MAINTENANCE OF GLUCOSE-SENSITIVE INSULIN SECRETION OF CRYOPRESERVED HUMAN ISLETS WITH UW SOULUTION AND ASCORBIC ACID 2 GLUCOSIDE

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FOOTNOTES

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Abbreviations: UW solutuion; University of Wisconsin solution, AA2G; ascorbic acid-2 glucoside, FBS; fetal bovine serum

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ABSTRACT

Background: Normal human islet cells are an ideal source for pancreas-targeted cell therapies, but availability of human donor pancreas for islet isolation is severely limited. To effectively utilize such scared donor organs for cell therapies, it is crucial to develop an excellent isolation, effective cryopreservation, and efficient gene transfer techniques for transportation of isolated cells. In the present study, we investigated the effect of University of Wisconsin (UW) solution and ascorbic acid-2 glucoside (AA2G) on cryopreservation of human islets. We also evaluated gene transfer efficiency of a lentiviral vector expressing E. coli LacZ gene, Lt-NLS/LacZ, in human islets.

Materials and Methods: Human islets were isolated with a standard digestion method at the University of Alberta. Isolated islets were transported to Japan for 40 hours and then subjected to cryopreservation experiments. The following preservation solutions were tested: 1) UW solution with 100 μg/ml of AA2G, 2) UW solution, 3) 100% fetal bovine serum (FBS), and 4) CMRL supplemented with 10% FBS. After 3 month-cryopreservation, islets were thawed and analyzed for viability, glucose-sensitive insulin secretion, proinsulin gene expression profile, and in vivo engraftment. The islets were also subjected to monolayer formation with 804G cell line-derived extracellular matrix (ECM), followed by Lt-NLS/LacZ transduction.

Results: Viability, morphology, glucose-sensitive insulin secretion, proinsulin gene expression, and monolayer formation efficiency of thawed cryopreserved islets were significantly better maintained by the use of UW solution. When AA2G (100 μg/ml) was combined with UW, such parameters were further improved. Adequate engraftment of UW+AA2G-cryopreserved human islets was achieved in the liver of nude mice. Efficient Lt-NLS/LacZ transduction was identified in monolayered islets

cryopreserved with UW solution with AA2G.

Conclusions: The present work demonstrated that combination of UW solution with AA2G (100 μg/ml) would be a useful cryopreservation means for human islets. Human islets monolayer-cultured with 804G-derived ECM were efficiently transduced with a lentiviral vector Lt-NLS/LacZ.

INTRODUCTION

The use of normal human islets in pancreas-targeted cell therapies is severely limited by worldwide shortage of donor pancreata. Considering the present status, it is of essence to efficiently utilize such scare donated pancreata for cell therapies. What remains to be seen for the resolution is the following: 1) establishment of an excellent isolation procedure for functional and uniform islet preparation, 2) development of an effective cryopreservation solution, and 3) establishment of a useful gene transfer technique for isolated and/or cultured islets. Therefore, in the present study we investigated an efficient cryopreservation solution and gene delivery tool. Development of an effective cryopreservation solution and gene transfer technique allows transportation and manipulation of primarily isolated islets between one center where islet isolation and/or culture can be performed and the other where such cells are actually utilized. University of Wisconsin (UW) solution[1, 2] was developed as an organ preservation solution for transplantation and we previously reported the usefulness of UW in a canine experiment of pancreatic islet cryopreservation.[3] AA2G is a potent antioxidant, and which is capable of supplying stable form of vitamin C in the culture medium and cryopreservation solution. Thus, we focused on combination of UW) solution and ascorbic acid-2 glucoside (AA2G) as a cryopreservation solution for human islets.[4- 9]

MATERIALS AND METHODS

Isolation of human islets. All the experiments performed here were approved by the Institutional Ethical Committee and were thus under the guideline. Human islets were isolated using defined protocols of enzymatic dissociation and purification using discontinuous gradients of Ficoll using a refrigerated Cobe 2991 machine at the Human Islet Isolation Laboratory of the University of Alberta. Isolated islets were shipped to Japan and then subjected to cryopreservation experiments. Purity and viability were routinely assessed by Dethizone stain and Acridine Orange (AO)/ Propidium Iodide (PI) assay, respectively, as previously reported [10].

Cryopreservation of primary isolated human islets. The transported human islets were carefully examined with stereomicroscope Stemi DV4 (Zeiss Co., Germany) to select viable islets demonstrating spherical shape and to exclude exocrine tissues and the resulting healthy islets were subjected to cryopreservation experiments (Figure 1). The following four preservation solutions were used: 1) UW solution (kindly provided by ViaSpan and Fujisawa Pharmaceutical Co., Ltd.) containing 100 μg/ml of ascorbic acid-2 glucoside (AA2G) (UW solution + AA2G) (Group A, n=5), 2) UW solution only (Group B, n=5), 3) 100% fetal bovine serum (FBS) (Sigma, St. Louis, MI) (Group C, n=5), and 4) CMRL1066 (Invitrogen, Carsbad, CA) supplemented with 10% FBS (Sigma) (Group D, n=5). Dimethylsulfoxide (DMSO) (Sigma) was added to each Group to be a final concentration of 10%. The cells were suspended in each cryopreservation solution with the final concentration of $1x10^3$ islets/ml, program-frozen to - 80 ^oC using a programmed freezing box (Stratagene, LaJolla, CA) and then cryopreserved in a nitrogen liquid tank for 3 months.

Viability of human islets after cryopreservation. Cell viabilities after cryopreservation were assessed by an AO/PI assay, as previously reported [10]. Thawed human islets after cryopreservation were suspended with CRMI medium supplemented with 10% FBS and seeded on 6-well plates at the density of 100 islets/well. The PI reagent was added to the cultures and visualized under UV light to evaluate the viability as ratios of red fluorescence-expressing dead cells to green fluorescence-expressing viable cells in the islets were compared among the Groups.

Measurement of glucose-sensitive insulin secretion. Using a static incubation method for assessing glucose-sensitive insulin secreting potential, as referred to stimulation index, the insulin levels of the culture medium was measured in thawed cryopreserved human islets for 3 months, as previously reported [10]. Thawed human islets after cryopreservation were seeded on 6-well plates at the density of 10 islets/well and maintained with RPMI medium supplemented with 10% FBS over night. Briefly, the culture medium was replaced with low glucose (50 mg/dl, 2.8 mmol) -containing RPMI medium (Invitrogen, Carsbad, CA) with bovine serum albumin (BSA) (Sigma) and HEPES (Sigma) for 1 hour, then with high glucose (450 mg/dl, 25 mmol) -containing RPMI for 1 hour, then changed to RPMI with low glucose (50 mg/dl) again. Each culture medium was collected for insulin measurement. Insulin detection analysis was conducted by Okayama Medical Laboratory (Kurashiki, Japan) according to the manufacture's protocol.

The gene expression of insulin in thawed cryopreserved human islets. To evaluate the gene expression of insulin in thawed human islets after 3-month cryopreservation, total RNAs were isolated from the islets cultured with low glucose-containing RPMI medium (50 mg/dl) for 1 hour and following 1-hour culture with high glucose (450

mg/dl) -containing RPMI using RNAzol (Cinna/BioTecx, Friendswood, TX) according to a manufacture's protocol. Reverse transcription was performed at 22°C for 10 min and then at 42°C for 20 min using 1.0 μg of RNA per reaction to ensure that the amount of amplified DNAs was proportional to those of specific mRNAs in the original samples. PCR was performed with specific primers in volumes of 50 μl containing 2.0 μg RT products under the manufacturer's guideline (PCR kit; Perkin-Elmer/Cetus, Norwalk, CT). The amplification reaction involved denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and at 72°C for 1 min using a thermal cycler (Perkin-Elmer, Foster City, CA). The PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining. The human GAPHD served as an internal control for the efficiency of mRNA isolation and cDNA synthesis. T Primers used were as follows: proinsulin (368 base pair (bp)), sense, 5'-GCCATCAAGCAGATCACTGTCC- 3', antisense, 5'-GGCTGCGTCTAGTTGCAGTAGTTC - 3', GAPDH (459 bp), sense, 5'-CAGCCGAGCCACATC-3', antisense, 5'- TGAGGCTGTTGTCATACTTCT-3'.

Monolayer-formation efficiency of thawed human islets. After cryopreservation, morphology and efficiency of monolayer formation of thawed human islets in each preservation solution were evaluated. To facilitate monolayer culture, thawed human islets were seeded on 6-well plates coated with an extracellular matrix (ECM) prepared from rat bladder 804G cells[11] (kindly provided by Dr. R. Oyasu, Northwestern University School of Medicine, Chicago, Illinois) at the density of 10 islets/well and cultured with the CMRL medium (Sigma) supplemented with 10% FBS, $1x10^{-7}$mol/L of insulin (Gibco BRL), 25 μg/L of EGF (Sigma), $1x10^{-6}$mol/L of dexamethasone (Sigma), penicillin $1x10^{5}$ U/L, and streptomycin $1x10^{5}$μg/L under 5% CO_2 at 37 °C. Cell morphology was carefully examined using an inverted phase

contrast microscope.

Lentiviral gene transfer in thawed human islets in monolayer cultures. Efficacy of lentivirus-mediated gene transfer was evaluated in cryopreserved human islets. A vesicular stomatitis virus G-protein (VSV-G)-pseudotyped lentiviral vector, LtV-NLS/LacZ (kindly provided by Dr. P. Leboulch, Harvard-Massachusetts Institute of Technology, Cambridge, MA, USA), encoding E. coli. LacZ gene tagged with nuclear localizing signal (NLS) was produced by three plasmids HPV 289, HPV 274 and YN15 and used for transduction experiment, as previously reported[12]. The thawed human islets cryopreserved with UW solution and AA2G (G1) were inoculated in 804G-derived ECM-coated dishes. When cell cultures developed monolayer formation, cells were subjected to transduction of LtV-NLS/LacZ for 12 hours, maintained with fresh medium for another 36 hours, and then stained with X-gal solution to detect NLS/LacZ activity, as previously documented[12].

Transplantation of thawed cryopreserved human islets in athymic nude mice. All procedures performed on animals were approved by the Okayama University Institutional Animal Care and Use Committee and were thus within the guidelines for humane care of laboratory animals. To evaluate an engraftment of thawed human islets cryopreserved for 3 months using UW and AA2G, islets were intraportally transplanted into the liver of 3 different male balb/c nude mice (KLEA Japan Inc., Tokyo, Japan), as previously reported[13]. Immediately after transplantation, animals underwent a daily intramuscular administration of FK506 (0.5mg/kg) (kindly provided by Fujisawa Pharmaceuticals Inc. Osaka, Japan). The animals were killed on day 3 after transplantation for histological examination and samples obtained were

fixed with 10 % formalin for Hematoxylin and Eosin (H.E) stain. The samples were also treated with 5% paraformaldehyde and embedded with Tissue-OCT compound for immunohistochemical examination for insulin stain. The liver specimen was treated with guinea pig polyclonal IgG antibody against insulin (DakoCytomation Co., Tokyo, Japan) under the manufacture's protocol.

Statistical analysis. Student's *t* test was applied to evaluate the significant difference among 4 groups. $p < 0.05$ was considered to be significant.

RESULTS

UW solution allowed almost normal morphology of cryopreserved islets. Three months after cryopreservation, human islets were thawed and maintained with CMRL medium. Morphology of thawed/cryopreserved islets in Group 1 and Group 2 was spherical (Figure 2- A, B). On the other hand, cryopreserved G3 and G4 islets were severely damaged and failed to maintain spherical shape (Figure 2- C, D).

UW solution maintained favorable viability of cryopreserved human islets. Mean viabilities of cryopreserved human islets assessed by an AO/PI assay were 68.3 % for UW solution+ AA2G (Group A, n = 5), 67.5 % for UW solution (Group B, n = 5), 43.2 % for FCS (Group C, n = 5), and 46.4 % for CMRL (Group D, n = 5) (Figure 3). The islet viability was maintained significantly better in UW solution-adopted Group A and Group B. Viability of UW+ AA2G- preserved islets was most favorable, which was compatible with that of islets after transportation (70 %), without significant difference with UW alone.

UW solution maintained favorable stimulation index. Glucose-sensitive insulin secretion, as expressed stimulation index, was measured to evaluate endocrine function of cryopreserved human islets. The stimulation index was 1.93± 0.5 for Group A, 1.17±0.6 for Group B, 0.74±0.27 for Group C, and 0.52±0.18 for Group D (p= 0.04048 for A vs. B, p= 0.001206 for A vs. C, p=0.001708 for A vs. D, p= 0.126962 for B VS. C, p= 0.053171 for B vs. D, and p= 0.064444C vs. D). Insulin secreting function of thawed/cryopreserved human islets was best maintained with UW and AA2G-utilized Group A islets (Figure 4).

Insulin gene expression was well maintained by UW solution. The gene expression of proinsulin assessed by RT-PCR was well maintained in the cryopreserved human islets with UW solution and AA2G (Figure 5). The finding was consistent with the results of the stimulation index.

Efficient lentiviral transduction in monolayered human islets. From on day 5 on, using 804G ECM favorable monolayer formation was observed in islets of Groups A and B in which UW solution was applied, indicating efficient cell adhesion on culture dishes and subsequent spread (Figure 6-A, B). In contrast, considerable number of floating dead cells was observed in FBS-preserved islets (Group C) and in CMRL-preserved islets (Group D). After lentiviral transduction, approximately 40 % of inner small-sized cells of these monolayered islets, which were considered to be endocrine cells with demonstrating enriched cytoplasmic granules, were positive for NLS/LacZ in an X-gal assay (Figure 6-C).

Human islets engrafted in the liver of the nude mice. To evaluate an engraftment of human islets cryopreserved with UW and AA2G, islets were transplanted into the liver

of nude mice. Healthy islets were observed in the branches of the portal vein by H.E stain (Figure 7-A) and the islets were positive for insulin (Figure 7-B).

DISCUSSION

Insulin dependent diabetes mellitus (IDDM) is still associated with high mortality despite of various intensive insulin treatments. Pancreas transplantation (PTX) is one of the curative therapies in the patients with IDDM, however, this procedure is highly costly, complex, and limited by scarcity of donor pancreata[14]. Currently, treatment of IDDM with islet transplantation (islet-Tx) has significant implications for organ replacement[15, 16]. Compared to PTX, islet-Tx is a technically much simpler procedure with less surgical stresses. However, this form therapy has overcome several issues, including resolution of donor shortage and establishment of efficient isolation technique and functional transportation and cryopreservation methods. To facilitate islet-Tx, it is of great value to develop an efficient cryopreserving technique of primary isolated human islets. Once such technology is established, freshly isolated islets can be stored for the future use and biological analyses. Toward such goal, we have focused on combination of University of Wisconsin (UW) solution and AA2G. UW solution was originally developed for hypothermic preservation of organs and is now widely used in clinical practice for cardiac, hepatic, and renal transplantation[1, 2]. UW solution is an intracellular-based preservative containing several important metabolic molecules, including hydroxylethyl starch, raffinose, and lactobiotine to stabilize cell membrane and to prevents edema of cells and acidosis[1, 2]. To reduce oxidant stress to cryopreserved cells (21,24), we utilized a vitamin C derivative, AA2G, because current work has demonstrated that apoptosis would progress during cryopreservation [17]. In organ preservation experiments using vitamins, investigators

reported that antioxidant vitamins decreased ischemic reperfusion injury, leading to protection of organ transplanted.[18, 19] Of these, ascorbic acid (AA) has been demonstrated to inhibit ischemic reperfusion injury by its potent reductive effect. However, application of AA to organ preservation solutions is difficult because AA is chemically unstable and rapidly deactivated when dissolved into water or exposed to air. Thus, we chemically constructed 2-O-alpha-glucopyranosyl-L-ascorbic acid (AA2G), which was converted into an active form of AA by α- glucosidase and cyclomaltodextrin glucanotransferase (CGTase) through transglucosylation which were ubiquitously present in all tissues.[4] A series of our experiments have showed that AA2G is considerably stable under high/ low temperature or oxidative conditions compared to naive AA. [4- 9] Therefore, unlike conventional vitamin C drugs, AA2G is much less affected by oxidation and can be easily stored in a considerably longer period of time. Based on such properties, we have continuously studied AA2G as a useful means for organ transplantation. [19, 20] In addition, our recent research on cold-preservation of porcine hepatocytes has demonstrated that the use of UW solution decreased caspase-3 activation during cryopreservation.[21] In this study, addition of 100 μg/ml of AA2G into UW solution allowed favorable conditions of thawed/cryopreserved human islets in terms of viability and glucose sensitive insulin secretion. Notably, after thawing, only islets cryopreserved with UW solution showed an efficient monolayer formation, which facilitated effective Lt-NLS/LacZ transduction. The observation encouraged a speculation that transduction of such human islets with immortalizing genes, such as simian virus 40 large T antigen[22] and human telomerase reverse transcriptase (hTERT) [23], could expand their populations. In the next step, in vivo transplantation of such cryopreserved islets would be desirable to confirm the in vitro results obtained in the current work. Islet functions should be addressed in experiments of much longer periods of cryopreserving time.

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FIGURE LEGENDS

Figure 1. Morphology of selected human islets. Viable human islets, demonstrating spherical shape, were selected when islets arrived at Okayama, Japan. The resultant healthy islets were nearly 100% positive for Dethizone stain and were subjected to cryopreservation experiments (A; phase contrast micrograph, B; Dethizone stain, original magnification x 100).

Figure 2. Morphological appearance of thawed human islets after cryopreservation. Three months after cryopreservation, human islets were thawed and cultured with RPMI medium. Human islets cryopreserved with UW solution showed normal spherical shape (A, B). In contrast, islets preserved with FBS and CMRL were collapsed and failed to maintain their normal shape (C, D) (A: UW solution+ AA2G, B: UW solution only, C: FBS, D: CMRL).

Figure 3. Viability of human islets after cryopreservation. The viabilities of cryopreserved human islets were 68.3 % for UW solution+ AA2G (A), 67.5 % for UW solution (B), 43.2 % for FBS (C), and 46.4 % for CMRL (D). Islet viability was maintained significantly better in UW solution-adopted Group A and Group B.

Figure 4. Glucose-sensitive insulin secretion. The stimulation index, indicating glucose-responding insulin secretion, was 1.93$\pm$ 0.5 for Group A, 1.17$\pm$0.6 for Group B, 0.74$\pm$0.27 for Group C, and 0.52$\pm$0.18 for Group D ($p < 0.05$ for A vs. B, C, and D). The well-maintained insulin secretion with glucose responsiveness was achieved in G1 human islets.

Figure 5. The gene expression of proinsulin. In an RT-PCR analysis, the gene expression of proinsulin was well maintained in the cryopreserved human islets with UW solution and AA2G (A: UW solution+ AA2G, B: UW solution only, C: FBS, D: CMRL).

Figure 6. Monolayer formation of cryopreserved human islets and subsequent lentiviral transduction of LacZ gene. A favorable monolayer formation was observed in islets of Group A in which UW solution and AA2G was applied (A). These islets were efficiently transduced with Lt/NLS-LacZ, demonstrating nuclear LacZ expression in approximately 40 % of islet cells.

Figure 7. Histology of the liver. Engraftment of thawed cryopreserved human islets with UW and AA2G was confirmed in the liver of in athymic nude mice. Almost normal structure of the transplanted islets was observed by H.E stain (A, original magnification x 100) and the cells were positive for insulin expression (B, original magnification x 100).