Diabetologia (1998) 41: 736–739

Diabetologia
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Transduction of non-dividing adult human pancreatic beta cells by an integrating lentiviral vector

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Summary Pancreatic islet cells are terminally differentiated endocrine cells and are refractory to stable infection by retroviral vectors, which require the breakdown of the nuclear membrane during cell division in order to insert the transgene into the host cell genome. Thus, attempts to render beta-cell allografts less immunogenic have had to rely on stable transfection of surrogate cells. Similarly, this problem has precluded the development of conditionally immortalized human beta cells for clinical allotransplantation. In this report, we demonstrate that adult human islet beta cells can be transduced by a new three-plasmid integrating lentiviral vector with an efficiency of $62 \pm 1.8\%$ at a multiplicity of infection (MOI) of 2.5 in vitro. This work makes genetic engineering of adult human pancreatic beta cells possible for the first time, allowing strategies to render beta-cell allografts non-immunogenic to be optimized and to creating conditionally immortalized human beta cells for clinical transplantation. [Diabetalogia (1998) 41: 736–739]

Keywords Lentiviral vector, retrovirus, human islet beta-cell, gene transfer, transplantation.

A major long-term goal of diabetes research is to re-establish normal glucose homeostasis in insulin-deficient patients by transplantation of insulin-producing beta cells [1]. The major unsolved problems of human beta-cell transplantation are overcoming allorejection, preventing recurrent autoimmunity, and expanding the limited supply of human tissue. Various approaches to these problems have shown promise in transgenic mice, but translation to adult human beta cells has been precluded because these terminally differentiated cells are refractory to stable infection by retroviral vectors [2], which require proliferation of the target cells and breakdown of the nuclear membrane during cell division in order to insert the transgene into the host cell genome. Thus, attempts to render beta-cell allografts less immunogenic have had to rely on stable transfection of surrogate cells. Similarly, this problem has precluded the development of conditionally immortalized human beta cells for functional study and clinical allotransplantation [3]

Recently, a new lentiviral vector system has been developed, based on the finding that human immunodeficiency virus (HIV) and other lentiviruses can infect nondividing cells [4]. It has been shown that this new vector system is able to efficiently and stably transfer genes into adult rodent brain and eye in vivo [5, 6]. In this report, we demonstrate the first successful transduction of adult human pancreatic islet beta cells, using lenti green fluorescent protein (GFP) and lenti-E. coli beta galactosidase gene (lacZ) vectors [4, 5].

Received: 26 January 1998 and in revised form: 17 March 1998

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Abbreviations: GFP, Green Fluorescent protein; lacZ, E. coli beta galactosidase gene; FCS, fetal calf serum; PBS, phosphate buffered saline; FITC, fluorescein isothiocyanate conjugate; CMV, cytomegalo virus; CRIP/MFG – cell packaging LINE, retroviral vector packaging cell line; MOI, multiplicity of infection.

Materials and methods

Preparation of human pancreatic islets. Human adult pancreatic islets were prepared at the Islet Transplantation Facility at the University of Giessen, Germany. Pancreata were obtained

within 8 h of death and islets were prepared as described previously [7]. In brief, following hypothermic perfusion via the abdominal aorta, organs were dissected in situ. After collagenase digestion, pancreata were digested in a continuous digestion-filtration device. Free islets were separated from nonislet tissue using a continuous Ficoll-Na-diatrizoate density gradient on a Cobe 2991 [7]. Islet purity of the preparations generally exceeded 80 %. Single islets were suspended in Connaught Medical Research Laboratory (CMRL)-1066 medium containing 10 % fetal calf serum (FCS), 1 % glutamine, 1 % N-2-hydroxyethyl piperagine N'-2 ethane sulphonic acid (HEPES), 1 % Pen/Strep, and incubated at 25 °C. The media were changed every week. The islet preparation was examined visually for bacterial or fungal contamination. Dithizone (0.02 % for 3 min) was used to detect contamination by non-endocrine cells (data not shown).

Preparation of human islet cells. To prepare single islet cells, approximately 5000 islets were digested in 8 ml of calcium-free phosphate buffered saline (PBS) containing 0.125 mg/ml of trypsin and 0.05 mg/ml ethylenediaminetetraacetic acid (EDTA) at 37 °C. The islet solution was rotated for 5 min at 37 °C and then placed on ice for 5 min to allow islets to settle. The supernatant containing the single islet cells was removed, 1 ml of FCS was added and the solution centrifuged in Connaught Medical Research Laboratory media for counting. The digestion cycle was repeated a maximum of four times to obtain additional cells.

Characterization of adult human islet cells. The purity of the isolated human islet cells was analysed by immunofluorescent staining using a guinea pig anti-porcine insulin antibody which is cross reactive against human insulin (Chemicon International, Temecula, Calif., USA). 10^5 cells per well were plated in 24 well plates the day before immunofluorescent staining. The cells were washed briefly with PBS, fixed in cold 4 % paraformaldehyde for 15 min, washed with PBS, and blocked with 10 % FCS/10 % goat serum/0.2 % triton in PBS for 30 min. Primary antibody, guinea pig anti-porcine insulin, (diluted 1:100) was added and the plate was incubated for 1 h. After washing with PBS, second antibody, fluroescein isothiocyanate conjugate (FITC) conjugated donkey anti-guinea pig IgG (diluted 1:100) was added and the plate was incubated for 1 h in the dark. After washing with PBS, the plate was examined using fluorescence microscopy.

Preparation of lentivirus. 293T cells were plated in 100 mm cell culture plates in Dulbecco's modified Eagle's medium (DMEM) containing 10 % FCS, 1 % Pen/Strep, and grown to 70 % confluency. A total of 40 µg of plasmid DNA was used for the transfection in the following proportions using Perfect Lipids no. 2 (Invitrogen Carlsbad Calif USA) according to the manufacturer's instructions: on to megato virus cmv abbreviation in 15 µg of pCMVΔR8.2, 20 µg of pHR'CMV-GFP or pHR'CMV-LacZ and 5 µg of pMD.G as described [4, 5]. These plasmids are gifts of Dr. D. Trono at the University of Geneva, Switzerland. Conditioned medium containing lenti-GFP or lenti-lacZ viruses were harvested 48 to 60 h after transfection, subjected to low-speed centrifugation, and filtered through 0.45 µmol/l filters. Viruses were further concentrated by ultracentrafugation as described. The viral preparation were frozen at –80 °C. For lenti-GFP, the 293T cells left on the plate were examined under fluorescence microscopy for percentage of green cells. For lenti-LacZ, the 293T cells left on the plate were fixed with 1 % glutaraldehyde and stained with X-gal (see below). More than 80 % of the cells turned green (lenti-GFP) or blue (lenti-LacZ) indicating that the cotransfection of the three plasmids was successful. To titrate the viral particles in the conditioned medium, 5×10^5 of 293T cells per well were infected overnight at 37 °C in six-well plates with serial dilutions of conditioned medium supplemented with polybrene (8 µg/ml]. The medium was replaced, the cells further incubated for 36 h, and expression of GFP or β-galactosidase scored by fluorescence microscopy or X-gal staining. Titres were calculated by counting the number of the foci of green or blue cells per well and dividing the number by the dilution factor.

Preparation of retrovirus. Retroviral producer cells, retroviral vector packaging cell line-ecoli beta galactosidase gene (CRIP/MFG-LacZ), were from Dr. R.C. Mulligan. CRIP/MFG-LacZ cells were grown to confluency as described and the supernatants containing MFG-LacZ viral particles were centrifuged at low speed, filtered through a 0.45 µmol/l filter and frozen at –80 °C. 293T cells were used to titre each preparation as described above.

Infection of human islet cells with lentiviral and retroviral particles · 10^6 single adult human islet cells were plated in 60 mm polylysine-coated plates 2 days before infection. Viruses were added to the CMRL-1066 medium supplemented with polybrene (8 µg/ml) at different multiplicity of infection (MOIs) and the cells were infected overnight. The media was replaced and the cells further incubated at 37 °C. Expression of the GFP reporter gene was examined 5 days after infection. Cells were washed with PBS, fixed with 4 % paraformaldehyde for 15 min at room temperature and washed again with PBS. Fixed cells were fluorescence-immunostained using guinea pig anti-porcine insulin antibody (Chemicon International, Temecula, Calif., USA) as the first antibody and Rhodamine-Red conjugated donkey anti-guinea pig IgG (Jackson Immuno Research West Grove PA USA) as the second antibody. Uninfected islet cells in separate plates were fixed and stained under the same conditions, and used as negative controls. Green cells and red cells of the same field were photographed using a fluorescence microscope. Two images (green and red) were scanned using Adobe Photoship Programme (Apple Computer B cuper + INw, Calif USA) and were then matched to give the double exposure image. Expression of the β-galactosidase was scored by X-Gal staining. The cells were washed twice with PBS, fixed with 1 % glutaraldehyde at room temperature for 5 min, washed again with PBS and stained with X-gal/Fe solution for 4 h at 37 °C. Uninfected islet cells in separate plates were fixed and stained under the same conditions, and used as negative controls. To avoid background of the endogenous β-galactosidase of islet β-cells, X-gal staining was done for 4 h.

Quantitation of infection efficiency and statistical methods. Data of infection efficiency were analysed using the descriptive statistics analysis software of Microsoft Excel.

Results

Pancreatic islets were prepared from human pancreas using Ricordi's automated method [7]. Since pancreatic islets contain four major cell types, alpha, beta, delta and pancreatic polypeptide, the purity of islet preparations was determined before viral infection. Single cells were prepared from human islets and plated onto cell culture dishes. An immunofluorescence assay using antibody against porcine insulin was used

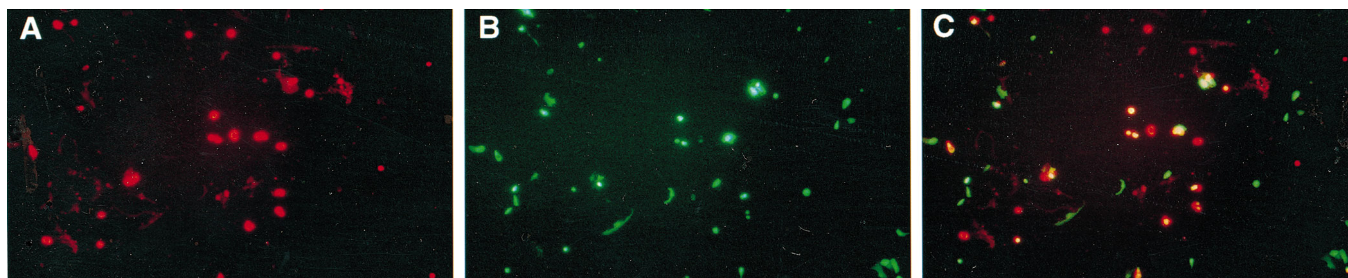

Fig. 1. Single human islet cells plated in polylysine-coated plates were infected with lenti-GFP viruses at a MOI of 2.5 in the CMRL-1066 medium supplemented with polybrene overnight. Five days after infection, cells were washed with PBS, fixed with 4% paraformaldehyde and immunostained using guinea pig anti-porcine insulin antibody and Rhodamine-Red conjugated donkey anti-guinea pig IgG. Red cells (A) and green cells (B) of the same field were photographed using a fluorescence microscope (100x). Two images (green and red) were scanned using Adobe Photoship Program and were then matched to give the double exposure image (C). **A**, human islet β-cells (red) positive to insulin antibody. **B**, human islet cells (green) infected by lenti-GFP vectors. **C**, human islet β-cells infected by lenti-GFP vectors (orange)

to identify the major cell type of these single cells. After immunostaining, the number of total cells and the number of insulin-producing cells were counted under the fluorescence microscope. 864 cells in 11 groups were counted and the average percentage of insulin-producing beta-cells was obtained. The results indicated that 82 ± 2.5% of the single cells prepared from human islets were insulin-producing beta-cells.

The transducing capacity of the lentiviral preparation in human islet beta-cells was first assayed quantitatively using lenti-GFP virus [5]. Single islet cells were prepared from human islets and plated in polylysine-coated 6 or 12-well plates and infected overnight with lenti-GFP viral particles at a MOI of 2.5. The infected human islet cells were examined under the fluorescence microscope every day. Green cells started to appear 2 days after infection. Five days after infection, the number of green cells reached its maximum. 909 cells in seven groups were counted and the average percentage of green (infected) islet cells was 68 ± 2.6% (data not shown). In order to quantitate the infection efficiency of beta-cells, cells were fixed with 4% paraformaldehyde and analysed by immunofluorescent assay using a guinea pig anti-porcine insulin antibody which is cross reactive against human insulin. Rhodamine-Red conjugated donkey anti-guinea pig IgG was used as the second antibody. The plate was examined using fluorescence microscopy for red (beta-cell) and green (lenti-GFP infected) cells, and photographed. If one cell was both red and green, it was a lenti-GFP infected beta-cell. 360 cells were counted in six separate fields. As illustrated in Figure 1, 62 ± 1.8% of the beta-cells (red cells in Fig. A) were transduced by lenti-GFP viral particles and expressed green fluorescent protein (green cells in Fig. B). Double exposure rendered lenti-GFP infected beta-cells orange and clearly distinguishable from the uninfected beta-cells (Fig. 1C). Since integration is a necessary step for the expression of transgenes delivered by both retroviral and lentiviral vectors [4], these results indicated that the lentiviral particles could deliver and integrate the GFP reporter gene into the genome of adult human beta-cells efficiently.

Although beta-cells are differentiated endocrine cells and do not proliferate in culture, a recent study on beta-cell regeneration suggested that differentiated beta-cells still possess the potential to replicate and divide under certain conditions [8]. To exclude the possibility that those infected beta-cells were dividing at the time of infection, human islet cells were then infected with a retroviral vector which only transduces dividing cells and the results were compared with that of the lentiviral vector which transduces both dividing and non-dividing cells. MFG-LacZ viral particles were prepared from retroviral producer cells, CRIP/MFG-LacZ (a gift from Dr. R.C. Mulligan). 293T cells were used as a control of dividing cells in this experiment. 293T cells and single human islet cells were infected with lentiviruses and retroviruses separately with the same MOI of 2.5. 293T cells were stained with X-gal 3 days after infection and the human islet cells were stained after 5 days. Uninfected cells were stained as negative controls. Figure 2 shows that lentivirus can transduce both 293T cells (dividing cells) and isolated human islet cells (non-dividing cells) and retrovirus can only transduce dividing 293T cells. The results clearly indicate that the human islet beta-cells infected by lentiviruses were non-dividing cells.

Discussion

Our long-term goal is to establish conditionally immortalized, non-immunogenic human islet beta-cell lines that can ultimately be used for transplantation therapy of diabetes mellitus. As an initial step towards this goal, we have shown that a lentiviral expression system successfully delivered the GFP and LacZ reporter genes into single non-dividing human

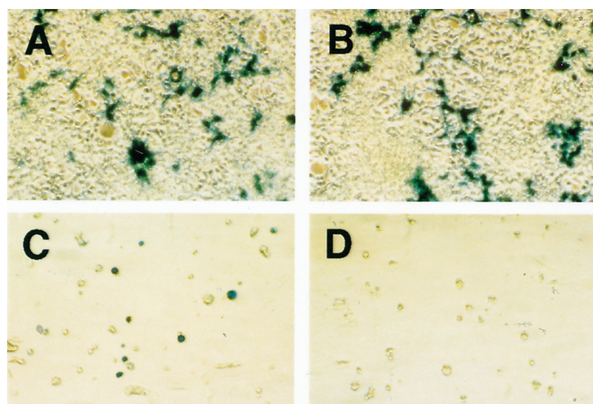

Fig. 2. Viruses were prepared and titrated. Single human islet cells (C,D) and 293T cells (A,B) plated in polylysine-coated 6-well plate were infected overnight with viruses (A and C for lenti-lacZ, B and D for retro-lacZ) at MOI of 2.5 in CMRL-1066 medium supplemented with polybrene (8 μg/ml). The medium was replaced, the cells further incubated, and expression of β-galactosidase scored by X-Gal staining 5 days after infection. Cells were photographed with a research microscope under phase-contrast. Different groups of cells are shown in each photograph (100 ×). A, 293T cells infected with lentiviral-lacZ. B, 293T cells infected with retroviral-lacZ. C, human islet cells infected with lentiviral-lacZ. D, human islet cells infected with retroviral-lacZ

islet beta-cells in vitro. Since it has been well established in retrovirus that integration is an obligatory step for retroviral gene expression [4], our results also demonstrate that the delivered reporter genes were integrated into the genome of adult human islet beta-cells.

This is the first time that adult human islet beta cells have been successfully transduced with an integrating vector. Fetal human islet cells have been successfully transduced with retroviral vectors, since these immature islet cells are still proliferating [9]. However, the incomplete differentiation and extremely limited availability of these cells make their use impractical for generating sufficient quantities of genetically engineered beta cells for ultimate clinical application.

The integrating three-plasmid lentiviral system shown in this report to transduce adult human beta cells has been shown previously to transfer genes into adult rodent eye and brain in vivo [6,10]. However, since genes deleted from lentiviral vectors may be superfluous for nuclear transport of the preintegration complex in some cell types, but essential in others, the ability of such vectors to transduce a given cell type must be empirically determined for each cell type. Similarly, the duration of expression of such vectors must be individually assessed in each tissue type. The expression of the lentivirus vector has been detected in neurons for up to 6 months [5]. This suggests that unlike retroviral vectors whose expression is silenced due to integration into a region of the host genome where transcription is downregulated [11], integrating lentiviral vectors may overcome the problem of gene silencing by integrating into stably open chromatin. The duration of transgene expression in adult human beta cells transduced with this vector remains to be determined and cannot be assumed a priori.

The two major obstacles to using adult human beta cells as transplantation therapy for diabetes mellitus are (a) the need to avoid the use of toxic immunosuppressive drugs to prevent allograft rejection (and in Type 1 patients, autoimmune disease recurrence), and (b) the need to provide much larger amounts of donor human tissue than are currently available from cadaveric sources [3]. The data obtained in this study demonstrate that adult human beta cells can now be successfully transduced with integrating lentiviral vectors, making it possible for the first time to optimize genetic engineering strategies to render beta-cell allografts non-immunogenic and to create stable, conditionally immortalized beta-cell lines for eventual clinical application.

Acknowledgements. We would like to thank Dr.Didier Trono at the University of Geneva, Switzerland for kindly providing the three plasmid lentiviral expression systems. This work was supported in part by Interdisciplinary Program Project grant no. 892001 from the Juvenile Diabetes Foundation International.

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