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Administration of tauroursodeoxycholic acid enhances osteogenic differentiation of bone marrow-derived mesenchymal stem cells and bone regeneration

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ABSTRACT

It is known that osteogenic differentiation of mesenchymal stem cells (MSCs) can be promoted by suppression of adipogenesis of MSCs. We have recently found that the chemical chaperone tauroursodeoxycholic acid (TUDCA) significantly reduces adipogenesis of MSCs. In the present study, we examined whether TUDCA can promote osteogenic differentiation of bone marrow-derived mesenchymal stem cells (BMMSCs) by regulating Integrin 5 (ITGA5) associated with activation of ERK1/2 signal pathway and thereby enhance bone tissue regeneration by reducing apoptosis and the inflammatory response. TUDCA treatment promoted *in vitro* osteogenic differentiation of BMMSCs and *in vivo* bone tissue regeneration in a calvarial defect model, as confirmed by micro-computed tomography, histological staining, and immunohistochemistry for osteocalcin. In addition, TUDCA treatment significantly decreased apoptosis and the inflammatory response *in vivo* and *in vitro*, which is important to enhance bone tissue regeneration. These results indicate that TUDCA plays a critical role in enhancing osteogenesis of BMMSCs, and is therefore a potential alternative drug for bone tissue regeneration.

1. Introduction

Tissue engineering to regenerate damaged or defective tissue is a promising alternative to current clinical treatments [1–2]. In previous decades, many clinicians and researchers working in the tissue engineering field have reported meaningful results regarding the possibility of inducing tissue regeneration using biomaterials and cells, including synthetic/natural proteins and growth factors [2–5]. Although advances in tissue engineering have improved the clinical treatment of specific tissue defects, the broad application of tissue engineering is limited by obstacles such as the functional and morphological maintenance of newly-formed tissue.

Bone tissue engineering has been developed using various approaches, such as biodegradable synthetic/natural polymer composites, bone inductive/conductive materials, and genetically modified cells, for therapeutic applications [6–7]. However, the engineered tissues for bone repair often lack vascularity and mechanical strength, and it is difficult to engineer the complex bone micro-architecture [8]. One major issue is that adipose tissue usually forms along with the engineered or newly regenerated bone tissue [9–11]. Likewise, several studies reported that the decrease in bone volume in age-related osteoporosis is usually accompanied by an increase in bone marrow adipose tissue [12–14]. For successful bone tissue regeneration, adipose tissue formation at defective bone tissue must be reduced.

Recombinant bone morphogenetic protein (BMP)-2 has a powerful osteoinductive capacity. Since 2002, BMP-2 has been available for orthopedic use, particularly anterior lumbar interbody fusion in the United States [15–16], and has been linked with bone tissue regeneration [17–18]. However, additional culture and treatment steps requiring large amounts of expensive growth factors and cytokines, such as BMP-2 and dexamethasone, which may be cytotoxic and inflammatory, are required prior to the implantation of bone grafting materials in order to achieve therapeutic effects [19–20]. Due to their short half-life and high cost, treatment with cytokines cannot solve the fundamental problems associated with bone tissue regeneration [3]. Therefore, new drugs need to be discovered to enhance bone tissue regeneration.

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The chemical chaperone tauroursodeoxycholic acid (TUDCA) has been approved by the USA Food and Drug Administration and in Europe. TUDCA is widely used to treat cholelithiasis and cholestatic liver disease [21–22] because it can effectively attenuate endoplasmic reticulum (ER) stress, which is induced by the accumulation of misfolded proteins in the ER, in liver tissue [23–24]. Recent reports revealed that TUDCA can also decrease the lipid content of adipocytes and reduce the body mass of obese humans [25–26]. More recently, we reported that TUDCA significantly decreases *in vitro* adipogenic differentiation of mesenchymal stem cells (MSCs) and *in vivo* adipose tissue formation when injected [27]. It was reported that osteogenic differentiation of mesenchymal stem cells (MSCs) can be promoted by suppression of adipogenesis of MSCs [28].

TUDCA can inhibit ER stress-induced apoptosis by modulating the binding of pro-apoptotic molecules to the mitochondria and the mitochondrial release of cytochrome C [29–32]. In addition, TUDCA treatment dramatically decreases inflammatory processes such as innate immunity colitis and acute pancreatitis [33–34]. [35]. Indeed, several studies reported that TUDCA can inhibit or attenuate apoptosis and inflammation in various diseases and traumatic injuries [34,36]. In the early phases of tissue regeneration, inflammatory cells such as monocytes and macrophages, which participate in the inflammatory response, undergo apoptosis resulting in delayed tissue healing and regeneration. [35]. Thus, it is important to prevent inflammation and minimize pro-inflammatory signaling in order to enhance bone tissue regeneration.

In the present study, we hypothesized that TUDCA treatment would effectively enhance osteogenic differentiation of mesenchymal stem cells (MSCs) and bone tissue formation by reducing apoptosis and inflammation at the implantation site. The effect of TUDCA on bone tissue regeneration has not been previously studied. We compared TUDCA with the clinically available BMP-2 recombinant protein in terms of bone tissue formation.

2. Materials and methods

2.1. Isolation and culture of mouse bone marrow mesenchymal stem cells (mBMMSCs)

mBMMSCs were isolated from 8 weeks old of male Balb/c mice as previously described [37]. After 8 h non-adhesive cells were washed out with Dulbecco's Phosphate Buffered Saline (DPBS, Gibco BRL, Gaithersburg, MD). The commercial mBMMSCs in immune response experiments were purchased from Cyagen Biosciences (OriCell™, Guangzhou, China) and approval for their use in this project was obtained from the CHA University of Institutional Review Board. mBMMSCs were cultured in the proliferation medium, Dulbecco's modified Eagle medium (DMEM, Gibco BRL) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco BRL) and 100 units/ml penicillin (Gibco BRL) in humidified air with 5% (v/v) CO_2 at 37 °C. The proliferation medium was changed every two days.

2.2. In vitro osteogenic differentiation of mBMMSCs

Osteogenic differentiation of mBMMSCs was induced at a cell density of 2×10^4 cells/cm^2 in osteogenic medium (DMEM supplemented with 10%(*v*/*v*) FBS, 1% GlutaMAX (Gibco BRL), 0.2 mM ascorbic acid (Sigma), 10 mM glycerol 2-phosphate (Sigma) and 100 units/ml penicillin (Gibco BRL)) with the presence or absence TUDCA for 21 days after media change, respectively, and as follows:

Group 1: no TUDCA supplementation during osteogenic medium,
Group 2: 10 nM TUDCA supplementation during osteogenic medium,
Group 3: 100 nM TUDCA supplementation during osteogenic medium,
Group 4: 300 nM TUDCA supplementation during osteogenic medium.

Each proliferation and differentiation medium was changed every two days.

For detection of Calcium deposits, differentiated mBMMSCs were fixed in 10% formalin for 30 min at room temperature (RT). After fixation, 2% (v/v) of Alizarin Red S staining solution was added to fixed mBMMSCs and incubated at RT in the dark for 45 min. The staining solution was removed and washed three times with deionized water. The microscopic images were obtained using microscope (IX71 inverted microscope, Olympus, Tokyo, Japan). Alizarin Red S was extracted using 10% (v/v) of cetylpyridinium chloride (CPC) for the quantification of calcium deposits and the absorbance was measured at 562 nm using microplate reader (Molecular devices, CA, USA).

2.3. MEK-1/2 inhibition of mBMMSCs during in vitro osteogenic differentiation

Osteogenic differentiation of mBMMSCs was induced at a cell density of 2×10^4 cells/cm^2 in osteogenic medium (as previously described) with the presence or absence TUDCA for 3 days after media change, respectively, and as follows:

Group 1: no TUDCA supplementation during osteogenic medium,
Group 2: 300 nM TUDCA supplementation during osteogenic medium,
Group 3: 300 nM TUDCA supplementation during osteogenic medium with 20 μM U0126 (MEK-1/2 inhibitor, Abcam).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR

Total RNA was extracted from transfected cells using TRIzol (Invitrogen) and 2 μg of total RNA was used for cDNA synthesis with RT-PreMix (Bioneer, Daejeon, Korea). PCR was performed with PCR-PreMix (Bioneer) under standard PCR conditions: BCL2-associated X protein (Bax), B cell leukemia/lymphoma 2 (Bcl-2), interleukin 1 beta (IL-1β), tumor necrosis factor-alpha (TNFα), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in Supplementary Table 1. PCR cycles consisted of an initial denaturation step at 94 °C for 5 min, followed by 32 amplification cycles consisting of 30 s of denaturation at 94 °C, 30 s of annealing at 62 °C, and 1 min of extension at 72 °C. Last, a final extension was performed at 72 °C for 10 min. PCR products were analyzed by UV irradiation on a 1.2% agarose gel stained with ethidium bromide. For quantitative real-time PCR analysis, gene-specific primers were designed to amplify runt-related transcription factor 2 (Runx2), IL-1β, TNFα and GAPDH. Primer pairs are as follows: Runx2 (5-TCC ACA AGG ACA GAG TCA GA-3, 5-TGG CTC AGA TAG GAG GGG TA-3), IL-1β (5-CAA CCA ACA AGT GAT ATT CTC CAT G-3, 5-GAT CCA CAC TCT CCA GCT GCA-3), TNFα (5-GCG GTG CCT ATG TCT CAG-3, 5-GCC ATT TGG GAA CTT CTC ATC-3) and GAPDH (5-ACA TCG CTC AGA CAC CAT G-3, 5-TGT AGT TGA GGT CAA TGA AGG G-3). All amplifications were performed in a final reaction mixture (20 μl) containing 1 final concentration of SYBR supermix, 500 nmol/l of gene-specific primers, and 1 ml of template, using the following conditions: an initial denaturation at 95 °C for 1 min, followed by 45 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 15 s, with a final extension at 72 °C for 5 min. After amplification, the baseline and threshold levels for each reaction were determined using the company's software package (Exicycler 96; Bioneer). For validation of polymerase chain reaction (PCR), amplified products were separated on 1% agarose gels and visualized by ethidium bromide staining.

2.5. Western blot analysis

Cells were washed with DPBS, lysed with 200 μl of RIPA buffer, and centrifuged at 13,000 rpm for 20 min. Supernatant was collected in 1.5 ml tube. Protein concentration was determined using BCA assay kit