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Allogeneic Bone Marrow Supports Human Islet β Cell Survival and Function Over Six Months

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Abstract

In this study, we established a new strategy for human islet longevity utilizing co-cultured human islet with allogeneic whole bone marrow (BM). The cultured islets' function and survival have been evaluated by multiple methods including assessment of insulin-secretion in response to high-glucose-challenge by ELISA and morphological evaluation of cell growth. Human islet only culture was used as control. Results show that BM increases islet survival and function with the eventual formation of pancreatic endocrine tissue capable of sustaining β cell function. BM cells were shown to be capable of stimulating pancreatic endocrine tissue growth.

Introduction

Pancreatic transplants provide promise for a cure of diabetes, but are limited by multiple factors not the least of which is tissue availability [1]. Alternatively, the Edmonton Protocol for β -cell islet transplantation can be effective, but islet viability is a restrictive factor as indicated by the limited number of islet transplants that have been accomplished thus far [2]. Difficulties in maintaining viable, functional islets following isolation from cadaveric sources represent a significant roadblock among the critical factors needed for successful β -cell islet transplants. The failure to maintain and expand islet tissue in vitro further restricts the availability of these tissues for patients.

The islet isolation process itself results in considerable damage to the islets and the supportive microenvironment. Many efforts have been made to develop methods and materials to preserve islet function during isolation [3-5]. Several studies have examined cellular signaling transduction pathways [6], generation of inflammatory factors [7, 8] and improvement of vascularization of transplanted material [9] with the ultimate goal of improved clinical response. Recent evidence supports the ability of BM cells to repair non-hematopoietic tissues, including CNS, renal, pulmonary and skin tissue, and possibly participate in tissue regeneration in these organs [10-12]. We proposed to utilize BM to generate a microenvironment favorable for the repair and longevity of human islets, which

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would be a valuable approach for long-term islet survival/function, leading to successful islet transplantation.

The reparative and regenerative effects of allogeneic BM, when placed in culture with isolated human islets are examined in this report. Co-culture with BM demonstrated the ability to repair damaged human islets resulting in functional insulin release from islets in long term culture. More work is needed to explore the mechanisms of how allogeneic BM supports islets. This finding generates a new possibility in which the use of a recipient's BM in co-culture with donor islets may then lead to an improved source of islet-based tissue for delivery to diabetic patients with the ultimate goal of a long-term correction of blood glucose abnormalities.

Methods

Human pancreatic islets

Human islet tissue, from normal donors, was obtained from Islet Resource Centers (ICRs) in the ICR Basic Science Islet Distribution Program, Human Islet Laboratory, University of Pennsylvania (Philadelphia, PA), Joslin Diabetes Center (Boston, MA) and City of Hope National Medical Center (Duarte, CA), University of Miami, School of Medicine, Diabetes Research Institute, (Miami, FL). The use of these cells is approved by the Institutional Review Board (IRB) at Roger Williams Hospital and the ICRs Committees.

Human BM

Human BM from normal donors was obtained under a separate Roger Williams Hospital IRB approved protocol. Bone-marrow mononuclear cells were isolated by Ficoll-Paque™ Plus (Amersham Biosciences; Amersham, UK) per manufacturer directions. Cells were then washed twice with 10% fetal calf serum (FCS) in phosphate buffered saline (PBS), resuspended in culture medium (see below). Trypan blue staining was used (manufacturer) to assess for cell viability.

Allogeneic BM co-culture with human islets

Human islets were received from Islet Cell Resource Centers (ICRs as described above) within 48 hours after harvest from cadaveric donors. The purity of islets in total isolated tissue was 90% as assessed by dithizone identification and viability was > 95% as determined by trypan blue dye exclusion after islets arrived. Islets were placed in culture at a number of 50 islet equivalents (IEQs) per ml with 1×10^6 allogeneic BM cells/ml. Cultures were maintained in RPMI 1640 (GIBCO) supplemented with 10% heated inactivated Fetal Bovine Serum (HiFBS, GIBCO), 5.5 mM glucose, 10 mM HEPES, and 1% P/S.

Evaluation of islet function

Islet function was evaluated by measurement of insulin release with or without a glucose challenge. The media was changed twice per week, and media insulin was assayed as basal insulin release. High-glucose challenge was performed once a week as follows: the media was collected and cultured cells were washed once with islet culture RPMI medium. Media was then replaced with high-glucose (20 mM) RPMI 1640 for 30 minutes. The media was then collected and stored at -80°C for insulin assay.

Human insulin assay

Insulin concentrations in the specimens (cell culture media or cultured tissue extracts) were measured using a Human Insulin ELISA Kit (Linco Research, St. Charles, MO) according to

the manufacturer's instructions and insulin concentrations were calculated using KC Junior[®] microplate reader software (Bio-Tek Instruments, Inc.) [13].

Fluorescent Immunohistochemistry

Cells grown on chamber slides were fixed with 3% paraformaldehyde, followed by exposure to 10% normal goat serum. The slides were blotted without washing, primary antibodies and a second fluorescent color and/or third antigen-detecting antibody were applied. When the process was finished, the slide was coverslipped using a fluorescent mount medium. The samples were evaluated and photographed using a confocal fluorescent microscope [14].

Statistical evaluations

All data is presented as the mean $\pm$ SEM and analyzed by the Analysis of Variance (ANOVA) followed by a student-t test, unless otherwise indicated. Sigma Plot (SPSS Inc. Chicago, IL) was used to draw graphs. All data represents the results of three independent experiments, unless otherwise indicated.

Results

BM cells preserve islet configuration

BM cells co-cultured with isolated islets appear to encircle these islets and to form a monolayer surrounding islet structures within the first 6 days of culture (figure 1a). These changes continue in long term culture (figure 1.b) and are associated with the maintenance of islet function as measured by an insulin response to glucose challenge (see below). In contrast, islet only cultures rapidly lose their morphology with cells spilling out of the islets (figure 1c) within the first week of culture, and continue to undergo a gradual degenerative process resulting in a non-functional monolayer of cells (figure 1d).

BM supports human islet function in long-term culture

Functional analysis of the cultures was performed by evaluating basal insulin release and insulin secretion in response to a high glucose challenge. Basal insulin release was detected in islet/BM co-cultures for up to 204 days (figure 2.a). Within the first 21 days of culture there was less basal insulin release in islet/BM co-cultures than that seen in islet only culture. By day 28 in islet/BM co-cultures, basal insulin release increased slightly and became stable until day 140, where gradual increase in basal insulin release was noted until day 204 (termination of experiment). In islet only cultures, basal insulin release levels gradually decreased with spikes of high insulin levels on days 15 and 28 and became negligible by day 49 and remained barely detectable. In conjunction with the morphologic studies, these findings indicate that the spikes in insulin release noted in islet only cultures on days 15 and 28 may have been due to leakage of insulin from dying β cells within the cultured islets. In a similar fashion, the relative low basal insulin release noted in the first 21 days of islet/BM co-culture may have been due to a paucity of dying β cells which achieved functional stabilization. The evaluation of insulin secretion in response to glucose challenge (20 mM) indicated that the surviving β cells in the islet/BM co-cultures appear to retain their function as determined by stable insulin release and the functional rebound seen during the period from 140 and 204 days. This change in insulin release noted after day 140 may be reflective of reorganization and possible rebuilding of islet structures, as will be discussed below in long-term cultures.

BM stimulation of human islet growth and reconstitution of endocrine tissue

After long-term co-culturing of islets with BM, a three-dimensional tissue structure was formed (figure 3.a) which can be viewed without microscope. Frozen tissue sections (5 μ m),

prepared from these three-dimensional structures, were stained with hematoxylin and eosin (figure 3. b.) and revealed islet like structures within this material. Many of the cells within the islet like structures stained for β cells (as determined by antibodies detecting proinsulin) while α cells, stained with anti-glucagon antibody, were less in number as revealed by fluorescence immunohistochemistry (figure 3. c.). The three-dimensional structures in these long-term cultures (190 days) do not appear to be diminished in basal insulin secretion (figure 3. d.) and in the insulin response to glucose challenge (figure 3. e.).

Discussion

The present results indicate that allogeneic human marrow cells allow for the preservation of islet's β -cell function through co-culturing with such islets. The direct evidence for this rests in the observation that co-cultured islets continue to demonstrate insulin release in response to high glucose challenge for more than six months with the preservation of islet morphology. These cultured islets ultimately grew into pancreatic like tissue with vigorous insulin release and in response to glucose challenge in vitro. In previous studies, cultured human islets have generally lost function and viability within 3-4 weeks, [17] as did islet only culture control in these studies. These observations suggest immediate strategies for islet cell transplantation using a co-culture with allogeneic (possibly recipient) BM cells.

Evaluation of these co-cultures revealed that allogeneic BM aggregated around the human islets and formed a capsule like structure surrounding the islets with apparent overall preservation of islet morphology. In islets cultured without BM cells, islet morphology soon became disrupted with many cells streaming from the structure and onto the culture dish. Even in early cultures at four weeks, islets co-cultured with BM demonstrated an increased number in insulin positive cells (supplemental data). Although insulin release can be viewed as β cell activity, the glucose challenge test truly represents islet function and survival since only living/vital β cells are able to respond to a glucose challenge (1 hour) while the constitutive release of insulin could partially result from the leakage of damaged β cells. The observation that BM enables islet survival and function for extended periods suggests that BM may provide a favorable microenvironment allowing for repair of islet injury and support of islet cell function.

Monitoring individual islets in culture revealed that BM co-cultured islets grow more vigorously compared to islets cultured without BM. In long term islet only cultures, (6 months) no viable islet cells could be detected. Evaluation of individual islets co-cultured with BM revealed a defined distribution pattern for α and β cells with α cells mostly restricted to a centralized location. The islets became incorporated into a three dimensional matrix. Functional activity in regards to constitutive insulin release and response to glucose challenge remained intact in these three dimensional cultures, indicating that they may represent an organotypic model of human pancreatic endocrine tissue.

BM reconstituted islets not only possess better function in vitro but also are functional as seen in the normalization of hyperglycemia *in vivo* following transplantation of BM co-cultured islets into the renal capsule of diabetic animals and such function is absent when cultured islets without BM are transplanted (supplemental data). This suggests that the strategy to generate an advantage by *in vitro* culture with BM can be translated into an *in vivo* application as recently reported in the direct utilization of human BM in animal studies [18]. Coculture BM with isolated human islets might become useful in the restoration and preservation of individual islets prior to transplantation. The advantages here include: preservation of islet morphology allowing for delivery via infusion; restoration of islet function leading to a reduced quantity of islets required per transplant; and, utilization of recipient BM for co-culture to potentially limit the alloantigens delivered. With further

refinement, three dimensional cultures might become useful as a potential tissue bank or in creating bioengineered constructs for alternative transplantation methods.

Initial studies comparing other cell sources besides BM, which included whole umbilical cord blood cells, mobilized peripheral blood cells, and isolated mobilized peripheral CD 34+ cells did not provide any benefit (data not shown) in short term culture (35 days). These observations have indicated that the cell(s) supporting the microenvironment may be unique to BM.

In summary, it appears that BM cells provide a supportive, nurturing milieu for β -cell islets, allowing for long-term survival, growth and function. These effects are probably due to repairing human islet injury and creating a microenvironment by the marrow tissue for pancreatic islet β -cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Islet morphology in short-term culture

(a) BM co-cultured with islets, Day 6 BM cells surround islet forming capsule like structure. (b) BM co-cultured with islets, Day 156. Intact islet morphology is seen in culture. (c) Islet only culture, Day 6. Cells spilling from islet, eventually to form monolayer (see following). (d) Islet only culture, Day 156. Complete loss of islet morphology with only monolayer of cells noted.

Figure 2. Bone marrow retains islet function in culture

(a.) Evaluation of baseline insulin release until 204 days culture and (b.) insulin secretion in response to a high glucose challenge (20 mM for 30 minutes) as measured for 204 days.

Figure 3. Pancreatic endocrine tissue generated from long-term BM co-cultures (a) shows tissue generated from islet in long-term bone marrow coculture (190 days), and the bar represents a length of 1 cm. The sample is about 1.8 cm long with clear three-dimensional structure. The histological structure of a 5 µm section from the frozen tissue in (b), stained with hematoxylin and eosin in (c), shows islet-like structure (arrow indicating) surrounded by porous scaffold-like tissue. Insulin content is 56307 uU/per mg protein (ELISA assay). The islet-like tissue contains primarily β cells and a few α cells. In (d) fluorescence immunohistochemistry reveals β cells with anti-human insulin antibody (red, b arrow), α cells with anti-human glucagon antibody (green, a arrow), and nucleus staining (DAPI, blue, magnification × 20). As indicated in Figures (e) and (f), evaluation of the tissue's insulin release and insulin in response to high glucose challenge before histological evaluation shows that the regenerated tissue has function. These data suggest that human islet tissue in coculture is generated not only because bone marrow facilitates islet aggregation but also because bone marrow stimulates islet cells to form islet tissues. B = bone marrow; I = islet * = p<0.05 vs. islet only culture.