Differentiation 82 (2011) 144–152

Contents lists available at ScienceDirect

Differentiation

journal homepage: www.elsevier.com/locate/diff

The simplest method for *in vitro* β-cell production from human adult stem cells

Dilli Ram Bhandari [a,b], Kwang-Won Seo [a,b,c], Bo Sun [a,b], Min-Soo Seo [a,b], Hyung-Sik Kim [a,b], Yoo-Jin Seo [a,b], Jurga Marcin [d], Nicolas Forraz [d], Helene Le Roy [d], Denner Larry [e], McGuckin Colin [d], Kyung-Sun Kang [a,b,c,*]

[a] *Adult Stem Cell Research Center, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea*
[b] *Laboratory of Stem Cell and Tumor Biology, Department of Veterinary Public Health, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea*
[c] *Research Institute for Veterinary Science, College of Veterinary Medicine, Seoul National University, Seoul, Republic of Korea*
[d] *Cell Therapy Research Institute, Parc Technologique de Lyon-Saint Priest, Woodstock Batiment Cedre 1, 97 Allee Alexandre Borodine, 69800 Saint Priest, France*
[e] *Department of Biochemistry and Molecular Biology, The University of Texas Medical Branch, Galveston, TX 77555, USA*

ARTICLE INFO

Article history:
Received 9 February 2011
Received in revised form
10 May 2011
Accepted 21 June 2011
Available online 22 July 2011

Keywords:
Diabetes
Differentiation
Duration
Insulin
Protocol
Stem cells

ABSTRACT

Diabetes mellitus is a challenging autoimmune disease. Biomedical researchers are currently exploring efficient and effective ways to solve this challenge. The potential of stem cell therapies for treating diabetes represents one of the major focuses of current research on diabetes treatment. Here, we have attempted to differentiate adult stem cells from umbilical cord blood-derived mesenchymal cells (UCB-MSC), Wharton's jelly-derived mesenchymal stem cells (WJ-MSC) and amniotic epithelial stem cells (AE-SC) into insulin-producing cells. The serum-free protocol developed in this study resulted in the differentiation of cells into definitive endoderm, pancreatic foregut, pancreatic endoderm and, finally, pancreatic endocrine cells, which expressed the marker genes SOX17, PDX1, NGN3, NKX6.1, INS, GCG, and PPY, respectively. Detection of the expression of the gap junction-related gene connexin-36 (CX36) using RT-PCR provided conclusive evidence for insulin-producing cell differentiation. In addition to this RT-PCR result, insulin and C-peptide protein were detected by immunohistochemistry and ELISA. Glucose stimulation test results showed that significantly greater amounts of C-peptide and insulin were released from differentiated cells than from undifferentiated cells. In conclusion, the methods investigated in this study can be considered an effective and efficient means of obtaining insulin-producing cells from adult stem cells within a week.

1. Introduction

Stem cell therapy represents a promising tool for the treatment of incurable diseases in the near future because of the unique characteristics of these cells related to the differentiation, regeneration, development, remodeling and replenishment of aged and diseased tissues. Stem cells derived from tissues other than embryos are referred to as adult stem cells. Tissues such as bone-marrow, blood, brain, and placenta are sources of adult stem cells. The umbilical cord, umbilical cord blood, and placenta become medical waste products following the birth of a baby, and these tissues represent rich sources of stem cells (Murphy et al., 2011; Magatti et al., 2008). Stem cells from these sources have the unique characteristics of being associated with a low risk of graft-versus-host diseases (GVHD), a lack of related ethical issues and easy availability (Zhao and Mazzone, 2011). In comparison, handling embryonic stem cells is more complex and is associated with ethical concerns. Because of the unique characteristics of stem cells from afterbirth byproduct origins, they have obtained a prominent position in the biomedical research field as limitless source of stem cells. Several clinical trials have already been performed in different diseases, including diabetes (Wei et al., 2003), spinal cord injuries (Kang et al., 2005), cardiac diseases (Copeland et al., 2009), lung injuries (Moodley et al., 2011), and neural diseases (Seo et al., in press), in animal models and in human trials.

Pancreatic β-cells are responsible for maintaining glucose homeostasis in the human body. Autoimmune destruction of

Abbreviations: AE-SC, amniotic epithelial stem cells; BSA, bovine serum albumin; CX36, connexin-36; DM, diabetes mellitus; EBM-2, endothelial cell basal medium-2; EGM-2, endothelial cell growth medium-2; FGF7, fibroblast growth factor7; GCG, glucagon; GVHD, graft-versus-host disease; hES, human embryonic stem cells; hUCB-MSC, human umbilical cord blood-derived mesenchymal stem cells; IHC, immunohistochemisty; INS, insulin; IPCs, insulin-producing cells; KGF, keratinocyte growth factor; mES, mouse embryonic stem cells; MSC, mesenchymal stem cells; NGN3, neurogenin 3; NKX6.1, NK6 homeobox 1; PDX1, pancreatic and duodenal homeobox 1; PPY, pancreatic polypeptide; SOX17, sex determining region Y-box 17; UCB, umbilical cord blood; UCB-MSC, umbilical cord blood-derived mesenchymal cells; UC-MSC, umbilical cord-derived mesenchymal stem cells; WJ, Wharton's Jelly; WJ-MSC, Wharton's Jelly-derived mesenchymal cells

* Corresponding author at: Adult Stem Cell Research Center, College of Veterinary Medicine, Seoul National University, 151-742 Seoul, Republic of Korea. Tel.: +82 2 880 1298; fax: +82 2 876 7610.
E-mail address: kangpub@snu.ac.kr (K.-S. Kang).

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doi:10.1016/j.diff.2011.06.003

β-cells causes diabetes mellitus (DM). It has been reported that 4–5% of world population suffers from DM (Shi et al., 2005). Therefore, there is an urgent need to develop reliable diabetes therapies. Testing blood glucose and injecting insulin represent life-saving therapies that have been practiced for a number of years. Other approaches, such as tissue- and cell-based therapies, have been employed to restore the functional β-cells. Among these methods, pancreatic islet transplantation is considered as a better means of treatment than insulin injection (Serup et al., 2001), but it is also associated with limitations of severe immunosuppression, a lack of sufficient numbers of islet donors and high economic costs. Current studies are focused on cell-based therapies, for which stem cells are the best option because of their therapeutic potential. In this regard, the development of limitless sources of stem cells represents a possible permanent way out for therapy of DM. Research in this field has been aimed at differentiating mouse embryonic stem cells (mES) (Shi et al., 2005; Lumelsky et al., 2001), human embryonic stem cells (hES) (Jiang et al., 2007b, 2007a; D'Amour et al., 2006), umbilical cord blood (UCB) (Gao et al., 2008), umbilical cord-derived mesenchymal stem cells (UC-MSC) (Wang et al., 2011), and AE-SC (Wei et al., 2003) into insulin-producing cells. The time required for differentiation into insulin-producing cells (IPCs) is variable in every study according to the methods of differentiation and cell sources used. Shortening the time required for differentiation is important to improve the efficiency of the differentiation process because it can save costs and diminish the possibilities of contamination. Because of their unique characteristics and the fact that they are abundantly available, UCB-MSC, Wharton's jelly-derived mesenchymal stem cells (WJ-MSC) and AE-SC were considered in the current study.

In the present study, we investigated whether the duration of differentiation of the most readily available and potentially unlimited source of stem cells in serum-free culture conditions could be shortened for the purpose of making their production more economic, efficient and time and labor saving.

2. Materials and methods

2.1. Cell isolation and culture

Human amniotic tissue was obtained from Guro Korea Medical Hospital (Seoul, Korea) under informed consent, and isolation and culture were performed with the approval of the Seoul National University Institutional Review Board (IRB no. 0611/001–002). The amniotic tissue was washed several times with PBS to remove blood and incubated with 0.05% trypsin–EDTA (Invitrogen, Carlsbad, USA) for 1 h. The amniotic epithelial cells were collected and suspended in standard culture media consisting of K-SFM (Keratinocyte-SFM) supplemented with 0.031 μg/μl human recombinant EGF, 12.4 mg/mL bovine pituitary extract (all from Invitrogen) and 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA).

Human umbilical cord blood-derived MSC (hUCB-MSCs) collection, isolation, and characterization was performed as described previously (Seo et al., 2009; Bhandari et al., 2011a, 2011b). For WJ-MSC isolation and culture, we followed the methods explained previously (Wang et al., 2004). In brief, after removing blood vessels from umbilical cord, mesenchymal tissues were scraped off with scalpel from the Wharton's jelly and centrifuged at 2000 rpm for 5 min at room temperature. The pellet was washed with serum-free DMEM and resuspended in 10 mL of DMEM at 2000 rpm for 5 min at room temperature. Then, this pellet was resuspended in 15 mL of DMEM containing 0.2 g/mL of collagenase and incubated for 16 h at 37 °C. Cells were washed, resuspended in 10 mL of DMEM containing 2.5% trypsin, incubated 30 min at 37 °C with agitation, washed and cultured in DMEM-HG containing 10% FBS in 5% CO_2 incubator. Cells were passaged before 90% confluency. After thawing, all UCB-MSC, WJ-MSC, and AE-SC were cultured in growth medium. Our growth medium contained Endothelial Cell Basal Medium-2 (EBM-2) (#CC-4176, Lonza, Walkersville, MD, USA) and the growth factor EGM-2 SingleQuots catalog# CC-4176 (Lonza) supplemented with 10% FBS (#16000-044, Invitrogen). Cells were detached with $1 \times 0.25\%$ trypsin–EDTA (#25200-072, Invitrogen) incubated for 5 min in an incubator and collected with phosphate-buffered saline (PBS), then spun down at the rate of 2000 rpm for 5 min. The supernatant was removed, and cells were resuspended with the above-mentioned medium and counted using a hemocytometer chamber. Subculturing was performed in the same medium with 1×10^6 cells per 100-mm diameter NUNC cell culture dish, and cell were cultured until 60–70% confluency.

2.2. In vitro differentiation of cells

All three cell lines, UCB-MSC, WJ-MSC, and AE-SC, were grown to 60–70% confluency in growth medium with 10% FBS. Then the medium was changed into differentiation medium. For differentiation, EBM-2 (Lonza) supplemented with EGM-2 (Lonza) was used as basal differentiation medium. Our differentiation protocol is divided into two stages. **Stage-1**: The 70% confluency cells were washed two times with PBS and cultured for 24 h in basal differentiation medium supplemented with 1 mM sodium butyrate (Sigma-Aldrich, St. Louis, MO, USA) and 50 ng/mL Activin A (R&D system). The next day, sodium butyrate was withdrawn. The medium was changed with fresh basal differentiation medium supplemented with 0.1–0.2% bovine serum albumin (Sigma-Aldrich) and 50 ng/mL Activin A for two more days. **Stage-2:** Basal differentiation medium was supplemented with 50 ng/mL KGF/FGF7 and 1% BSA to prepare stage-2 medium. Before changing into stage-2 medium, cells were washed twice with PBS. Control cells were grown in basal differentiation medium supplemented with 10% FBS. Images of the cells were collected at the end of each stage using an Olympus CKX41 microscope (Olympus, Tokyo, Japan).

2.3. RT-PCR

Total RNA was extracted with an easy-spin™ Total RNA Extraction Kit (iNtRON biotechnology, Sungnam, Korea) according to the manufacturer's instructions. cDNA synthesis was performed using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen) with 2 μg of total RNA and oligo dT. The primers used for each gene and the annealing temperatures and number of cycles are shown in Table 1. A Hot-Start Taq (#203603, Qiagen) kit was used for RT-PCR amplification. RT-PCR products were loaded into 1.5–2.5% agarose gels, stained with ethidium bromide and photographed under ultraviolet light in a Gene Flash (Syngene Bio Imaging, Cambridge, UK) machine.

2.4. Immunofluorescence staining and quantification

For immunofluorescence staining, 3×10^4–5×10^4 cells/well were cultured on a 4-well chamber slide and differentiated according to the differentiation protocol. At the end of differentiation, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with PBS twice, cells were incubated with blocking solutions (10% normal goat serum in PBS) overnight at 4 °C. Cells were washed two more times with PBS and then incubated with anti-mouse insulin antibodies (#ab8302, Abcam, Cambridge, MA, USA) at a 1:100 dilution and anti-rabbit C-peptide (#4020-01, Linco) at a dilution of 1:200 in 5% blocking solution at room temperature