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Three-dimensional differentiation of bone marrow-derived mesenchymal stem cells into insulin-producing cells

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ABSTRACT

Fibrin glue (FG) is used in a variety of clinical applications and in the laboratory for localized and sustained release of factors potentially important for tissue engineering. The aim of this study was to evaluate FG scaffold effect on differentiation of insulin-producing cells (IPCs) from bone marrow-derived mesenchymal stem cells (BM-MSCs). In this experimental study BM-MSCs were cultured and the cells characterized by analysis of cell surface markers using flow cytometry. BM-MSCs were seeded in FG scaffold (3D culture) and then treated with induction media. After induction, the presence of IPCs was demonstrated using gene expression profiles for pancreatic cell differentiation markers (PDX-1, GLUT-2 and insulin) and insulin detection in cytoplasm. Release of insulin by these cells was confirmed by radioimmunoassay. Expression of the islet-associated genes PDX-1, GLUT-2 and Insulin genes in 3D cultured cells was markedly higher than the 2D cultured cells exposure differentiation media. Compared to 2D culture of BM-MSCs-derived IPCs, the insulin release from 3D BM-MSCs-derived IPCs showed a nearly 3 fold ($p < 0.05$) increase when exposed to a high glucose (25 mM) medium. Percentage of insulin positive cells in 3D experimental group showed an approximately 3.5-fold increase in compared to 2D experimental culture cells. The results of this study demonstrated that FG scaffold can enhance the differentiation of IPCs from rats BM-MSCs.

1. Introduction

Diabetes mellitus is one of the most common chronic diseases which directly affect millions of people (Pandey, 2010). Type 1 diabetes is caused by autoimmune destruction of the pancreatic islet insulin-producing beta-cells. Insulin administration does not prevent long-term complications of the disease, as the optimal insulin dosage is difficult to adjust. Replacement of the damaged cells with regulated insulin-producing cells is considered the ultimate cure for type 1 diabetes. Transplantation of intact human pancreases or isolated islets has been severely limited by the scarcity of human tissue donors (Zalzman et al., 2005).

Many studies have been focused on how to develop renewable sources of islet-replacement tissue. Whereas some studies have shown the generation of insulin producing cells (IPCs) from progenitor cells of the pancreas (Bonner-Weir et al., 2000), liver (Yang et al., 2002, Liu et al., 2013), pluripotent embryonic stem cells (Lumelsky et al., 2001; Ebrahimie et al., 2014), and skin derived stem cells (Guo et al., 2009), the efficiency of in vitro generated IPCs is low.

Ianus et al. (2003) showed that labeled cells from the bone marrow were able to contribute, even if just partially, to the pancreatic endocrine lineage. It has been reported that 3D culture is important for the acquisition of mature IPCs (Takeuchi et al., 2014).

A 3D culture is advantageous to imitate the in vivo micro environment by enhancing cell-cell and cell-matrix interactions and subsequent cell signaling (Wang et al., 2007; Grayson et al., 2004; Schmeichel and Bissell, 2003; Mohr et al., 2006) To date, a variety of 3D cell culture systems have been developed and adopted for directing stem cell differentiation into various lineages (Levenberg et al., 2003; Liu et al., 2013; Mohr et al., 2006).

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It has been revealed that biomaterial scaffold can enhance differentiation of various cell types into IPC compared to those differentiated in 2D cultures (Vaithilingam et al., 2008; Wang et al., 2007; Ku et al., 2004; Kubo et al., 2004; Hebrok, 2012).

Fibrin glue (FG) is a natural fibrous protein involved in the clotting of blood. FG scaffolds can be engineered as a tissue substitute that is biocompatible and biodegradable (Ahmed et al., 2008). Proliferation and differentiation of the stem cells can be achieved in a fibrin matrix, and fibrin alone or in combination with other materials has been used as scaffolds to regenerate adipose tissue, bone, cartilage, etc. (Ahmed et al., 2008). In this study effect of FG on BM-MSCs differentiation into IPCs was investigated.

2. Materials and methods

2.1. Isolation of BM-MSCs

This study was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences. BM-MSCs cultures were prepared under sterile conditions. Briefly, the femur and tibiae of the Wistar rats were excised with special attention given to remove all connective tissue attached to bones. Bone marrow was extruded from these bones by flushing the BM cavity using a syringe with 20-gauge needle filled with culture medium (DMEM) supplemented with 10% fetal calf serum (FCS). The harvested BM-MSCs were gently pipetted to break up cell clumps in order to obtain cell suspension. After a homogenous cell suspension was achieved, the cells were centrifuged at 1200 rpm for 7 min and the cell pellet was resuspended in 3 ml of culture medium. The cell suspension was seeded in 25 cm plastic tissue culture flasks with 5 ml culture medium and maintained at 37°C in a humidified atmosphere with 5% CO_2. Cultures of BM-MSCs were inspected and refed every 3 days and passaged when the BM-MSCs have reached approximately 80% confluence. The mesenchymal population was isolated on the basis of its ability to adhere to the culture plate (Moradi et al., 2012; Wakitani et al., 1995; Barbash et al., 2003).

Expression of cell surface markers on the BM-MSCs culture prior to use of differentiation media were analyzed using flow cytometry. The cells were characterized with regard to a set of markers characteristic for BM-MSCs including CD44, CD105, CD45 and CD34 (Karaoz et al., 2009).

2.2. Experimental design

The BM-MSCs at passages three were used in this experiment. Four groups including two controls and two experimental groups were formed. The cells were cultured in DMEM as 2D control group. The seeded cells onto the FG scaffolds were cultured in DMEM and used as 3D control group. The cultured cells in IPC differentiation media used as 2D experimental group. The seeded cells onto the FG scaffolds were cultured in IPC differentiation media and used as 3D experimental group.

A three-stage protocol was used to induce IPC. Stage 1: The cells (1×105/ml) were cultured (37 °C, 5% CO_2) in serum-free high glucose DMEM (25 mmol/L) containing 0.5 mmol/L beta-mercaptoethanol (Invitrogen) for 2 days. Stage 2: The cells then were cultured in the medium containing 1% non-essential amino acids (Invitrogen), 20 ng/ml fibroblast growth factor (FGF, Sigma-Aldrich), 20 ng/ml epidermal growth factor (EGF, Sigma-Aldrich), 2% B27 (Invitrogen), 2 mmol/L L-glutamine and 10 ng/ml exendine-4 (Sigma) in 6-well plates for 8 days. Stage 3: The cells were cultured for an additional 8 days in new medium containing 10 ng/ml betacellulin, 10 ng/ml activin A, 2% B27, 10 mmol/L nicotinamide and 10 ng/ml exendine-4 (Sun et al., 2007).

Table 1
Sequences of genes.

Gene	Sequences	
	Forward	Reverse
PDX-1	AAACGCCACACACAAGGAGAA	AGACCTGGCGGTTCACATG
GLUT-2	CAGCTGTCTCTGTGCTGCTTGT	GCCGTCATGCTCACATAACTCA
Insulin	TCTTCTACACACCCATGTCCC	GGTGCAGCACTGATCCAC
Glucagon	GTAATGCTGGTACAAGGCAG	CCAGTTGATGAAGTCTCTGG
Somatostatin	CTGCATCGTCCTGGCTTTGG	TGCAGCCAGCTTTGCGTTCC
PAX-4	TGGCTTTCTGTCCTTCTGTGA	TCCAAGACTCCTGTGCGGTAG
MafA	CTTCAGCAAGGAGGAGGTCAT	GCGTAGCCGCGGTTCTT
β-Actin	ACCTGACAGACTACCTCATG	ATCGTACTCCTGCTTGCTGA
GAPDH	CTCTGGTGGACCTCATGGCCTAC	CAGCAACTGAGGGCCTCTCT

2.3. 3D culture

FG scaffolds (total volume: 400 μl) were made by combining fibrinogen (from bovine plasma) at a concentration of 10 mg/ml, 2.5 mM $CaCl_2$, and 2 NIH units/ml of thrombin (all from Sigma except where indicated). The scaffold was characterized by SEM (Willerth et al., 2006). Scaffolds were prewetted overnight (approximately 12 h) at 4 °C in culture medium consisting of DMEM supplemented with 10% FCS, 100 U/ml penicillin, 10 μg/ml streptomycin, and 0.1 mm nonessential amino acids. Following trypsinization, BM-MSCs at passage 3 ($1–1.5 \times 10^6$ cells/scaffold) were resuspended into 200 μl of culture medium and were seeded onto the FG scaffolds, and maintained for 3 days. The cell seeded scaffolds were then incubated in the IPC media.

2.4. Scanning electron microscopy (SEM)

Unseeded and seeded scaffolds were fixed with 2.5% glutaraldehyde buffered in 0.15 mol/L sodium cacodylate (pH 7.2, 20 °C, 1 h). After fixation, the cultures were repeatedly rinsed in cacodylate buffer. The cultures were dehydrated in a graded series of ethanol (50%, 70%, 95% and 100% alcohol) prior to critical point drying. The preparations were sputter-coated with gold-palladium before SEM.

2.5. Real-time polymerase chain reaction

Using the RNeasy Mini kit (Qiagen), RNA was isolated from the harvest cells according to manufacturer's instructions. cDNA was produced from the extracted RNAs using the cDNA synthesis kit based on the manufacturer's instructions (Fermentas, Canada). Primer sequences are shown in Table 1. Approximately 2 μl of cDNA was amplified in each 25 μl PCR reaction mix containing 12.5 μl of 2 × SYBR Green Master Mix (Fermentas, Canada), 0.2 μl of each 10 pmol forward and reverse primers (designed in primer 3 software, Table 1) and 10.1 μl DEPC water. PCR amplification was done in 40 cycles using the following program: 95 °C for 10 min, 95 °C for 15 s, 5 °C for 30 s and 60 °C for 34 s. Data were analyzed using the $2^{-\Delta\Delta CT}$ method. Gene expression in IPCs was normalized either to undifferentiated BM-MSCs or adult rat islets. Expression values were corrected for the housekeeping gene β-actin (Ebrahimie et al., 2014).

2.6. Radioimmunoassay (RIA)

The differentiated cells were plated in 24-well plates at 10^5 cells per well. The cells were preincubated for 1 h in glucose-free Krebs-Ringer bicarbonate (KRB), and incubated with KRB containing 5.56 mmol/L, 16.7 mmol/L and 25 mmol/L of glucose (glucose challenge) for an additional 1 h, respectively. The KRB media were collected and frozen at −80 °C until assay (Gabr et al., 2012). Insulin assay was performed by RIA using a commercially available rat RIA kit (Millipore) according to the manufacturer's instructions.