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Mutual effect of subcutaneously transplanted human adipose-derived stem cells and pancreatic islets within fibrin gel

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ABSTRACT

While subcutaneous tissue has been proposed as a potential site for pancreatic islet transplantation, concern remains that the microvasculature of subcutaneous tissue is too poor to support transplanted islets. In an effort to overcome this limitation, we evaluated whether fibrin gel with human adipose-derived stem cells (hADSCs) and rat pancreatic islets could cure diabetes mellitus when transplanted into the subcutaneous space of diabetic mice. Subcutaneously co-transplanted islets and hADSCs showed normalization of the diabetic recipient's blood glucose levels. The result was enhanced by co-treatment of fibroblast growth factor-2 (FGF2) in the fibrin gel. The hADSCs enhanced islet viability after transplantation by secreting various growth factors that can protect islets from hypoxic damage. Afterward, hADSCs could maintain islet viability by recruiting new microvasculature nearby the transplanted islets via overexpression of vascular endothelial growth factor (VEGF). The hADSCs did not directly differentiate into endothelial cells (no detection of biomarkers of human endothelial cells), but showed evidence of differentiation toward insulin-secreting cells (detection of human insulin). Mice receiving islet transplantation alone did not become normoglycemic. Collectively, co-transplantation of fibrin gel with islets and hADSCs will expand the indications for islet transplant therapy and the potential clinical application of cell-based therapy.

1. Introduction

Pancreatic islet transplantation is beneficial in the treatment of patients with type 1 diabetes mellitus [1,2]. The majority of islet transplantations are performed via the portal vascular system into the liver [3,4]. Most recipients initially achieve insulin independence after intrahepatic islet transplantation. Unfortunately, due to rapid apoptosis of transplanted islets, long-term glycemic control is difficult [5,6]. It is hypothesized that the parenchymal oxygen tension in the liver is below that of the pancreas, despite the liver having a dual arterial and venous vascular supply [7,8]. Procedure-related complications-hemorrhage and thrombosis are also risks with this treatment. As a result, many investigations have pursued alternative sites of islet transplantation in order to optimize islet engraftment and function, reduce transplantation mass necessary, and decrease immunogenicity [3,9].

Islet transplantation into subcutaneous tissue may have advantages in prolonging the longevity of the transplanted cells: i) the area is accessible with minimal invasiveness under local anesthesia [9–12], ii) it provides massive transplantable capacity, iii) and the tissue is removable if severe adverse events occur. The potential benefits have been tempered by the belief that the vasculature of the subcutaneous tissue may be inadequate to supply high oxygen tension, thereby resulting in abnormal insulin secretion and hypoxia-induced cell death [13]. While this is a potential limitation of this therapy, islet cells infused into the subcutaneous space have been shown to survive when methods to induce prevascularization with angiogenic factors and synthetic vascularizing devices to form vascular-rich beds [10,14–16], or a synthetic polymer scaffold to

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allow for cell attachment to the ectopic site are used [17–19]. These devices are difficult to apply clinically because the synthetic materials need to remain in the body for a prolonged period.

Human adipose-derived stem cells (hADSCs) are multipotent progenitor cells residing in the stromal-vascular fraction of adipose tissues. They are capable of differentiating into endothelial cells [20,21]. In a previous study, hADSCs improved reperfusion in a limb with ischemia secondary to femoral artery ligation due to their angiogenic effects [22–24]. Furthermore, hADSCs have been successfully used to support and augment soft tissue after lipotransfer by revascularizing the transferred adipose tissue [25]. hADSCs have also been demonstrated to have the immunomodulatory properties of bone marrow-derived stem cells, including the inhibition of T-cell proliferation, beta-cell function, and dendritic cell maturation [26–29]. These reports indicate that hADSCs could potentially inhibit the inflammatory response against transplanted islets and promote islet graft survival via the formation of a vascular-rich bed of subcutaneous tissue.

Here, we investigated whether such a mixture could enhance islet viability and functionality for long-term after subcutaneous transplantation. To this end, the viability and insulin secretion of islets accompanied with hADSCs was studied under normoxic and hypoxic conditions in both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Isolation of rat islets and hADSCs

Lewis rats purchased from Charles River (Orient, Seoul, Korea) were used as islet donors. Islets were isolated from adult male rats after intraductal collagenase injection (Type P, Roche Diagnostic, Mannheim, Germany), followed by digestion and purification by centrifugation on histopaque (1.077 g/mL; Sigma, St Louis, MO, USA). hADSCs were isolated from lipoaspirates collected from patients from whom informed consent was obtained, then cultured. hADSCs were cultured in α-minimum essential medium (α-MEM, Gibco BRL, Gaithersburg, MD, USA) that was supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco BRL), 100 units/mL of penicillin, and 100 μg/mL of streptomycin growth medium. All experiments were performed using hADSCs within five passages. To distinguish the islets from hADSCs and host cells after transplantation, the cytoplasmic membranes of islets were pre-labeled with the fluorescent probe cell tracker 1,1′-1,1-dioactadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen, Eugene, OR, USA). Islets were incubated in culture medium containing DiI dye (6.25 μg/mL) for 2 h. The labeled cells were washed twice with PBS.

2.2. Hypoxic culture of islets and hADSCs

Cells transplanted into ischemic regions are exposed to hypoxia immediately after transplantation due to a lack of initial vasculature and are prone to undergo apoptosis. Indeed, a high level of cell death has been observed within a few days after transplantation. To mimic *in vivo* ischemic condition, we cultured cells under specific hypoxic condition. To generate hypoxic culture conditions for the islets and hADSCs, cell culture dishes with islets or islets and hADSCs with or without fibroblast growth factor-2 (FGF2, 50 ng/mL daily addition, R&D Systems, Minneapolis, MN, USA) were placed in a hypoxic incubator (MCO-18M, Sanyo, Tokyo, Japan) containing 1% oxygen and 5% CO_2 at 37 °C without FBS for 48 h. 48 h after cell culture, conditioned medium was collected from each group for enzyme-linked immunosorbent assay (ELISA, R&D Systems Inc., Minneapolis, NE, USA) to detect insulin and vascular endothelial growth factor (VEGF). Islets were cultured under hypoxic condition with hADSC-conditioned medium with or without FGF2 supplement for 48 h to detect the effect of various factors secreted from hADSCs to islet viability and functionality.

2.3. Measurement of angiogenesis factors in hADSC conditioned media

hADSCs were cultured with α-MEM and its conditioned medium was collected at day 4. Angiogenesis factors in the conditioned medium were measured using Proteome Profiler™ Human Angiogenesis Antibody Array (R&D Systems) according to the manufacturer's instructions. Conditioned medium was diluted and mixed with detection antibody cocktail and incubated with a capture antibody arrayed membrane overnight at 4 °C. After washing, the membrane was incubated with HRP-conjugated streptavidin. Chemiluminescence (Kodak, Rochester, NY, USA) was used for signal detection.

2.4. Transplantation of fibrin gel having islets and hADSCs mixture

Diabetes was induced in five-week-old female athymic mice (6-week-old, Orient, Seoul, Korea) with a 200 mg/kg streptozotocin (STZ, Sigma) injection. Mice were anesthetized with xylazine (10 mg/kg) and ketamine (100 mg/kg). Three days after STZ injection, the mice were randomly divided into four experimental groups ($n = 8$ for each group). Islets (8×10^2 islets were suspended in 0.2 mL fibrin gel (Greenplasts; Greencross PD Co., Yongin, Korea)), islets (8×10^2 islets) and hADSCs (8×10^5 cells suspended in 0.2 mL fibrin gel) with or without 25 μg FGF2 (FGF2-loaded in fibrin gel) were transplanted subcutaneously into the dorsal area of the diabetic mice. Blood samples from the tail vein were obtained for blood glucose determination. Body weight was measured every three days. The physiological status of angiogenesis in the transplanted islet cells was evaluated at three weeks after treatment. Negative controls were mice with transplantation of fibrin gel only without islet cells. All animal experimentation was approved by the Institutional Animal Care and Use Committee at Hanyang University (No. HY-IACUC-11-056) and Seoul National University (No. SNU-110310-4).

2.5. Measurement of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) ratio of islets

Changes in the ATP/ADP ratio have been used to evaluate the levels of cell death and viability. Increased levels of ATP and decreased levels of ADP have been recognized in proliferating cells. In contrast, decreased levels of ATP and increased levels of ADP are recognized in apoptotic cells. The ATP/ADP ratio assay kit (Abcam, Cambridge, UK) was used to detect the ATP/ADP ratio in the islets following transplantation (islets culture only, islets and hADSCs co-culture with or without FGF2 under normoxic and hypoxic conditions). The ATP/ADP ratio was measured according to manufacturer instructions.

2.6. Western blot analysis

The mice tissue samples from transplanted regions were lysed using a Dounce homogenizer (50 strokes, 4 °C) in ice-cold lysis buffer (15 mM Tris HCl (pH 8.0), 0.25 M sucrose, 15 mM NaCl, 1.5 mM $MgCl_2$, 2.5 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 2 mM NaPPi, 1 μg/mL pepstatin A, 2.5 μg/mL aprotinin, 5 μg/mL leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.125 mM Na_3VO_4, 25 mM NaF, and 10 μM lactacystin). Protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL, USA). Equal protein concentrations from each sample were mixed with sample buffer, loaded, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% (v/v) resolving gel. Proteins were separated by SDS-PAGE were transferred to an Immobilon-P membrane (Millipore Corp., Billerica, MA, USA) and then probed with antibody against insulin (rat insulin detection; Cell Signaling, Inc. Danvers, MA, USA, human insulin detection; LifeSpan Biosciences, Inc. Seattle, WA, USA), human VEGF (Millipore Corp.), hypoxia-induced factor 1 alpha (HIF-1α, R&D Systems), rat caspase-9 (Thermo scientific pierce antibodies, Rockford, IL, USA), human caspase-3 (LifeSpan Biosciences), and human nucleus antigen (HNA, Chemicon, Temecula, CA, USA), for 1 h at room temperature. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, Santa Cruz) for 1 h at room temperature. The blots were developed using an enhanced chemiluminescence detection system (Amersham Bioscience, Piscataway, NJ, USA). Luminescence was recorded on X-ray film (Fuji super RX, Fujifilm Medical Systems, Tokyo, Japan) and bands were imaged and quantified with an Imaging Densitometer (Bio-Rad, Hercules, CA, USA).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

Cell or tissue samples were homogenized and lysed in TRIzol reagent (Invitrogen). Total RNA was extracted with chloroform (Sigma) and precipitated with 80% (v/v) isopropanol (Sigma). After the supernatant was removed, the RNA pellet was washed with 75% (v/v) ethanol, air-dried, and dissolved in 0.1% (v/v) diethyl pyrocarbonate-treated water (Sigma). RNA concentration was determined by measuring the absorbance at 260 nm with a spectrophotometer. Reverse transcription was performed using 5 μg of pure total RNA and SuperScript™ II reverse transcriptase (Invitrogen), followed by PCR amplification of the synthesized cDNA. PCR consisted of 35 cycles of denaturing (94 °C, 30 s), annealing (58 °C, 45 s), and extending (72 °C, 45 s), with a final extension at 72 °C for 10 min. PCR was followed by electrophoresis on 2% (w/v) agarose gel and visualization with ethidium bromide staining. PCR products were analyzed using a gel documentation system (Gel Doc 1000, Bio-Rad). β-actin served as the internal control. The RT-PCR results were quantified with an Imaging Densitometer (Bio-Rad).

2.8. Immunohistochemistry

The mice tissue samples from retrieved from transplanted regions were harvested at 3 weeks after treatment. The samples were embedded in an optimal cutting temperature compound (O.C.T. compound, TISSUE-TEK® 4583, Sakura Finetek USA Inc., Torrance, CA, USA) and frozen then sliced into 10 μm thick sections at −22 °C. All