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Fibrin scaffold enhances function of insulin producing cells differentiated from human umbilical cord matrix-derived stem cells

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ABSTRACT

Tissue engineering is a new strategy which proposed to treat numerous human diseases nowadays. Three dimensional (3D) scaffolds fill the gap between two dimensional cell culture (2D) and animal tissues through mimicking the environmental behaviors surrounding the cells. In this study, hUCMs into insulin producing cells in fibrin scaffold were differentiated compare to conventional culture condition. Differentiation rate was estimated by real time PCR, immunocytochemistry (ICC) and the chemiluminesence (CLIA) and enzyme immunoassay (EIA). Real time PCR's results showed an increasing expression in *NKX2.2, PDX1* and *INS* (producing the hormone insulin) genes in fibrin scaffold. Furthermore ICC analysis exhibited that insulin and pro-insulin proteins were more in fibrin scaffolds. CLIA and EIA on insulin and C peptide secretion indicated that both of groups were sensitive to the glucose challenge test but significant higher response was observed in fibrin scaffold (6.5 fold in 3D, 1.8 fold in 2D culture). It could be concluded that differentiation of hUCM cells into insulin producing cells in fibrin scaffold 3D culture system is much more efficient than 2D conventional culture system.

1. Introduction

Diabetes mellitus (DM) is the most debilitating and life threatening metabolic disorder in the modern societies (Fauci, 2008). Statistics issued by WHO show that DM will be the seventh leading cause of death worldwide by 2030 (Wild et al., 2004). Unfortunately, medications, herbal remedies, islet cell transplantation, and recently stem cell transplantation, although have excreted some impact on the control of the blood glucose but could by no means withstand DM and its complications (Wright and Pohajdak, 2001). Stem cell therapy has opened a new window through which DM problems might be overcome. However, stem cell differentiation into insulin producing cells (IPC) is in its infancy period and requires more precise investigations before being utilized for the treatment of DM in humans (Aguayo-Mazzucato and Bonner-Weir, 2010). Tissue engineering is a new strategy proposed for tissue injury and disease treatment (Ma et al., 2012). Three dimensional (3D) scaffold fills the gap between two dimensional cell culture (2D) and animal tissues by mimicking the behaviors surrounding environment (Yamada and Cukierman, 2007). 3D system provides a suitable model for investigating mechanical force of surrounding environment (Meng et al., 2014). The most general types of materials utilized as scaffold are metals such as titanium; synthetic organic materials such as polymers; synthetic inorganic materials such as hydroxyapatite, and natural organic materials such as collagen and fibrin (Meng et al., 2014). Fibrin has widely been used as a natural hydrogel in tissue engineering applications (Osathanon et al., 2008). It is a biologic network which is assembled by polymerization of fibrinogen; a glycoprotein which is normally present in human blood plasma, and essential for wound healing and other biological phenomena (Blombäck et al., 1994). Fibrin gel can serve as a transport system for many kinds of mesenchymal cells (Bensaid et al., 2003; Pelaez et al., 2009). Previous studies have shown successful proliferation and differentiation of stem cells in fibrin scaffold (Catelas et al., 2006). *In vitro* differentiation of different stem cell into the IPCs, although functionally available, has encountered a great challenge that insulin is insufficiently secreted by the IPCs, and the number of IPCs in a given volume is

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scanty for a successful transplantation (Seyedi et al., 2016). In the present study we aimed to evaluate the differentiation capacity of hUCM cells; an immunocompetent type of mesenchymal stem cells with embryonic origin, into IPCs in 3D and 2D environment. To assess this hypothesis, we determined gene expression, proteins that are involved in IPC differentiation and insulin secretion by Immunocytochemistry (ICC), insulin and C peptide secretion by chemiluminesance and finally the response of IPCs in 2D and 3D culture systems to glucose challenge test.

2. Materials & methods

All the materials were purchased from Sigma Company (Sigma, Basel, Switzerland) unless those otherwise stated. Ethical approval has been granted by the Institutional Ethical Review Board with approval number of 69-1780 at Kerman University of Medical Sciences, Kerman, Iran.

2.1. hUCM isolation and culture

In this study were used frozen batches of hUCMs that their mesenchymal properties has been confirmed by Flowcytometry on CD34, CD45, CD73, CD105 and CD90, and their stemness potential has been confirmed by osteogenic and adipogenic differentiation (Seyedi et al., 2016). After thawing and viability assessment, the cells were cultured in DMEM/F12 supplemented with 10% FBS, (Gibco), 100 IU/mL P/S and 2 μg/mL amphotericin B. The culture media was refreshed every 72 h.

2.2. hUCMs differentiation into IPCs

In the 2D group 2 × 105 viable hUCM cells at passage 4–6 were cultured in a 25-cm^2 tissue culture flask. After 80% confluence, the medium was replaced by pancreatic medium. In the fibrin scaffold group, 2 × 105 viable hUCM cells were suspended in 50 μL fibrinogen. The suspension was aspirated into an insulin Syringe with cut end, and 30 μL thrombin was then added. After several pipetting, the insulin Syringe was incubated for 10 min inside a 37 ○C, CO_2 incubator. The fibrin clot containing stem cells were pushed out of the syringe. Fibrin clot was transferred in 24 well culture plate and the pancreatic medium was added. The culture continued for 14 days and the medium was refreshed every 3 days. We used a previously described three steps pancreatic induction medium with few modifications (Seyedi et al., 2015) as follows.

Stage 1, DMEMF12 + 10% FBS for 3 days; stage 2, neural conditional medium (NCM) for 5 days, and stage 3, serum-free DMEM/F12 medium with 17.5 mM glucose, 10 nM pentagastrin, 10 mM nicotinamide, 10 nM exendin-4, 100 pM hepatocyte growth factor, B-7 serum-free supplement (Gibco) and 1% penicillin/streptomycin.

The cells in 2D culture were assessed daily by an inverted microscope (Nikon Ts100, Japan) but in 3D culture, it could not be performed due to the opacity of the fibrin clot.

2.3. Gene expression

Total RNA was extracted using Total RNA purification kit (Jena Bioscience). DNA contamination was eliminated by exposure of RNA samples to DNase I (Fermentas). cDNA was synthesized using 100 ng of total RNA by Accupower Cycle Script RT Premix kit (Bioneer) at 20 °C for 1 min and 46 °C for 4 min in 12 cycles, followed by inactivation at 95 °C for 5 min. Real time PCR was performed by using real time RT-PCR machine (Qiagen, UK, Rotor Gene 6000) and the primers that are listed in Table 1. Two microliters forward and reverse primers, 2 μl cDNA, 6 μl DEPC water and 10 μl Accutpower 2× green star qPCR master mix (Bioneer) were placed in an opaque tube. Every reaction was carried out in duplicate. The mixture was placed in real time RT-PCR machine and 45 cycles were run. Each cycle consisted of 15 s of denaturation at 95 °C and 40 s annealing/extension at 58–60 °C. In all the experiments, a no template control (NTC) was used. At the end of real time PCR reaction, thermal cycler drew a melting curve and reported CT for every sample. After ΔΔCT calculating, gene expression was computed in every sample by $2^{-\Delta\Delta CT}$ formula.

Table 1
Primer sequence for real time PCR.

Gene		Primer sequence	Size (bp)
NKX2.2	F	ATGTAAACGTTCTGACAACT	228
NKX2.2	R	TTCCATATTTGAGAAATGTTTGC	
PDX1	F	CTGTGCTCCAGTTCCACACT	163
PDX1	R	ACAGCCTCTACCTCGGAACA	
Insulin	F	TTCTTCTACACACCCAAGAC	192
Insulin	R	CTAGTTGCAGTAGTTCTCCA	
GAPDH	F	TGCACCACCAACTGCTTAGC	87
GAPDH	R	GGCATGGACTGTGGTCATGAG	

2.4. Immunocytochemistry analysis of insulin and proinsulin

In the monolayer group, the induced cells were cultured on glass slide and fixed by 4% paraformaldehyde. In the fibrin group, after histological preparation and paraffin blocking, 5 μm sections were prepared. Tris-EDTA buffer was applied as antigen retrieval for insulin and EDTA buffered was used as antigen retrieval for proinsulin. Both groups were incubated with PBS containing 0.5% Triton X-100 and 10% goat serum for 45 min at 37 °C. They were then incubated with primary guinea pig anti-insulin antibody (Abcam1:100, Ab7842) and Anti-proinsullin antibody (Ab7761, 1:200) at 4 °C for 24 h. On the following day, the cells were washed three times with PBS. Incubated for 45 min at room temperature with Alexa-fluor 488 Goat anti-guinea pig IgG (Ab150185) and Alxa-flour goat anti mouse IgG (Invitrogen). The cells nuclei were visualized by 5 μg/mL of Hoescht 33258 for 10 min. The slides were assessed under a fluorescence-inverted microscope equipped with a digital camera (DP71, Olympus, Japan).

2.5. Glucose challenge test

The induced cells were cultured in low glucose DMEM medium supplemented with 2% FBS for 12 h. For detection of insulin secretion and glucose sensitivity test, the cells in 2D and 3D systems were exposed to high and low glucose medium (25 mM and 3 mM) for 2 h, respectively. The supernatant was collected and the insulin and C-peptide were measured by human insulin CLIA kit (KA2801, abnova) and C-Peptide EIA Kit (k4757, Biovision) respectively, as recommended by the manufacturer. Cells with no pancreatic induction were used as negative control, and human Langerhans pancreatic cells were used as positive control.

2.6. Statistical analyses

Experiments were repeated three times. The data were analyzed by one-way ANOVA and Tukey post-hoc test. A difference of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Morphological changes of the IPCs

In the 2D group, under pancreatic induction, gradual changes were observed in the induced hUCM cells; spindle shaped adherent hUCM cells shifted into round adherent cells. These cells tended to