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Valproic acid promotes differentiation of hepatocyte-like cells from whole human umbilical cord-derived mesenchymal stem cells

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ABSTRACT

Mesenchymal stem cells (MSCs) are mesoderm-derived cells that are considered a good source of somatic cells for treatment of many degenerative diseases. Previous studies have reported the differentiation of mesodermal MSCs into endodermal and ectodermal cell types beyond their embryonic lineages, including hepatocytes and neurons. However, the molecular pathways responsible for the direct or indirect cell type conversion and the functional ability of the differentiated cells remain unclear and need further research. In the present study, we demonstrated that valproic acid (VPA), which is a histone deacetylase inhibitor, induced an increase in the expression of endodermal genes including CXCR4, SOX17, FOXA1, FOXA2, GSC, c-MET, EOMES, and HNF-1β in human umbilical cord derived MSCs (hUCMSCs). In addition, we found that VPA is able to increase these endodermal genes in hUCMSCs by activating signal transduction of AKT and ERK. VPA pretreatment increased hepatic differentiation at the expense of adipogenic differentiation. The effects of VPA on modulating hUCMSCs fate were diminished by blocking AKT and ERK activation using specific signaling inhibitors. Together, our results suggest that VPA contributes to the lineage conversion of hUCMSCs to hepatic cell fate by upregulating the expression of endodermal genes through AKT and ERK activation.

1. Introduction

Human embryonic stem cells (hESCs) and mesenchymal stem cells (MSCs) have ability to self-renew and differentiate into many different cell types. hESCs are derived from the inner cell mass of preimplantation embryos and are capable of giving rise to almost all cell types (Biswas and Hutchins, 2007). We have previously demonstrated that neurons, pancreatic and hepatic cells derived from ESCs improved organ functions after transplantation into animal model of diseases (Kim et al., 2002; Shim et al., 2007; Woo et al., 2012). In contrast hESCs, MSCs, which are derived from fetal or adult mesodermal tissues, have limited proliferative and differentiation capacities and are considered a source of somatic cells for the treatment of several diseases or injuries, including fracture nonunions, osteogenesis imperfecta, hypophosphatasia, and osteoarthritis (Gupta et al., 2012; Undale et al., 2009; Zhang et al., 2012). Recently, human umbilical cord-derived MSCs (hUCMSCs) have received much attention as an ideal cell source for clinical use since they have many advantages, including easy procurement, lower risk of viral contamination and tumor formation, non-invasive accessibility, low immunogenicity, and abundant availability (Heijnen et al., 2012). The umbilical cord harbors different types of MSC-like populations in several compartments, including Wharton's jelly, the subendothelium of umbilical veins, and the perivascular region, and most previous studies have focused on hUCMSCs derived from entire umbilical cord tissues or from Wharton's jelly (Can and Karahuseyinoglu, 2007).

Recent studies have expanded application to include the ability to induce differentiation of MSCs *in vitro* into cells of ectodermal and endodermal origins (Charbord, 2010; Dhanasekaran et al., 2013). In addition, previous studies have reported differentiation of mesodermal MSCs into several endodermal cell types beyond their embryonic lineages, including hepatocytes (Campard et al., 2008; Seo et al., 2005; Talens-Visconti et al., 2006). In general, the differentiation of MSCs into hepatocytes has been induced by serum-free differentiation medium containing several growth factors and cytokines, such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), oncostatin M (OSM), and dexamethasone (DEX) (Campard et al., 2008; Seo et al., 2005; Talens-Visconti et al., 2006). However, contrary to embryonic stem cells (ESCs), the hepatic differentiating ability of MSCs is limited with low efficiency. Hepatocyte-like cells derived from MSCs showed low expression levels of mature hepatocyte related genes and proteins. Also, secreted albumin, gamma-glutamyl transpeptidase, and CYP

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activity were not detected in previous studies (Campard et al., 2008; Seo et al., 2005).

To overcome these obstacles, many studies have reported the hepatic differentiation using small molecules (less than 5000 molecular weight), including trichostatin A, sodium butylate, and valproic acid (VPA) as non-viral agents based on their safe and convenient differentiation across a broad lineage spectrum (Chen et al., 2009; Snykers et al., 2007, 2009). VPA is a histone deacetylase inhibitor and is used as an antiepileptic and anticonvulsant drug for various disorders such as schizoaffective disorders, social phobias, and neuropathic pain (Talens-Visconti et al., 2006). In recent reports, VPA significantly improved the *in vitro* hepatic differentiation of mouse ESCs and mouse bone marrow stromal stem cells (Chen et al., 2009; Dong et al., 2009). These studies demonstrated that VPA treatment could regulate the gene expression patterns of fibroblast growth factor receptors (FGFR-1IIIc and FGFR-2IIIc) and c-MET in mouse bone marrow stem cells (BMSCs). The results suggested that the upregulated gene expression of FGFR and c-MET induced by VPA may significantly contribute to hepatic differentiation (Chen et al., 2009). However, the molecular mechanism of VPA on hepatic differentiation from stem cells is not well known and few studies have tested the ability of VPA to differentiate human MSCs into hepatocytes.

The purpose of this study was to investigate the effects of VPA on hepatic differentiation of hUCMSCs and to suggest possible mechanisms of VPA contributing to the lineage conversion from hUCMSCs to hepatic cell fate.

2. Materials and methods

2.1. Isolation and culture of hUCMSCs

Human umbilical cord derived mesenchymal stem cells were obtained from HurimBioCell Inc. after normal deliveries. Tissue collection for research was approved by the institutional review board of HurimBioCell Inc. The cords were drained of all blood and washed in saline with 1% penicillin-streptomycin (GIBCO®, Grand Island, NY, USA). Whole umbilical cord tissues were minced into very fine fragments without removing the vessels. Minced tissues were directly plated in a culture flask. After 7 days, the tissues were removed and the medium was replaced. When cells were confluent, they were subcultured using a trypsin solution. The cells were then seeded into culture flasks in MSC-qualified medium (Invitrogen, MesenPro RSTM medium, Carlsbad, CA, USA) at 1×10^6 cells/cm^2.

2.2. Treatment of small molecules

Small molecules used in the present study were VPA (Sigma–Aldrich, St. Louis, MO, USA), PD0325901 (PD, Stemgent, Cambridge, MA, USA), LY294002 (LY, Stemgent), MK-2206 (Selleckchem, Houston, TX, USA), API-2 (Sigma–Aldrich), bromocriptine (Sigma–Aldrich) and fisetin (Sigma–Aldrich). All small molecules were added in serum-free medium, high-glucose DMEM/F12 supplemented with 1 mM nonessential amino acids (GIBCO®), and 0.1 mM β-mercaptoethanol (Sigma–Aldrich). hUCMSCs were pretreated with 10 mM VPA, 1 μM PD, 50 μM LY, 2 μM MK-2206, 10 μM API-2, 10 μM bromocriptine and/or 20 μM fisetin for 6 h to induce differentiation.

2.3. Differentiation procedures

2.3.1. Adipogenic and osteogenic differentiation

hUCMSCs at passage 3–5 were plated at a density of 1×10^4 or 3×10^4 cells/cm^2 in expansion medium for 24 h, and then cultured in STEMPRO® adipocyte and osteocyte differentiation medium (GIBCO®) for 4 weeks. After 4 weeks, lipid vesicles and calcium deposition were evaluated by Oil Red O and Alizarin Red staining. Quantitative analysis of Oil Red O positive cells was measured using a microplate spectrophotometer (520 nm).

2.3.2. Chondrogenic differentiation

hUCMSCs at passage 3–5 were cultured by making droplets of 8×10^4 cells/droplet in culture medium for 24 h. The next day, cell pellets were transferred to suspension dishes and cultured in STEMPRO® chondrogenesis differentiation medium (GIBCO®). Proteoglycans of chondrocytes were stained by Alcian Blue dye after 4 weeks.

2.3.3. Hepatic differentiation

hUCMSCs at passage 3–10 were seeded at a density of 3×10^4 cells/cm^2 in plates coated with rat tail collagen type I (Sigma–Aldrich) in MesenPro RSTM medium (GIBCO®). Hepatic differentiation was induced by culturing hUCMSCs in ITS medium (Kim et al., 2003). Supplemented with 20 ng/ml hepatocyte growth factor (R&D systems, Minneapolis, MN, USA), 10 ng/ml oncostatin M (Sigma–Aldrich) and 10^{-6} mol/L dexamethasone (Sigma–Aldrich) for 15 days. Hepatic differentiation was confirmed by immunofluorescence staining with anti-human albumin and Periodic Acid Schiff stain (PAS).

2.3.4. Flow cytometry

Undifferentiated and VPA-treated hUCMSCs were analyzed by flow cytometry for cell surface and cytosolic antigen expression. Undifferentiated cells, at the third passage, were incubated with fluorescent isothiocyanate [FITC]-conjugated antibodies for against monocyte marker (CD14; BD Bioscience, San Jose, CA, USA), B-lymphocyte marker (CD19; BD Bioscience), endothelial cell marker (CD31; BD Bioscience), hematopoietic stem cell marker (CD34; BD Bioscience), lymphocyte marker (CD45; BD Bioscience), mesenchymal stem cell markers, CD44 (BD Bioscience), CD73 (eBioscience, San Diego, CA, USA), CD90 (BD Bioscience), CD105 (eBioscience), and CD146 (BD Bioscience), or mouse immunoglobulin G isotype control (eBioscience) for 30 min at 4 °C. VPA treated cells were dissociated and resuspended with PBS with 1% FBS. After washing, the cells were analyzed by FACS-Calibur (BD Bioscience) with FlowJo software (http://www.flowjo.com/; Tree Star Inc., Ashland, OR, USA).

2.4. Determination of cell viability and proliferation

2.4.1. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay

For the viability assay, 5×10^3 cells/well were plated in 96-well plates and cultured with MesenPro RS™ medium for 48 h. The culture medium was changed to serum-free medium with/without VPA (Sigma–Aldrich) for different times (3, 6, 12, 24, 48, 72, 96 h). The medium was then removed and MTT reagent (Sigma–Aldrich) was added to the cells and incubated at 37 °C in 5% CO_2 and 95% air. After 2 h incubation, the MTT solution was removed, and 100 μl/well of dimethyl sulfoxide (DMSO; Sigma–Aldrich) was added. The absorbance of the reduced form of MTT was measured at 550 nm and 650 nm in a Microplate Spectrophotometer (Bio-Tek Inc., Winooski, VT, USA).

2.4.2. 5-Ethynyl-2′-deoxyuridine (EdU) incorporation assay

For measuring cell proliferation using EdU experiments, hUCMSCs were seeded at 1.5×10^5 cells/well in 6-well plates and cultured for a day. Then the medium was changed to serum-free medium containing 10 μM EdU, with or without VPA (Sigma–Aldrich) for 6 h. EdU incorporation was performed and analyzed using the Click-iTH EdU Imaging Kit (Invitrogen). After EdU incorporation, the