Ultrasound in Med. & Biol., Vol. ■■, No. ■■, pp. ■■–■■, 2018

0301-5629/$ - see front matter

https://doi.org/10.1016/j.ultrasmedbio.2018.01.005

ULTRASOUND IRRADIATION COMBINED WITH HEPATOCYTE GROWTH FACTOR ACCELERATE THE HEPATIC DIFFERENTIATION OF HUMAN BONE MARROW MESENCHYMAL STEM CELLS

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(*Received* 31 *October* 2017; *revised* 3 *January* 2018; *in final form* 12 *January* 2018)

Abstract—This study investigated the impact of ultrasound (US) irradiation on the hepatic differentiation of human bone marrow mesenchymal stem cells (hBMSCs) induced by hepatocyte growth factor (HGF) and the possible mechanisms. We treated hBMSCs, using HGF with and without US irradiation. Cell viability and stem cell surface markers were analyzed. Hepatocyte-like cell markers and functional markers including α-fetoprotein (αFP/AFP), cytokeratin 18 (CK18), albumin (ALB) and glycogen content were analyzed at the time point of day 1, 3 and 5 after treatment. The involvement of Wnt/β-catenin signaling pathway was evaluated as well. The results showed that the US treatment at 1.0 W/cm^2 or 1.5 W/cm^2 for 30 s or 60 s conditions yielded favorable cell viability and engendered stem cell differentiation. At day 5, the expressions of AFP, CK18, ALB and the glycogen content were significantly elevated in the US-treated group at both messenger ribonucleic acid and protein levels (all $p < 0.05$), in comparison with HGF and control groups. Among all the US treated groups, the expression levels of specific hepatic markers in the (1.5 W/cm^2 for 60 s) group were the highest. Furthermore, Wnt1, β-Catenin, c-Myc and Cyclin D1 were significantly increased after US irradiation (all $p < 0.05$), and the enhancements of c-Myc and Cyclin D1 could be obviously impaired by the inhibitor ICG-001 ($p < 0.05$, $p < 0.05$), in accordance with decreased ALB and CK18 expression and glycogen content (all $p < 0.05$). In conclusion, US irradiation was able to promote the hBMSCs' differentiation mediated by HGF *in vitro* safely, easily and controllably. The activation of Wnt/β-catenin signaling pathway was involved in this process. US irradiation could serve as a potentially beneficial tool for the research and application of stem cell differentiation. (E-mail: lianfang_du@126.com)

Key Words: **Stem cells, Differentiation, Ultrasound, Hepatocyte growth factor, Signaling pathway, Hepatocyte.**

INTRODUCTION

Bone marrow mesenchymal stem cells (BMSCs) are multipotent progenitor cells that have self-renewal capacity, long-term viability and differentiation potential toward diverse cell types. They are easily obtained and manipulated, rejection can be avoided by autologous transplantation and no ethical issues are linked to their use (Zaher et al. 2014). As BMSCs can differentiate into hepatocyte-like cells *in vitro*, have the potential to migrate into the injured liver and improve liver function, they have become an important tool for studying the genesis and development of the liver or the related drug research, which also brings to light cell therapy of liver failure (King and Newsome 2014; Russo and Parola 2012). However, it is reported that the differentiation ability of BMSCs *in vitro* could be gradually decreased as the donor age or cell passage number increases and the *in vitro* cultivation is prolonged (Kretlow et al. 2008; Li et al. 2011; Morrison and Scadden 2014). Therefore, investigating an efficient way of promoting differentiation of BMSCs *in vitro* is of great importance not only for research but also for clinical use.

It has been shown that ultrasound (US) irradiation was able to improve the osteogenic and chondrogenic differentiation abilities of BMSCs coupling with high polymer material or growth factors (Choi et al. 2013; Guha Thakurta et al. 2015; Marvel et al. 2010; Wang et al. 2014). For the hepatic differentiation of BMSCs, hepatocyte growth factor (HGF) was regarded as one of the most important inducing factors in the bone marrow microenvironment. HGF could be used alone or in combination with other cytokines to induce BMSCs into hepatocyte-like cells (Chivu et al. 2009; Snykers et al. 2009). In this study, we investigated the effect of coupling the US irradiation with the HGF in stimulating the differentiation of BMSCs into hepatocyte-like cells.

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MATERIALS AND METHODS

Cell culture and treatment

We purchased human bone marrow mesenchymal stem cells (hBMSC) from ScienCell Research Laboratories (San Diego, CA, USA) and cultured with preconfigured compound medium including mesenchymal stem cell medium (ScienCell Research Laboratories), 10% fetal bovine serum (ScienCell Research Laboratories), 1% mesenchymal stem cell growth supplement (ScienCell Research Laboratories) and 1% penicillin/streptomycin solution (ScienCell Research Laboratories). Aliquots containing 5×10^4 cells were inoculated in 24-well plates (Costar, Cambridge, MA, USA). The cells were then incubated in a culture incubator (Sanyo, Moriguchi, Osaka, Japan) at 37°C in a humidified atmosphere (5% CO_2/95% air) and allowed to grow to 80%–85% confluence before transfection. Passage 2–3 hBMSCs were used in all experiments.

To investigate the involvement of the Wnt signaling pathway in hepatic differentiation of hBMSCs under HGF and US exposure, the cells were pretreated with Wnt signaling inhibitor ICG-001 (10 μM) (Selleckchem, Houston, TX, USA) for 30 minutes, followed by the independent treatment of HGF with and without US exposure. Culture medium was refreshed every other day. Five days later, total cell protein or ribonucleic acid was harvested from hBMSC cells and analyzed for Wnt 1, β-Catenin, Cyclin D1, c-Myc, Albumin (ALB), Cytokeratin 18 (CK18), Alpha-Fetoprotein (αFP/AFP) and glycogen content.

US irradiation treatment

We used a therapeutic US apparatus, Physioson (Physiomed Elektromedizin AG, Schnaittach, Bavaria, Germany), with 1-MHz frequency, 100-Hz pulse repetition frequency, 20% duty cycle and 25 mm^2 cross-sectional area. The experimental facilities were fixed in a supporter with a hole on the upper surface where the transducer could be placed. The culture plate was put onto the surface of the transducer. We coated 1- to 3-cm-thick couplants to form the conductive pathway of US waves from transducer to cells. Immediately after the addition of 20-ng/mL HGF (Sigma-Aldrich, St. Louis, MO, USA) into culture plate wells, the cells were irradiated with US (US). The experimental parameters were adjusted in the range of 0.5–1.5 W/cm^2 of US intensity, 30–120 seconds of US exposure time, respectively.

After optimizing US parameters, the cells were divided into the following six groups: (i) the blank group (only BMSCs), (ii) the HGF group (BMSCs treated with HGF but no US exposure), (iii) the US1 group (BMSCs treated with HGF and 1.0 W/cm^2 & 30 s US exposure), (iv) the US2 group (BMSCs treated with HGF and 1.0 W/cm^2 & 60 s US exposure), (v) the US3 group (BMSCs treated with HGF and 1.5 W/cm^2 & 30 s US exposure) and (vi) the US4 group (BMSCs treated with HGF and 1.5 W/cm^2 & 60 s US exposure).

Cell viability and cell surface markers expression

Cell viability was determined using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Kumamoto, Japan). An aliquot containing 5×10^4 cells was inoculated into each well of a 24-well plate (Costar, Cambridge, MA, USA). The culturing medium was replaced with CCK-8 transfection solution (10 μL/mL HBSS) in each well 24 h and 48 h after the US treatment. The supernatants were transferred to a 96-well plate (Costar) 1 h after the replacement. The optical density of each well was measured at 450 nm, using a SpectraMax 190 Microplate Reader (Molecular Devices, San Francisco, CA, USA) and cell viability was calculated according to optical density 450 values, background absorbance (from wells without cells) was subtracted from all values.

Adherent cells were detached with 0.25% trypsin 48-h later, suspended in phosphate-buffered saline (PBS) at 10^6 cells/mL and incubated for 60 min at 4 °C with monoclonal antibodies of CD34-APC (BD Biosciences, Franklin Lakes, NJ, USA), CD45-PE (BD Biosciences), CD29-FITC (BD Biosciences) and CD105-PE (BD Biosciences). After incubation, the excess of antibodies was washed with filtered PBS. The fluorescence-activated cell sorting (FACS) analysis was performed using a flow cytometer (BD Biosciences), which was calibrated according to the manufacturer's recommendations, then analyzed with FlowJo 7.6.1 software (Tree Star, Ashland, OR, USA). Each assay included at least 10,000 gated events.

Immunofluorescence staining

After the treatment of HGF with or without optimal US for 5 d, the cells cultured on glass coverslips were sequentially incubated with 4% paraformaldehyde (Sigma-Aldrich), 0.5% triton X-100 and 0.5% bovine serum albumen and PBS. The primary antibodies used in this study included mouse antibody against AFP (1:100) (Cell Signaling Technology, Danvers, MA, USA), rabbit antibody against ALB (1:100) (Proteintech, Rosemont, IL, USA) and rabbit antibody against CK18 (1:100) (Proteintech) and incubated the cells overnight at 4 °C. The secondary antibody Goat anti-Rabbit IgG (H + L) (Thermo Fisher,Waltham, MA, USA) and Goat anti-Mouse IgG (H + L) (Thermo Fisher) immersed the cells for 1 h with a dilution of 1:500. Rhodamine phalloidin (1:100 dilution) (Invitrogen, Carlsbad, CA, USA) and DAPI (1:2000 dilution) (Sigma-Aldrich) stained the cells for 20 min. The coverslips overlaying the cells were washed with PBS between each incubation step and then imaged, using a confocal laser scanning microscope (Leica Microsystems,