Lycium Barbarum Polysaccharide Induce the Neuronal Differentiation of Mesenchymal Stem Cell for Potential Application in Neural Regeneration

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ABSTRACT

Lycium barbarum has been considered as a valuable old herbal medicine that has been widely used in Asian country. Lycium barbarum polysaccharides (LBPs), a major component, are proved to possess a variety of beneficial effects including neuro-protection, anticancer properties and anti-aging as well as immune modulation. However, the biological functions and inductive mechanisms of LBP towards the mesenchymal stem cell (MSC) differentiation process have not been fully investigated. In this study, we found that the expression of specific marker of neuron cells including β-tubulin III, NF200 and synapsin was up-regulated when cultured with LBP after 7 days during differentiation of bone marrow-derived MSCs to neuron-like cells, while the pluripotency marker such as Oct4 and Klf4 were down-regulated. By using the staining of phalloidin, the cytoskeleton of the stem cells induced by LBP exhibited lengthening character, resulting in the induction effect of LBP towards BMSCs. Overall, we demonstrate that the directional differentiation process of BMSCs can be partially regulated by LBP. We conclude that this strategy may provide opportunities for physician to improve the effectiveness of stem cell therapy for the treatment of degenerative cerebrovascular disease.

Key Words: Mesenchymal stem cell, Neural regeneration, Lycium barbarum polysaccharide, Tissue engineering

Introduction

Brain impairment may develop to the damage of neural tissue immediately after the initial injury, and then result in permanent neurologic deficit. Most of current studies focus on the damage control, which is also regarded as supportive measure. Therefore, there is an emergent need to repair the dysfunctional neural tissues by introducing novel regenerative strategy (Loane and Faden, 2010). It is well known that the transplantation of stem cells has been thought as a promising therapy for treating the central nervous system (CNS) diseases such as Parkinson's, spinal cord injury, and stroke as well (Calore et al., 2018; Meira et al., 2017; Boltze et al., 2017). Woodbury and Sanchez first reported the potential application of stem cells and harvested from rat bone marrow to differentiate into the neural cells (Schultz and Lucas, 2006). Kim and his colleagues have further successfully proved the expression of neurofilament in human adult MSCs could be induced by the growth factors (Kim et al., 2002). However, the current strategy for the induction of hPSC differentiation mainly focus on the utilization of biochemical factors, which could be easily affected by the in vitro culture conditions, leading to a relative prolonged differentiation process, and lower differentiation effectiveness (Chandrabose et al., 2018). Additionally, the potential damage derived from these chemical inducers cannot be ignored (Nicolas et al., 2017).
Lycium barbarum, named as *Gou qi zi* or *Lycium barbarum*, is a member of Solanaceae family and originated from China. Lycium barbarum polysaccharide (LBP), which is approximately 40% by dry mass of Lycium barbarum, has been considered as the most effective component as polysaccharides (Chang and So, 2008). Previously, LBP has been reported to regulate the immune function (Gan et al., 2003), repair the liver damage (Ha et al., 2005), acts against the aging and oxidation (Ho YS et al., 2010; Li XM et al., 2007), and alleviate the side-effects of chemotherapy (Gong et al., 2010). Although the widespread study of LBP (Li SY et al., 2011; Lu and Zhao, 2010), the study focusing on the inductive mechanism of LBP on the differentiation into the neural like lineage have remained unexplored.

Few studies address the induction of BMSCs differentiation into the neuron cells by using traditional Chinese herbs alone, especially in Lycium barbarum polysaccharide. Hence, our study was aimed to elaborate the inductive effects of LBP on the neural differentiation of BMSCs and whether this strategy could be utilized as a potential alternative for the treatment of central nervous system (CNS) disorders over CNS derived neural stem cells. In our study, we utilized Lycium barbarum polysaccharide as a bio-inducer and explored whether a traditional Chinese medicine LBP would increase the viability of BMSCs and further drive their directional differentiation, thereby enhancing the neural regeneration.

**Materials and Methods**

**BMSCs isolation, culture and characterization**

All the experiments were performed according to the guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee (IACUC) of Three Gorges University, China.

Briefly, the mesenchymal stem cells derived from Sprague-Dawley (SD) rats bone marrow and then transfected with green fluorescent protein (GFP) (passage 2) were purchased from Cyagen Biosciences Inc (USA) and cultured in serum-free MSC growth medium (StemPro-MSC-SFM CTS™, Gibco, Invitrogen, USA) supplemented with 1% penicillin-streptomycin at 37°C with 5% CO₂ and 100% humidity. Cells with 3-4 passages were used for the further experiments. Osteogenic differentiation, adipogenic differentiation and chondrogenic differentiation (Differentiation Kit, Gibco) were performed to characterize the stem cells. Briefly, the cells were plated in 6-well plates (6 × 10⁴ cell/well), and the differentiation medium was added according to the manufacturer’s instructions and changed three times per week. 14 days later, the chondrocytes and adipocytes were stained with Alcian blue and Oil Red O, respectively, and after 28 days, the osteocytes were stained with Alizarin red.

**Purification of LBP**

LBP purified from Lycium barbarum was harvested in Ningxia province of China. The purification process of LBP consists of hot-aqueous extraction, centrifugation, filtration, ethanol precipitation, lyophilization, gradient DEAE-Cellulose (Pharmacia, Piscataway, NJ) (Wong et al., 1994) and subsequent filtration by Amicon 20 kD filtering instrument (the upper chamber was collected). It has been demonstrated that LBP purified by the integrated process will be composed of 5 glycoconjugates (LbGp1-LbGp5) (Peng and Tian 2001).

**Assessment of the viability, morphology, and proliferation of BMSCs after the induction of LBP**

At day 1, 3, 5 and 7 after being cooperating with LBP (1 g/L), a CCK-8 assay was assessed. BMSCs that were cultured with standard medium (without LBP) served as control. To assess the viability of BMSCs, on days 3,7,11, and 16 after administrated with LBP (1 g/L), the cells were incubated with calcine AM (1 mM) for 1 h and next incubated with propidium iodide (1 ug/mL) for 5 min. Finally, the cells were imaged under the fluorescence microscopy (Leica, Germany).

**Real-time quantitative PCR**

BMSCs were cultured in 6-well plates (either with standard medium or LBP) for 7 days. Then, Total RNA was extracted through the Takara MiniBEST Universal RNA Extraction Kit (Japan) according to the manufacturer’s instructions. cDNA was synthesized (PrimeScript™ II First Strand cDNA Synthesis Kit, Japan) by following the manufacturer’s protocol. The running conditions of PCR started with an initial denaturation for 1 min at 95°C, followed by total 40 cycles of 15 s at 95°C, 20 s at 58°C and 20 s at 72°C. After normalization by housekeeping gene ß-actin, the expression of genes of interest (shown in Table 1) was measured by using the 2^-ΔΔCt method.

**Western blotting analyses**

Total protein was isolated from BMSCs administrated with or without LBP on day 7. Equal amounts of
protein from cell were digested and then loaded onto a 5% SDS gel (Aspen, China). Next, they were transferred to a polyvinylidene fluoride (PVDF) membrane. 5% BSA were used to block the extra protein and then the samples were incubated with primary antibodies against β-tubulin III (1:500), NF-200 (1:500) and β-actin (1:10 000) overnight at 4°C. Additionally, anti-Oct4 (1:100) and anti-Klf4 (1:100) were also assessed. Finally, an HRP-conjugated secondary antibody (1:10 000) was added and the Immobilon Western Chemiluminescent HRP Substrate system (Millipore, USA) were used. All of the antibodies were purchased from Abcam Inc (United Kingdom).

Immunofluorescence

BMSCs cultured with or without LBP on day 7 were harvested and fixed in 4% paraformaldehyde for 25 min. Next, the samples were stained with primary antibodies against β-tubulin III (1:100), RT-97 (1:200), NF-200 (1:500), Synapsin (1:500) and β-actin (1:10 000) overnight at 4°C. Additionally, anti-Oct4 (1:100) and anti-Klf4 (1:100) were also analyzed. After incubation with Cy3-conjugated secondary antibodies (1:50, Aspen, China), samples were added with DAPI to stain the cell nuclei.

Enzyme-linked immunosorbent assay (ELISA)

The level of cytokines in the serum-free growth medium of BMSCS and the supernatants of BMSCS cultured with LBP, including bFGF, VEGF and TGF-β in the induction medium (Fig. 2A) and supernatants of BMSCS cultured with LBP after 7 days (Fig. 2B) were calculated by ELISA. The results indicated that the level of growth factors in the serum-free medium and supernatants are negligible to induce the BMSCS differentiation compared with induction medium (p < 0.001). For the biocompatibility, compared with standard culture without LBP, BMSCS cultured with LBP proliferated slightly over seven days (Fig. 2C). Additionally, BMSCS cultured with LBP maintained favorable viability (more than 85.7%) for up to 16 days (Fig. 2D).

Moreover, in comparison with culture without LBP (Fig. 3), BMSCS showed a spindle-shaped morphology with the presence of LBP, showing the elongated cytoskeleton as well (Fig. 3).

Effect of LBP culture on BMSC stemness

The stemness-related gene expression including Oct4 (fold change, 0.443 ± 0.038, p<0.001), and Klf4 (fold change, 0.632 ± 0.042, p<0.001) decreased when BMSCS were cultured with LBP compared with BMSCS cultured without LBP (Fig. 4A). Additionally, western blotting and immunofluorescence staining also proved the RT-PCR results (Fig. 4B, C, D).

Results

Characterization of stem cell surface markers of BMSCS

Notably, harvested BMSCS with passage 3 exhibited loose-bodied, fibroblast-like or flattened morphology (Fig. 1A-C). Cells were viable even being stored at -80°C after a longer period, demonstrating a high vitality and a capability to be able to rehabilitate quickly. Furthermore, our results indicated that these cells were able to differentiate into osteocytes, adipocytes and chondrocytes (Fig. 1D-F).

LBP biocompatibility

To evaluate the potential possibility of serum-free medium upon the differentiation of BMSCS and assess the possible autocrine function of BMSCS, the concentration of growth factors and inducers including bFGF, VEGF and TGF-β in the induction medium (Fig. 2A) and supernatants of BMSCS cultured with LBP after 7 days (Fig. 2B) were calculated by ELISA. The results indicated that the level of growth factors in the serum-free medium and supernatants are negligible to induce the BMSCS differentiation compared with induction medium (p < 0.001). For the biocompatibility, compared with standard culture without LBP, BMSCS cultured with LBP proliferated slightly over seven days (Fig. 2C).

Statistical analysis

All of the values are expressed as mean ± SD. Statistical significance was measured using the Student's unpaired t test (two-tailed). A p value <0.05 was considered statistically significant.
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**Figure 1. Characterization of BMSCs.** (A) and (B) BMSCs exhibited large, flattened or fibroblast-like morphology. (C) Fluorescent microscopy revealed morphology of GFP+ BMSCs cultured in plates at passage 3. Additionally, these cells were capable of differentiating into osteocytes, adipocytes and chondrocytes (D-F).

**Figure 2. Biocompatibility of LBP.** The concentration of growth factors and inducers including TGF-β, bFGF and VEGF in the induction medium were compared with serum-free medium (A) and supernatant of BMSCs cultured with LBP after 7 days (B) by ELISA. (C) A CCK-8 assay was used to assess BMSCs proliferation over 7 days. (D) The result of Live/dead assay of BMSCs cultured with LBP for 16 days. Data is given as the mean ± SD. ***p< 0.001
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**Figure 3. BMSCs morphology, viability and proliferation cultured with LBP.** In comparison with culture without LBP, BMSCs exhibited a 3D spindle-shaped morphology with the presence of LBP, showing the elongated cytoskeleton as well.

**Figure 4. Effect of LBP on BMSC stemness.** (A) qRT-PCR analysis of Oct4 and Klf4 gene expression in the presence of LBP versus standard culture without LBP. The data of standard culture were considered as 1. (B) Western blotting of stemness genes. (C) Quantification of western blotting. (D) Immunofluorescent staining demonstrated that BMSCs cultured with LBP expressed diminished levels of stem cell-related genes. Data is given as the mean ± SD, ***p<0.001

NF200 (0.734 ± 0.029, p<0.001), synapsin (0.7156 ± 0.0284, p<0.001) (Fig. 5B,C). Notably, qPCR analysis and immunofluorescence staining results showed the same trend (Fig. 5A,D).

**Discussion**

Although *Lycium barbarum* polysaccharide (LBP) has been investigated during the past decades, few studies have assessed the BMSCs differentiation induced directly by LBP. This study clearly proved that BMSCs maintained their viability in the presence of LBP and therefore differentiated into the favorable cell types without adding soluble inducers. Thus, we concluded that the LBP is able to act as inductive stimuli that is equivalent to other inducers to regulate stem cells directional commitment for neural regeneration. This is a crucial finding for neurological physician, given that most patients with nerve injury are lack of sufficient local inducers to drive transplanted BMSCs to differentiate into the favorable cells that are beneficial for neuron regeneration.
LBP has been thought to be a valuable traditional herbal medicine that is widely used according to Chinese pharmacopoeia (Liang et al., 2018). It can provide vitamins, lutein, betacarotene, zeaxanthin and is rich in polysaccharides (Amagase et al., 2009), making it as a promising and effective agent against aging, neuro-degeneration and inflammation (Ho YS et al., 2007; Gao K et al. 2015; Li XM, 2007). In the nervous system, LBPs has been proved to repair the neuronal loss, which is attributed to β-amyloid peptide (Ho YS et al., 2007; Yu MS et al., 2007) or glutamate excitotoxicity (Ho YS et al., 2009), etc. In addition, Lau et al., (2012) previously demonstrated that LBPs could enhance neurogenesis in area of the hippocampus and subventricular zone.

So far, the study focusing on the induction role of LBP on the differentiation into the neural like lineage have not been assessed. Previous studies have proved that culturing of BMSCs with the presence of LBP would promote their proliferation while maintain their stemness properties (Wang X et al., 2018), but here we demonstrated that LBP is capable of stimulating BMSCs to differentiate into favorable cells types such as neural like cell, which is in contrast to the previous findings. Notably, N-acetylglucosamine is the major component of Lycium barbarum (Lee CJ et al., 2004). It has been demonstrated that the pluripotency marker Oct4 of BMSCs is required to be O-GlcNAcylated with the presence of N-acetylglucosamine for BMSCs differentiation (Jang et al., 2012). Therefore, our findings lead us to raise the hypothesis that N-acetylglucosaminylase, the major ingredient of LBP, may drive the differentiation of BMSCs rather than maintaining the cells with pluripotency character. In our study, on day 7, the assessment of stemness markers of BMSCs including Oct4 and Klf4 suggested that the pluripotency and self-renewal status of BMSCs have been changed, leading to the completely differentiation of them into the neuron cells, which play important supporting roles in the neural regeneration (Tran et al., 2018).

In this study, we measured the representative markers of neuron cells, including β-tubulin III, NF-200 and Synapsin (Wang R et al., 2017; He B and Nan G, 2016). After the culture with LBP’s induction, LBP enhanced the expression of the above key factors of neuron cells, suggesting the favorable differentiation of the stem cells under in vitro culture in the absence of any traditional inducers. Normally, the induction of differentiation of BMSCs is achieved by extra soluble factors (Vater et al., 2011; Lu HF et al., 2012), however, the relative short-term availability and complex interactions limited the further application of this strategy (Faia-Torres et al., 2015). Thus, our findings may pave a new and simple way for the induction of BMSCs’ direct differentiation into the neuron cells’ like linage without any extra inducers.
Here, we prove that the directional differentiation process of BMSCs can be partially regulated by the traditional Chinese herbs, LBP, in the absence of soluble stimuli. We believe that our findings demonstrate a particular property of LBP as an optimized milieu for administrating BMSCs fate and driving their directional differentiation, suggesting a promising way to ultimately utilize the potential of BMSCs for neural regeneration.

Conclusion

Herein, a green agent, Lycium barbarum polysaccharide, was used to induce the differentiation of BMSCs for neural regeneration in the absence of soluble inducers. In addition to the excellent biocompatibility, LBP induced BMSCs to differentiate directionally into neuron cells, leading to the enhanced neural regeneration. Thus, we believe that this strategy provides opportunities to enhance the effectiveness of stem cell therapy for the treatment of degenerative cerebrovascular disease. Further optimization of the LBP to reinforce its properties should further advance BMSC-based therapy.

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