resistant to injury.

Heme oxygenase-1 (HO-1) is the rate-limiting enzyme in heme degradative pathway that catalyzes oxidation of heme into biliverdin, carbon monoxide (CO), and free iron[6], and it has been described as a ubiquitous inducible stress protein capable of cytoprotection via radical scavenging and apoptosis prevention. As HO-1 is inducible, not expressed constitutively, gene transfer can provide effective, targeted, and relatively persistent expression of HO-1. The aim of this study was to investigate the effects of HO-1 gene transfection mediated by recombinant adenovirus on the viability of cultured rat islet and explore the potential value of HO-1 gene in islet

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transplantation.

MATERIALS AND METHODS

Animals

Ten male SD rats weighing 250-300g were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Sciences.

Main reagents

Collagenase P(Roche, USA), Newborn calf serum (Gibco, USA), RPMI-1640 medium (Gibco, USA), fetal calf serum(Gibco, USA),Ficoll400(Pharmacia, Sweden), Dithizon (Sigma, USA), Acridine orange (Sigma, USA), Propidium iodide (Sigma, USA), HEPES(Sigma, USA), insulin radioimmunoassay kit (Tianjin Jiuding Biotech Co, Ltd, China), BCA detection kit(Pierce Biotechnology, USA), murine antihuman HO-1 monoclonal antibody(Stressgen, USA), AP-conjugated rabbit antimice polyclonal antibody(Promega, USA), and NBT+BCIP staining kit (Tianjin Haoyang Biological Manufacture CO, LTD, China) were prepared.

Islet isolation, culture and identification

The SD rats were anaesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) and the abdominal cavity was cut open. The main bile duct was located and clamped at both ends. Ten milliliters of collagenase P solution(1 mg/ml, pH7.8) was injected into the duct and then the distended pancreas was surgically resected, and incubated at 38℃ for 15 min. The digested gland was vigorously shaken for 10s and the digestion was stopped by Hanks solution (4℃) with 10% Newborn calf serum (NCS). The tissue was filtered through a 600μm screen, and then washed by Hanks solution twice. Islets were purified by centrifugation at 3 000 rpm for 20 min on discontinuous Ficoll gradients. After several washes with Hanks solution, islets were suspended in RPMI-1640 medium containing 10% fetal calf serum (FCS), 20 mmol/l HEPES, 100 U/ml of penicillin and 100 mg/ml of streptomycin at 37℃ in a humidified atmosphere of 95% air and 5% CO_2. Dithizone (DTZ) is a zinc-chelating agent known to selectively stain pancreatic beta cells because of their high zinc content, so islets purity was assessed by dithizone staining with islets counted and scored for size. An algorithm was used for the calculation of 150μm-diameter islet equivalent number(IEQ).

Determination of living cells

The fluorescent dye, containing 10 μl AO(670 μmol/L) and 1ml PI (750 μmol/L), was used at 1:10 dilution. After islets were washed twice with Hanks solution, fluorescent dye was added to each well. Ten minutes later, islets were analyzed under a fluorescence microscope. Living cells were identified by a green staining (AO), whereas dead cells showed a brown-red staining(PI).

Generation of recombinant adenovirus

Recombinant adenovirus containing human HO-1 gene was generated by using the AdEasy System. The HO-1 gene was cloned into the shuttle vector pAdTrack-CMV, and the resultant plasmid was linearized with Pme I digestion. The linearized plasmid was co-transfected into Escherichia coli BJ5183 cells with an adenoviral backbone plasmid, pAdEasy-1. Recombinants were selected, and recombination was determined by restriction endonuclease analysis. The linearized recombinant plasmid was transfected into HEK-293 cells, and high-titer viral stocks were prepared. Viral titers were determined by plaque assay and expressed as plaque forming units per ml (pfu/ml). Ad-EGFP was generated using the same system and supplied by Institute of Genetics of Fudan University. Viral titers of Ad-hHO-1 and Ad-EGFP were 1.96×10^9 and 1.99×10^9 pfu/ml, respectively.

Gene transfer to islets

Islets were incubated in serum-free culture medium containing Ad-HO-1 and Ad-EGFP vectors at the multiplicities of infection(MOI) of 20 at 37℃ for 4h with agitation every 1 hour. MOI was calculated on assumption that islets contained 1000 cells. After infection, islets were washed twice with culture medium and incubated for at least 48 h before further analysis to allow for transgene expression. Control islets were mockly infected. Mockly infected islets underwent a similar procedure, but were not exposed to virus during the incubation period and were not incubated with any vectors.

Western blot analysis

Islet cells (48h post-infection) were washed with cold PBS and lysed in 2% SDS, Tris-HCl 60 mmol/L (pH 6.8) buffer, incubated at 95℃, sonicated in a water bath at 37℃ and centrifuged at 12 000 r/m for 15 min. Assessment of the total protein content was carried out with BCA detection kit. Aliquots corresponding to 100 μg of protein were subjected to a 15% SDS-PAGE pre-cast gel electrophoresison and transferred electrophoretically to nitrocellulose membrane. The membranes were incubated with 5% nonfat dry milk in TBS (20 mmol/L Tris, 500 mmol/L NaCl, pH 7.5) overnight at 4°C to block non-specif-

ic binding. Subsequently, the membranes were immunoblotted with 1:200 mice anti-human HO-1 monoclonal antibody followed by incubation with 1:1000 AP-conjugated rabbit anti-mice polyclonal antibody and NBT+BCIP detection.

Glucose stimulation assay

Whole islets were washed with serum-free and glucose-free medium (twice), and incubated in first low(2.8 mmol/l) and then high(16.7 mmol/l) concentration of glucose in culture medium. The static incubation assay was performed in a 24-well flat-bottomed culture plate with 30IEQ/well and 3 duplicate wells for each islet group. Supernatant from each well was collected per hour, and the concentration was measured using insulin radioimmunoassay kit. Stimulation index(SI) was calculated by dividing the insulin respo nse to high glucose incubation by the insulin response to low glucose incubation.

Statistical analysis

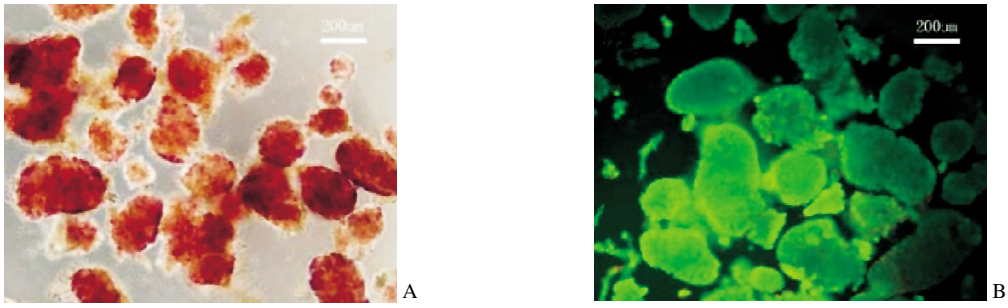
The data were expressed as mean ± SD. Statistical and graphical analysis was performed with software of SPSS. Analysis was performed using two-tailed Student's *t* test where appropriate.

RESULTS

Purity and viability of freshly isolated rat islets

The purity of freshly isolated islets was above 95% calculated from the ratio of dithizone stained cells to dithizone nonstained cells as the percentage of total cell number (***Fig 1A***). As living cells were identified by green fluorescence (AO stained), and dead cells were identified by a brown-red fluorescence (PI stained), the viability of freshly isolated islets was above 95% calculated from the ratio of green stained to red stained cells as the percentage of total cell number(***Fig 1B***).

Transduced gene expression

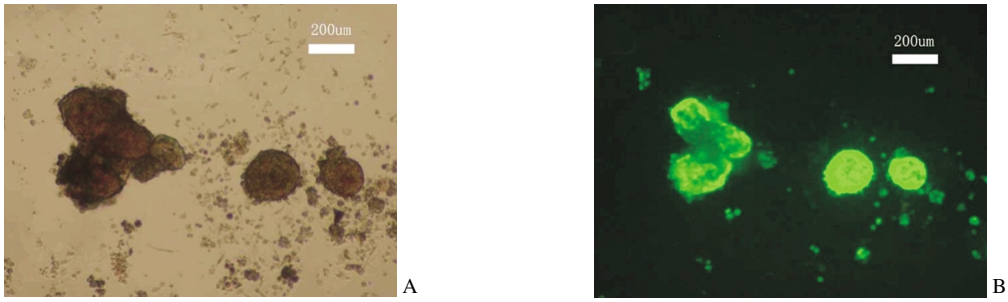


A B

A:islets stained with Dithizon(× 100); B:islets stained with AO/PI(× 100)

Fig 1 Purity and viability of isolated rat islets

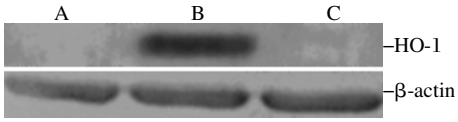
Typical microphotographs of rat islets transfected with Ad-EGFP at MOI of 20 were taken at 48h post-transfection are shown in ***Fig 2***. ***Fig 2A*** showed the Ad-EGFP-infected islets under normal light. ***Fig 2B*** showed the same sample under fluorescence light, and the green fluorescence was intense. ***Fig 3*** showed that human HO-1 protein was detected in the Ad-HO-1-infected islets but not in uninfected or Ad-EGFP-infected islets. Therefore, adenovirus mediated gene transfer was successful.



A B

A:Ad-EGFP-infected islets under normal light (× 100); B:Ad-EGFP-infected islets under fluorescence light(× 100)

Fig 2 Transduced EGFP gene expression

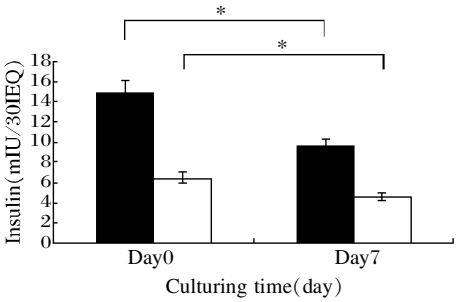


A:control group; B:Ad-HO-1 group; C:Ad-EGFP group

Fig 3 Western blot for human HO-1 protein in transfected islets

Effects of culture on rat islet viability

Glucose-stimulated insulin secretion was used to assess the viability of cultured rat islets. As shown in ***Fig 4***, a significant reduction in glucose-stimulated secretion of insulin by rat islets was observed after the islets were cultured for 7 days. In other word, islet viability decreased conspicuously after 7-day culture.



The insulin release upon different concentrations glucose stimulation decreased conspicuously after 7 days culture. ■, 16.7 mmol/l glucose; □, 2.8mmol/l glucose. $^*P < 0.05$.

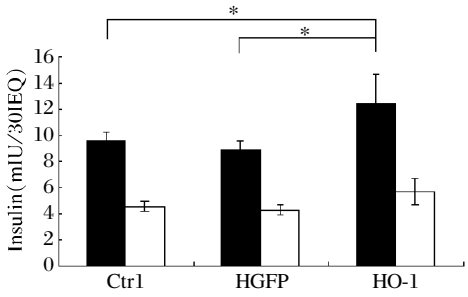
Fig 4 Effect of culture on glucose-stimulated insulin release

Protective effects of HO-1 gene transfer on viability of cultured islet

After 7-day culture, insulin secretions of uninfected(control islets), Ad-EGFP infected, and Ad-HO-1 infected islets were measured in response to low (2.8 mmol/L)and then high (16.7 mmol/L)concentrations of glucose. As shown in ***Fig 5*** and ***6***, the insulin release upon high level glucose stimulation and stimulation index (SI) of Ad-HO-1-infected islets were significantly higher than those of Ad-EGFP-infected islets and control islets ($P < 0.05$).Therefore, HO-1 gene transfer could protect viability of cultured islet.

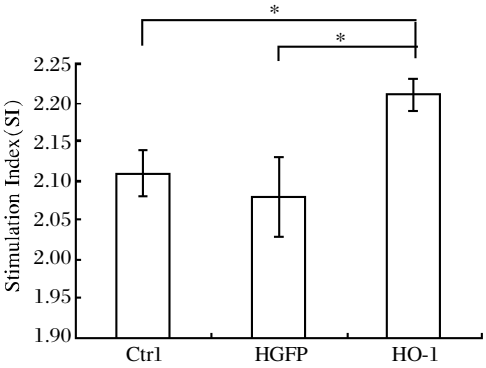
DISCUSSION

Recent a dvances in clinical islet transplantation have allowed patients with type 1 diabetes to become insulin independent [1], but the procedure is limited since islets from two or more donors per recipient are typically required. This limitation is in part due to quality/viability of transplanted islets. Once isolated, non-vascularized pancreatic islets are easily damaged in *in vitro* culture by hypoxia,activation of free radicals, apoptosis and diffusion-limited avail-



The insulin release upon low concentration glucose stimulation wasn't statistically significant different among different group of the cultured islets, but the insulin release upon high concentration glucose stimulation of Ad-HO-1-infected islets was significantly higher than those of Ad-EGFP-infected islets and control islets. ■,16.7mmol/l glucose; □,2.8 mmol/l glucose. $^*P < 0.05$.

Fig 5 Effect of HO-1 gene transfer on insulin release of cultured islets



The SI of Ad-HO-1-infected islets was significantly higher than those of Ad-EGFP-infected islets and control islets. $^*P < 0.05$.

Fig 6 Effect of HO-1 gene transfer on stimulation index(SI) of cultured islets

ability of nutrients, resulting in decreased number and function. Islet losses as high as 30% to 50% have been reported after 48 hours of culture [7]. Therefore, approach towards protecting islets viability would facilitate islet transplantation.

Pancreatic islets, as a cellular graft, are especially suited for gene therapy applications, as they can be infected *ex vivo* and then transplanted with minimal systemic exposure of the recipient to the vector. Therefore, it is feasible to transfer genes of therapeutic utility *ex vivo* to islet cells to protect islet viability.

HO-1 system is thought to exert antioxidant, antiapoptosis, maintenance of microcirculation, and anti-inflammatory function. HO-1 is not expressed constitutively, and it has been demonstrated that overexpression of HO-1 by chemical induction can protect islet cells from apoptosis and improve *in vivo* function after transplantation[8]. Compared with chemical induction, gene transfer can provide effective, targeted, and relatively persistent expression of HO-1.

Adenoviral vectors can efficiently delivery genes to pancreatic islets[9]. In addition, adenovirus vectors can be produced in high titers and there is no risk of insertional mutagenesis as their genomes are not integrated into chromosomes. It has been demon strated that adenoviral vector at a lower transfecting dose (MOI 10 and 100) provided effective transfer of marker genes without inducing significant apoptotic cell death and without impairing cell functions[9]. In our study, islets were infected with recombinant adenovirus vectors at MOI of 20. According to fluorescence photographs taken at 48h post-transfection, the expression of EGFP was intense. Furthermore, human HO-1 protein was detected by western blot in Ad-HO-1- infected islets at the same time. This demonstrated that recombinant adenovirus was efficient to deliver exogenous genes into rat islets *in vitro*, and the exogenous gene expressions were efficient.

Even though many human islet transplant protocols culture islets for only 24 to 72 hours, islets were cultured for 7 days in this study. This culture period was selected to minimize the effects of isolation factors on islet function, while maximizing the effects of culture. After 7-day culture, the insulin release upon either low or high concentration of glucose stimulation was significantly lower than that of freshly isolated islets ($P < 0.05$), this was consistent with previous reports showing that islets degraded during *in vitro* culture. Nevertheless, the insulin release upon high concentration glucose stimulation and stimulation index(SI)of Ad-HO-1-infected islets were significantly higher than those of Ad-EGFP-infected islets and control islets($P < 0.05$). This demonstrated that HO-1 gene transfer conferred protective effects on islet viability during *in vitro* culture. It was probably related to diverse effects of the different end products of heme catabolism. Biliverdin was subsequently reduced into bilirubin, a powerful anti-oxidant, and it could inhibit the generation of reactive oxygen species [10]. CO had been reported to have a cytoprotective role in different systems [11-13], and CO was not only a stimulator of insulin release but was also a trigger of the transients of $[Ca^{2+}]i$ assumed to coordinate the secretory activity of β-cells [14]. Iron induced the synthesis of ferritin reported as having a cytoprotective role in endothelial cells[15].

As glucose-stimulated insulin release is a favorable marker to assess the biological viability of islet, this study reported on the protective effect of HO-1 gene transfection on the viability of cultured rat islets. Strategies aimed at HO-1 gene transfer to islet might result in better outcome in islet transplantation.

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