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The effect of 3-hydroxybutyrate on the *in vitro* differentiation of murine osteoblast MC3T3-E1 and *in vivo* bone formation in ovariectomized rats

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Abstract

3-hydroxybutyrate (3HB), one of the degradation products of microbial biopolyesters polyhydroxyalkanoates (PHA), is a high energy metabolic substrate in animals. This study evaluated the effects of 3HB on growth of osteoblasts *in vitro* and on anti-osteoporosis *in vivo*. Alkaline phosphatase (ALP) assay, Van Kossa assay and Alizarin S red staining were used to study *in vitro* differentiation of murine osteoblast MC3T3-E1 cells. The intensity of *in vitro* cell differentiation measured in ALP was in direct proportion to the concentration of 3HB when it was lower than 0.01 g/L. Calcium deposition, a strong indication of cell differentiation, also showed an obvious increase with increasing 3HB concentration from 0–0.1 g/L, evidenced by Alizarin red S staining and Van Kossa assay. RT-PCR also showed significantly higher expression of osteocalcin (OCN) mRNA in MC3T3-E1 cells after 3HB administration. *In vivo* study using female Wistar rats (3 months old, $n = 80$) allocated into normal, sham-operated or ovariectomized (OVX) group that led to decreasing bone mineral density (BMD), bone histomorphometry and biomechanics compared with normal and sham groups, had demonstrated that 3HB increased serum ALP activity and calcium deposition, decreased serum OCN, prevented BMD reduction resulting from OVX. All these led to enhanced femur maximal load and bone deformation resistance, as well as improved trabecular bone volume (TBV%). In conclusion, 3HB monomer containing PHA can be effective bone growth stimulating implant materials.

Keywords: 3-hydroxybutyrate; PHA; Osteoblast; Osteoporosis; Polyhydroxyalkanoate

1. Introduction

3-hydroxybutyrate (3HB) containing polyhydroxyalkanoates (PHA) has shown to be effective as bioimplant materials that stimulate tissue regeneration including bone and cartilages tissues [1–4]. 3HB is also one of the main ketone bodies primarily produced in the liver from degradation of long-chain fatty acids and transported through plasma to peripheral tissues [5]. The role of 3HB as an energy source and lipogenic precursor has already been recognized [6]. Recently, 3HB has been employed to treat traumatic injuries that benefit from elevated levels of ketone bodies such as hemorrhagic shock [7,8], extensive burns [9], myocardial damage [10], and cerebral hypoxia, anoxia, and ischemia [11]. Furthermore, 3HB has been found to be able to reduce death rate of human neuronal cell model culture for Alzheimer's and Parkinson's disease [12] and to ameliorate the appearance of corneal epithelial erosion through suppression of apoptosis [13]. 3HB was also reported to be able to correct defects in mitochondrial energy generation [14]. Additional advantages for 3HB include good tolerance by humans and a short half-life *in vivo* [15]. There have been already several potential therapeutic applications reported for 3HB [16].

3HB is a degradation product of some PHA, for which we have demonstrated good biocompatibility in tissue engineering applications [2,17–19]. The foremost ability among these properties is to support high levels of cell and tissue growth [4,20–25]. It is speculated that monomers

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released from PHA degradation contribute to improve tissue regeneration [26], which was supported by the fact that 3HB presence activated Ca^{2+} channel and increased calcium influx in the cultured cells [26]. Also, it was found that 3HB suppressed the death of cell line L929 when cultured at high density [26]. It was also found that 3HB prevented apoptosis induced by serum withdrawal [27]. While the underlying mechanism is still being investigated, it is proposed that 3HB possibly fuels the mitochondria and thereby promotes cell growth to high density which requires extensive energy and nutritional supplies [14].

Previous results showed that PHA copolyester (PHBHHx) consisting of 3HB and 3-hydroxyhexanoate promoted the growth and differentiation of osteoblasts. Could the degradation product of PHBHHx, namely 3HB, contribute to this phenomenon? If the answer is yes, 3HB could be a candidate to treat osteoporosis.

Currently, treatments for osteoporosis mainly depend on suppressing osteoclast activity and bone resorption. Although these treatments can decrease the frequency of fracture and increase bone mineral density (BMD), the long-term suppression of bone resorption seems to be ineffective for bone remolding [28]. The uses of growth factors, hormones or fluoride compounds to stimulate bone formation encountered side effects that limit their applications [29]. It is therefore, significant to develop efficient and safe treatments for osteoporosis that is attributed to reduced osteoblast activity and bone formation [30–32].

In this study, for the first time, 3HB was used to investigate its effect on *in vitro* growth and differentiation of murine osteoblast MC3T3-E1 cell line, a well-accepted model for osteogenesis study [33]. The *in vivo* effect of 3HB on osteoporosis was also studied using ovariectomized (OVX) rats, an effective osteoporosis animal model [34].

2. Experiment

2.1. Materials

Murine osteoblastic MC3T3-E1 cells were generously provided by Professor Rong-qing Zhang, Department of Biological Sciences and Biotechnology of Tsinghua University, Beijing, China. Fetal bovine serum (FBS) was purchased from Hyclone (UT, USA), streptomycin from Amresco (Solon, OH), DL-3-hydroxybutyrate sodium salt (3HB) and penicillin from Sigma Chemical Co. (St. Louis, MO). All other culture media were purchased from Gibico-BRL (Gaithersberg, MD). TRIzol reagent and DEPC were purchased from Invitrogen (CA, USA). RNeasy Mini Kit with Rnase-Free DNase set was from Qiagen (California, USA). Reverse transcription reagents were from Tiangen Co., Ltd. (Beijing, China). Ex Taq DNA polymerase was purchased form TaKaRa (Dalian, China). The female Wistar rats were purchased from Beijing Vital River Experimental Animals Co. Ltd. under license no. SCXK (Beijing) 2002-0003.

2.2. Culture of murine osteoblast MC3T3-E1 cells

The cells of murine osteoblast MC3T3-E1 were grown in Dulbecco's modified Eagles medium (DMEM, Gibico) supplemented with 10% (v/v) FBS (Hyclone, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. Incubation was conducted in a CO_2 incubator (5% CO_2, 95% air) (MCO-15AC, SANYO Co. Ltd., Japan) at 37 °C. The cells were subcultured every 2 or 3 days in the presence of 0.25% (w/v) trypsin plus 0.02% (w/v) ethylenediaminetetraacetic acid tetrasodium salts solution (EDTA) (Gibico).

2.3. Assay of alkaline phosphatase (ALP) activity

MC3T3-E1 cells were seeded in 48-well plates (10^4 cells/well) containing DMEM medium plus 10% FBS. After the cells attached on the bottom of the wells, the culture medium was changed to DMEM + 10%FBS medium containing 10 mM disodium β-glycerophosphate (β-GP) (Sigma, St. Louis, MO, USA), 0.15 mM ascorbic acid (Sigma) and 10^{-8} M dexamethasone (Sigma) [35]. Simultaneously, different concentrations of 3HB sodium salt were added to the culture medium in the wells. After 21-day cultivation, the cells were washed twice with phosphate buffered saline (PBS) and harvested in 200 μL/well of lysis buffer (pH 8.2, 10 mM Tris-HCl, 2 mM $MgCl_2$ and 0.05% Triton X-100). Cells were lysed through 3 cycles of freezing and thawing. Aliquots were reserved for protein analysis. 300 μL of 8 mM *p*-nitrophenyl phosphate (Sigma) in 0.1 M sodium carbonate buffer (pH 10) containing 1 mM $MgCl_2$ was added to the reaction mixture, which was incubated at 37 °C for 30 min (total 500 μL). The reaction was stopped by adding 50 μL of 1.0 N NaOH/well [35]. The yellow sample solutions containing *p*-nitrophenol as the reaction product were measured at wavelength of 405 nm using a microplate reader (Versamax, Molecular Device, USA). A standard curve was prepared using *p*-nitrophenol (Sigma). Total protein content of cell lysates was measured according to Lowry et al. [36], and was expressed as the protein content of the cell lysate.

2.4. Calcification assay

The calcium deposition of MC3T3-E1 cell culture was studied using Alizarin red S staining solution. The Alizarin red S solution was freshly prepared: briefly, 0.1 ml of 28% ammonia solution in 100 ml distilled water was added to the solution of Alizarin red S (1 g in 100 mL distilled water), the pH was adjusted to approximately 6.4. The cells were cultured for 21 days under the same conditions as that of the ALP assay [35]. After incubation, the cell cultures were washed three times with Dulbecco's PBS without calcium and magnesium salts (PBS(−)). Subsequently, the cells were fixed by addition of 10% formalin dissolved in PBS(−) solution for 1 h. After the cell fixing process, the cell cultures were washed three times with distilled water and stained by Alizarin red S solution for 15 min. The redundant stains were removed by washing the cell culture twice with distilled water. Digital images (DC 300F, Leica, Germany) of Alizarin red S stained cultures were obtained and the number of the calcification nodules was calculated by averaging six values of different sights under the microscopic counting (DM IRB, Leica, Germany).

2.5. The Van Kossa assay

The cells were cultured for 21 days under the same condition as that of the experiment of ALP assay. After incubation, the cells ware washed with 150 mM NaCl twice and fixed with 10% formalin dissolved in PBS(−) solution for 1 h. After the fixing process, the cell culture was treated with 100 μL of 5% $AgNO_3$. This treatment lasted for 30 min under ultraviolet radiation. Following the removal of the $AgNO_3$ solution, the culture medium was washed with PBS(−) twice followed by addition of 5% $Na_2S_2O_3$ into the plate and sustained for 10 min [37]. After washing the plate with distilled water twice, the cell culture was stained with Neutral Red for 10 min. The redundant stains were removed and the digital images of the stained cultures were obtained (DM IRB, Leica, Germany).

2.6. RT-PCR study on mRNAs encoding osteocalcin (OCN)

RT-PCR was used to detect the mRNAs encoding OCN [38]. The cells were cultured for 21 days under the same condition as that of the