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Ginkgo biloba extract promotes osteogenic differentiation of human bone marrow mesenchymal stem cells in a pathway involving Wnt/β-catenin signaling

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ABSTRACT

Human bone marrow derived mesenchymal stem cells (BM-MSCs) are a novel cell source used in stem cell therapy to treat bone diseases owing to their high potential to differentiate into osteoblasts. Effective induction of osteogenic differentiation from human BM-MSCs is critical to fulfill their therapeutic potential. In this study, Ginkgo biloba extract (GBE), a traditional herbal medicine, was used to stimulate the proliferation and osteogenic differentiation of human BM-MSCs. The present study revealed that GBE improved the proliferation and osteogenesis of human BM-MSCs in a dose-dependent manner in the range 25–75 mg/l, as indicated by alkaline phosphatase (ALP) activity and calcium content. However, such effect was decreased or inhibited at 100 mg/l or higher. The dose-dependent improvement in osteogenesis of human BM-MSCs by GBE was further confirmed by the dose-dependent upregulation of marker genes, osteopontin (OPN) and Collagen I. The increased osteoprotegerin (OPG) expression and minimal expression of receptor activator of nuclear factor-κB ligand (RANKL) suggested that GBE also inhibited osteoclastogenesis of human BM-MSCs. Further mechanistic study demonstrated that the transcriptional levels of bone morphogenetic protein 4 (BMP4) and runt-related transcription factor 2 (RUNX2) in the BMP signaling, β-catenin and Cyclin D1 in the Wnt/β-catenin signaling, increased significantly during GBE-promoted osteogenesis. Meanwhile, loss-of-function assay with the signaling inhibitor(s) confirmed that the BMP and Wnt/β-catenin signaling pathways were indispensable during the GBE-promoted osteogenesis, suggesting that GBE improved osteogenesis *via* upregulation of the BMP and Wnt/β-catenin signaling. The present study proposed GBE to be used to upregulate the osteogenic differentiation of human BM-MSCs for new bone formation in BM-MSC-based cell therapy, which could provide an attractive and promising treatment for bone disorders.

1. Introduction

Bone homeostasis is finely tuned in the human body by two dynamic processes, bone formation by osteoblasts and bone resorption by osteoclasts. The homeostasis may be disrupted by inflammation and oxidative stress [1,2]. Chronic exposure of inflammation and oxidative stress may result in bone loss, osteoporosis, bone fragility (fracture), and cause many different bone diseases including rheumatoid arthritis (RA) and ankylosing spondylitis (AS) [1,3–7]. There are studies and reports demonstrating that inflammation and oxidative stress inhibit osteogenesis of mesenchymal stem cells (MSCs) in bone formation by inhibiting cell growth, proliferation, differentiation of osteoblasts and their progenitors, and further calcium mineralization. In the regulation of osteogenesis, many signaling pathways are involved in, such as the MAPK, BMP, Wnt, and NF-κB signaling [8].

Stem cell therapy with MSCs has gained more and more popularity for the treatment of bone diseases over the years in clinical

Abbreviations: GBE, Ginkgo biloba extract; BM-MSCs, bone marrow derived mesenchymal stem cells; ALP, alkaline phosphatase; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; BMP4, bone morphogenetic protein 4.

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practices due to their high potential to differentiate into osteoblasts [9]. In addition, MSCs can be readily obtained from patients themselves, easily expanded *ex vivo*, and well controlled for clinical uses [9–11]. However, the inflammatory and oxidative microenvironment in the location of bone impairments has a toxic effect on the proliferation and osteogenesis of the transplanted MSCs. For example, transplanted bone marrow MSCs (BM-MSCs) are likely to die shortly after transplantation because they are subjected to the unfamiliar and noxious microenvironment. It was reported that approximately over 80% of transplanted cells died within the first 24 h after transplantation [12] due to inflammation and oxidative stress [13].

Anti-inflammatory and anti-oxidative properties of Ginkgo biloba extract (GBE) has sparked our interest in its application as a therapeutic reagent for bone diseases, especially in stem cell therapy with MSCs. Ginkgo biloba leaves is a traditional Chinese herbal medicine for over 2000 years. It is also on the market for more than 1 century as an herbal medicine in Europe and dietary supplement in US [14]. GBE has been widely used for the treatment of cerebrovascular insufficiency, peripheral vascular insufficiency and cognitive impairment that is associated with aging and neurodegenerative disorders such as Alzheimer's disease (AD) due to its protective effects against hypertension, thrombosis, inflammation, oxidative stress and infection [15–21]. Specifically, GBE reduces oxidative stress [17,20–22] and inhibits expression of inflammatory cytokines, such as TNF-α, NF-κB, p65, IL-6, IL-1β, and IL-10 in different cell and animal models [23–25], which are tightly associated with bone diseases. In addition, GBE was able to promote growth and proliferation of different types of cells, such as neural stem cells, endothelial progenitor cells and cochlear hair cells [14,22,26–29].

However, the effect of GBE on osteogenic differentiation of human BM-MSCs in new bone formation for bone repair was poorly understood. In this study, GBE effect on the proliferation and osteogenesis of human BM-MSCs was investigated and possible underlying mechanisms were further explored. It was revealed that GBE promoted proliferation and osteogenesis of human BM-MSCs in a dose-dependent manner in the range of 25–75 mg/l. Mechanistic study showed that such improvement by GBE in osteogenesis was *via* upregulation of the BMP and Wnt/β-catenin signaling. Taken together its protective properties against inflammation and oxidative stress, our present study highlighted GBE as a therapeutic candidate for the treatment of bone diseases, especially in stem cell therapy with MSCs giving the fact that bone diseases are associated with an inflammatory and high oxidative microenvironment.

2. Materials and methods

2.1. Human BM-MSC culturing

Human BM-MSCs were isolated and expanded according previously published literature [30,31]. Briefly, bone marrow aspirate (2.0–3.0 ml) was collected from osteotomy sites from patients, who signed an informed consent form. The aspirate was mixed gently with α-minimum essential medium (α-MEM) in a total volume of 10 ml, containing 15% embryonic stem cell qualified-fetal bovine serum (ES-FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 18% Chang B and 2% Chang C (Irvine Scientific, Santa Ana, CA, USA). Cells were cultured in a humidified CO_2 incubator at 37 °C for 5 days. After debris and blood were removed carefully with serum-free α-MEM, cells were cultured until 80–90% confluent before 1:3 splitting in α-MEM, containing 15% ES-FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 18% Chang B and 2% Chang C 20%. The medium was changed every 2 days. Cells of passage number 6 (P6) and 9 (P9) were used. The protocol was previously reviewed and approved by the ethics committee of Fourth Military Medical University. The GBE is a standardized formula that contains active gradients of 6% ginkgolides and bilobalides. 100 mg GBE contains 3.08 mg of ginkgolides A, B and C, and 2.91 mg of bilobalide. The GBE presented in the mediums for the whole culturing period.

2.2. Induction of osteoblast differentiation

In the study, osteoblast differentiation was inducted by 10 nM dexamethasone (Dex) (Sigma, USA). After 6 or 9 passages, human BM-MSCs were seeded into a 96-well plate at a confluency of 80–90% in osteogenic media of α-MEM containing 15% ES-FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 18% Chang B, 2% Chang C and 10 nM Dex. The osteogenic medium was changed every 2 days.

2.3. Flow cytometry

To analyze cell surface marker proteins, protocols in other previously published studies were adopted and followed. Briefly, the human BM-MSCs were seeded at a concentration of $2–3 \times 10^6$ cells/well into a 24-well plate following a passage. The cells were cultured until 80–90% confluent. The cells were then rinsed with PBS solution containing 1% BSA (Sigma, USA) before isolated by trypsinization and resuspended in PBS solution. 50 μl of cell suspension was incubated with fluorochrome-conjugated monoclonal antibody for 1 h in the dark, washed three times with PBS, and analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). The antibodies used in the experiments were CD29-FITC (Serotec, Oxford, UK), CD44-FITC (Serotec), CD45-FITC (Serotec), CD34-FITC (Serotec). Mouse fluorochrome-conjugated isotype control IgG antibodies (Serotec) were used in the experiments as a negative labeling control.

2.4. Alkaline phosphatase (ALP) activity assay and ALP staining

Alkaline phosphatase (ALP) is a cell marker of osteoblasts. Its level can be used as an indicator of osteoblast cell number differentiated from human BM-MSCs. In the study, the human BM-MSCs were cultured in a 96-well plate at 80–90% confluency for osteogenesis. The osteogenic medium was changed every 2 days. For ALP activity assay at day 7, cells were washed with TB buffer twice (20 mM Tris, pH7.5, 150 mM NaCl) before being lysed with 100 μl lysis buffer (TB buffer, plus 0.1% Triton). After centrifugation at 12,000 rpm for 20 min at 4 °C, 45 μl of supernatant was incubated with 100 μl of ALP substrate p-nitrophenyl phosphatase (pNPP) liquid substrate system (Promega, Madison, WI, USA) at 37 °C for 10–60 min depending on the ALP activity in the extracts, and the absorbance at 405 nm was measured on a 96-well plate reader with the substrate system according to the manufacturer's instructions. ALP activity was normalized to total protein. Meanwhile, ALP staining was performed at day 7 using an ALP staining kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Cells were fixed by 10% formalin for 1 h at room temperature before being stained for 5–20 min with chromogenic substrate in the kit depending on the ALP expression level in the cultured cells.

2.5. Calcium accumulation assay with alizarin red-sulfate (ARS) staining

For ARS staining, human BM-MSCs were plated into a 96-well plate at confluency of 80–90% and cultured for 24 days in osteogenic medium with or without treatment. The medium was changed every 2 days. After GBE treatment, the medium was removed and the cells were washed for three times with PBS and fixed in 70%