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Effects of bone marrow on the microenvironment of the human pancreatic islet: A Protein Profile Approach

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ABSTRACT

Stem cells are a new therapeutic modality that may support the viability and function of human organs and tissue. Our previous studies have revealed that human allogeneic bone marrow (BM) sustains pancreatic β cell function and survival. This paper examines whether BM creates a microenvironment that supports human pancreatic islets in vitro by evaluating 107 proteins in culture media from BM, islet, and islet/bone marrow (IB) with mass spectrometry. Proteins were considered up- or down-regulated if p-values < 0.05 and fold change was greater than 2 fold I VS. IB. In addition, proteins identified that were uniquely found in islets co-cultured with bone marrow, but not in islets or bone marrow. A 95% protein probability was used as a threshold. Twenty three proteins were upregulated, and sixteen proteins were downregulated. The function of each protein is listed based on the protein database, which include structural proteins (9 upregulated, 4 downregulated); anti-protease and anti-endopeptidase enzymes (8 upregulated); cation binding proteins (6 up-regulated). Six proteins were uniquely identified in islet co-cultured with bone marrow. Three are anti-proteases or anti-endopeptidases, and 1 is a structural protein. These findings suggest that BM, by changing culture media proteins, may be one of mechanisms to maintain human islet function and survival.

1. Introduction

Diabetes mellitus type I presents with hyperglycemia due to insulin insufficiency. Autoimmune destruction of the pancreatic islets requires lifelong dependence on insulin injections (Nathan, 2015; Ismail-Beigi, 2012). Islet transplantation is a promising therapy for type I diabetes. Unfortunately, this is limited by the supply of cadaveric donors and the limited viability of islets (Shapiro et al., 2017; Corritore et al., 2016; Pellegrini et al., 2016). Sustaining human islet function and survival after transplantation is a significant challenge. BM stem cells have been applied towards this challenge (Luo, 2012). In Luo et al., 2007, islets were co-cultured in vitro with bone marrow (IB).

The limited viability is likely due to the disruption of the microenvironment. The microenvironment of a pancreatic islet consists of the microvasculature (Luo et al., 2011), growth and transcription factors (Milanesi et al., 2012; Luo et al., 2011), oxygen content (Lo et al., 2013), angiogenesis factors (i.e., VEGF) (Brissova et al., 2006; Pepper et al., 2013), and extracellular matrix (ECM) proteins (Cheng et al., 2011; Thiovelt et al., 1985). It also includes nerve fibers and endothelial cells (de Koning et al., 1998). The ECM proteins do not merely provide a mechanical scaffold for the islets, but also provide molecular signals, bind growth factors, up-regulated hormone pathways, immune up- and down-regulation, promote wound healing, and facilitate cell migration (Cheng et al., 2011; Bornstein et al., 2002; Thiovelt et al., 1985; Luo et al., 2009, 2011).

Stem cells offer a new approach to improving islet viability and function after procurement and transplantation (Lysy et al., 2016; Noguchi, 2012). Adult BM stem cell consists of many classes of stem cells, such as the mesenchymal stem cells and BM is particular practical because of its availability, procurability, low tumorigenicity, and minimal ethical controversy (Ilgun et al., 2015).

Multiple types of studies support the role of BM in supporting β cell insulin secretion. BM improves islet viability and insulin secretion in vitro (Luo et al., 2007; Luo and Luo, 2005). BM improves hyperglycemia in the diabetic animal model (Hao et al., 2013; Milanesi et al., 2012; Zhao et al., 2008; Hess et al., 2003; Banerjee et al., 2005). Clinically, patients who receive bone marrow

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Abbreviations

BM	bone marrow
ECM	extra cellular matrix protein
I	islet only
IB	islets co-cultured with bone marrow
SCF	stem cell factor
FC	fold change
STRING	Search Tool for the Retrieval of Interacting Genes/ Proteins

transplants experience improved hyperglycemia (Xiaoxia et al., 2014; Dave et al., 2014; Bhansali et al., 2014, 2014b; Wang et al., 2015).

BM supports β cell mass, viability, and function through multiple mechanisms (Luo et al., 2007). This includes trans-differentiation (Luo et al., 2009), paracrine communication (Milanesi et al., 2012; Gao et al., 2014), promotion of neo-vascularization (Milanesi et al., 2012; Shin et al., 2015; Rafii and Lyden, 2003; Luo et al., 2011), and immune-modulation (Luo et al., 2011; Yu et al., 2017).

In this study, we hypothesized that BM creates a specific microenvironment that sustains human islet function and survival. We examine how BM modifies the proteins in the culture media. To explore the full gamut of proteins, label-free mass spectrometry is ideal given its wide-scale analysis. The difference from the most mass spectrometry studies examine the intracellular protein profile (Ahmed, 2010; Ortsäter and Bergsten, 2006; Bergsten, 2009); in this study, we examined the microenvironment by analyzing the culture media. We characterize the function of the proteins that are up- and down-regulated; and the proteins that are expressed in islet co-cultured with BM.

2. Material and methods

2.1. Human pancreatic islets

Human pancreatic islets were received from 2 to 3 normal donors from the Integrated Islet Distribution Program (IIDP) from 30 to 50 donors/yearly, University of Pennsylvania (Philadelphia, PA, USA), Massachusetts General Hospital (Boston, MA, USA) and City of Hope National Medical Center (Duarte, CA, USA). The use of these cells was approved by the IRB (Institutional Review Board) at Roger Williams Medical Center and the ICR Committees.

Human pancreatic islets were received in ice bags within 48 h after procurement from each healthy cadaveric donor. The purity of islets in the total isolated tissue was 75–90% as assessed by dithizone identification and viability was > 95% as determined by Trypan blue dye exclusion.

2.2. Human bone marrow

Human bone marrow cells were collected from normal donors under a separate Roger Williams Medical Center IRB approved protocol. BM mononuclear cells were isolated by Ficoll-Paque Plus (Amersham Biosciences, Amersham, UK) per manufacturer directions to eliminate blood erythrocytes. Briefly, cells were mixed with PBS 1:1 and loaded on top of Ficoll in 2:1. Multiple donors were used for this experiment. The ages of BM donors range from 28 to 35 year. Cells were centrifuged and isolated in three layers and middle layer cells were removed carefully to new tubes and then washed twice with 10% FCS in PBS, re-suspended in culture medium. Trypan blue staining was used (Invitrogen Corp., Carlsbad, CA, USA) to assess for cell viability. Isolated BM consists of multiple populations including BM mesenchymal cells and stem cells.

2.3. Co-culture

Once islets were received, they were immediately co-cultured with BM. BM was isolated and placed in co-culture. Whole BM progenitors were used in co-culture at 1×10^6 allogeneic BM cells/mL. The ratio of islet equivalent to BM cells was $1{:}10^4$. This ratio was used because it was determined to be the optimum ratio (Luo et al., 2007) and was used in a previous study (Luo et al., 2011). BM was cultured in media 1 L Roswell Park Memorial Institute (RPMI), 20% fetal bovine serum (FBS), 10 mM HEPES, 2 gm sodium bicarbonate, 1.0% penicillin/streptomycin, 5.5 mM glucose, and 50 ng/mL of recombinant human stem cell factor (SCF).

Pancreatic islets were placed into 2 experimental groups: islets only (I), and islet co-cultured with bone marrow (IB). These were placed in a 6 well plate. There were three wells with islets only (I). There were six wells with islets co-cultured with bone marrow (IB). The six wells with islets co-cultured with bone marrow were split into 2 sets. Each set consists of 3 wells. The averages of the 2 sets are included in Table 1. The averages of all 6 wells are included as well.

Each well had islets plated at a density of roughly 50 islets per mL. Growth media in each well was changed twice a week. The cells were washed with media. Cultures were periodically examined under a Zeiss microscope (Zeiss, Oberkochen, Germany) to check for contamination or necrosis. Islets were incubated at 37 °C at 5.0% CO_2 with a pan of water to maintain humidity. BM and islets were co-cultured for 4 weeks.

2.4. Gel electrophoresis

A gel electrophoresis is performed on 20% FBS, an eluate of RPMI, and a flowthrough of RPMI. Running gel was created. Coomassie staining was used. A ladder is used (Fig. 1).

2.5. Protein preparation, MS analysis, and peptide database analysis

At the end of four weeks, culture media from each well were procured. Protein concentrations were measured using Bradford reagent. Samples were denatured with urea (8 M) and incubated for 1 h at 56 °C. Iodoacetamide (5 mM) was used to alkylate the samples. They were incubated for 1 h in the dark (at room temperature). Samples were diluted at a 1:4 ratio with 10 mM Tris (pH 8.0). They were incubated overnight at 37 °C with trypsin (1:100 protease:protein ratio). Trifluroacetic acid was used to adjust the pH to 2.7. This was decanted after centrifugation (1000 g for 5 min). The left over peptides were desalted (Oasis HLB extraction cartridges) and dried (SpeedVac Concentrator).

Once dried, peptides were dissolved into Buffer E (5 mM ammonium formate, pH 2.65, 30% acetonitrile). They were loaded into a 1 mL Resource S (SCX) column after equilibration with Buffer E). The chromatography column was eluted with a linear gradient of 5 mM to 1.5 M ammonium formate (flow rate = 0.5 mL/min). 32 1 mL fractions were collected. Protein concentrations were measured with absorbance ($\lambda = 280$ nm). The fractions with the highest peptide concentrations were dried (SpeedVac Concentrator) and stored at −70 °C.

Samples were analyzed by a fully automated reverse phase chromatography followed by tandem mass spectrometry. Dry peptide-enriched mixtures were dissolved in 0.1% acetic acid in water. This was adsorbed to a C18 analytical column (360 μm outer