Effects of simvastatin on the osteogenic differentiation and immunomodulation of bone marrow mesenchymal stem cells

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Abstract. The present study aimed to investigate the effects of simvastatin on the bone differentiation capacity and immunological characteristics of bone marrow mesenchymal stem cells (BMSCs). BMSCs were isolated and cultured in medium containing 1.0 μmol/ml simvastatin. The alkaline phosphatase activity, mRNA expression levels of osteocalcin and bone sialoprotein, and calcium nodule formation were assessed to determine the osteogenic differentiation capability of BMSCs. To investigate alterations in the immunological properties of simvastation-treated BMSCs, the immunogenicity of these cells and the effect of BMSCs on phytohemagglutinin-stimulated lymphocyte proliferation were also assessed. Following treatment with simvastatin, the alkaline phosphatase activity, and mRNA expression levels of osteocalcin and bone sialoprotein were increased significantly in the BMSCs. In addition, von Kossa staining revealed a brown calcium-positive reaction zone in simvastatin-treated cells. Simvastatin-induced BMSCs revealed no affect on the proliferation of allogeneic lymphocytes, however, inhibited phytohemagglutinin-induced lymphocyte proliferation. Collectively, simvastatin promoted the osteogenic differentiation of BMSCs significantly without affecting their immunosuppressive properties.

Introduction

Malunion is a major problem during orthopedic trauma and the incidence of non-union is 5-20% (1). The non-union of fractures results in physical and psychological suffering of the patients and consumes a vast quantity of social health resources (2). Autologous bone grafting is the gold standard for the surgical treatment of non-union, however, it has a high risk of postoperative complications associated with the harvesting procedure used, including donor site morbidity. Therefore, it is not accepted by certain patients, although other treatments struggle to restore the physiological structure and function of bone (3). In recent years, research using bone marrow mesenchymal stem cells (BMSCs) has brought new hope to bone tissue engineering. For example, previous studies have confirmed that BMSCs can repair bone defects, promote fracture healing (4) and exert marked immunomodulatory properties (5).

As a 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor, simvastatin is the first-line treatment for hyperlipidemia, and is widely used worldwide as a result of its safety, effectiveness and low cost (6,7). Furthermore, simvastatin exerts anti-inflammatory effects, induces angiogenesis and promotes endothelial cell growth (8). It also regulates bone regeneration by promoting osteoblast function and inhibiting osteoclast cell function (9). However, the effects of simvastatin on the behavior of BMSCs in terms of osteogenic differentiation and immunomodulatory capabilities remain to be elucidated. The aim of the present study was to investigate the effects of simvastatin on the osteogenic differentiation and immunological properties of BMSCs.

Materials and methods

Isolation and cultivation of cells. The present study was approved by the Research Ethical Committee of Yidu Central Hospital, Weifang Medical University (Shandong, China), and adult bone marrow and peripheral blood were collected from healthy volunteers who had provided written informed consent. The BMSCs were isolated and cultured, as described previously (10). The bone marrow specimens were carefully added to 1.077 g/ml Ficoll separation medium (Ding-Guo Changsheng Biotechnology Co. Ltd., Beijing, China) and centrifuged at 896 x g at room temperature for 20 min to collect the mononuclear cells. The cells were washed three times with D-Hanks (Beijing Union Medical College, Beijing, China) and were seeded into 25 cm^2 flasks at a density of 10^5 cells/cm^2 in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum (Gibco), 2 mol/l glutamine (Beijing Union Medical College), 100 U/ml penicillin (Beijing Union Medical College) and 100 U/ml streptomycin (Beijing Union Medical College). The cells were incubated at 37˚C with 5% CO_2, and the medium was changed 24 h later to remove non-adherent cells. The medium was changed every 2-3 days, and the cells

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were subsequently digested with 2.5 g/l trypsin (Beijing Union Medical College) and passaged once the cells had reached 80% confluence. In the treatment group, simvastatin (1.0 μmol/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to the BMSC culture medium for different durations, as indicated. Human peripheral blood mononuclear cells (PBMCs) were extracted and cultured, as described previously (11).

Alkaline phosphatase (ALP) activity assay. The BMSCs were incubated with simvastatin (1.0 μmol/ml) for 1 or 7 days, and the cellular ALP activity was subsequently measured, according to a previous report (12). Briefly, the culture medium was discarded and the cells were washed three times with phosphate-buffered saline. Next, 100 μl Tris buffer (pH 7.4; Sigma-Aldrich) was added to each well, and the mixture was pipetted up and down. The cell suspension was further treated using ultrasonic vibration generator (SJBD-163; Shijiboda Co., Ltd., Shenzhen, China) until the cells were fully lysed. A total of 100 μl substrate was added to the cell lysates, incubated at 37˚C for 15 min, and quenched with 0.05 N NaOH. The absorbance at 405 nm was measured using a microplate reader (Type Spectramax Plus, Molecular Devices, Sunnyvale, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). The BMSCs were incubated with simvastatin (1.0 μmol/ml) for 14 days and the total RNA was extracted using an RT-PCR kit (Takara Bio, Inc., Dalian, China). Following extraction, the osteocalcin, bone sialoprotein and GAPDH (housekeeping gene) sequences were amplified using the following primers: Osteocalcin, forward: 5'-CATGAGAGCCCTCACA-3' and reverse: 5'-AGAGCGACACCCTAGAC-3'; bone sialoprotein, forward: 5'-CTATGGAGAGGACGCCACGCCTGG-3' and reverse: 5'-CATAGCCATCGTAGCCTTGTCCT-3'; GAPDH, forward: 5'-AGCCGCAAGCCGCATCTTCTTTTGCGTC-3' and reverse: 5'-TCATATTTGGCAGGTTTTTCT-3'. Following completion of the PCR reaction, the amplification products were separated on 1.5% agarose gels and were observed using a GelDoc XR+ gel imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

von Kossa staining. The BMSCs were incubated with simvastatin (1.0 μmol/ml) for 21 days and were subsequently stained using von Kossa to detect calcium. In brief, the cells were fixed with 95% alcohol for 30 min at room temperature after washing in phosphate-buffered saline (Beijing Union Medical College). The BMSCs were reduced in 2% silver nitrate solution (Sigma-Aldrich) under ultraviolet radiation for 20 min, followed by incubation with 5% sodium hyposulfite (Sigma-Aldrich) for 5 min. The cells were then observed under a light microscope (Type IX53, Olympus, Tokyo, Japan).

Immunogenicity of BMSCs. Following incubation with simvastatin (1.0 μmol/ml) for 7 days, the BMSCs were collected and seeded at a density of $5.0x10^4$ cells. Once the BMSCs had adhered to the dish, an equal quantity of allogeneic PBMCs was added and the cells were incubated at 37˚C with 5% CO_2 for 5 days. A total of $5.0x10^4$ allogeneic PBMCs cultured alone and co-culture of $5.0x10^4$ PBMCs from different individuals were used as controls. The proliferation of PBMCs was detected using a cell counting kit (CCK)-8 kit (Dojindo Co, Ltd., Kumamoto, Japan). A microplate reader (Molecular Devices) was used to measure the proliferation rate at a wavelength of 450 nm

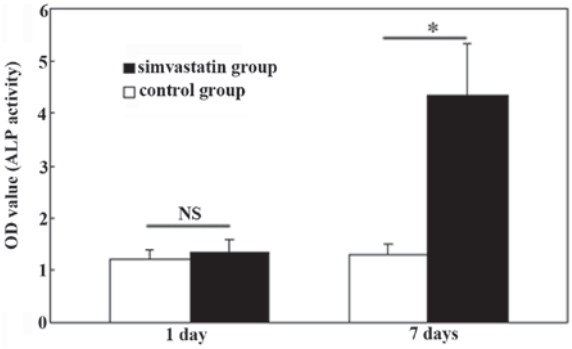

Figure 1. Treatment with simvastatin increases the levels of ALP. The activity of ALP was determined using an ALP activity assay. The activity was significantly higher in the simvastatin-treated BMSCs compared with the untreated BMSCs following co-culture for 7 days (*P<0.05). NS, no significant difference; BMSCs, bone marrow mesenchymal stem cells; ALP, alkaline phosphatase; OD, optical density.

Immunomodulatory properties of BMSCs. Following incubation with simvastatin (1.0 μmol/ml) for 7 days, the BMSCs were collected and seeded at different concentrations (2.5 or $5.0x10^4$, or $2.5x10^5$). Once the cells had adhered, $5.0x10^4$ PBMCs were added (BMSC:PBMC ratios of 1:5, 1:1 and 5:1) in the presence of a final concentration of 0.5 μg/ml phytohemagglutinin (PHA; Sigma-Aldrich), and the cells were incubated at 37˚C with 5% CO_2 for 5 days. The proliferation of PBMCs was then analyzed using a CCK-8 kit.

Statistical analysis. All data are expressed as the mean ± standard deviation from at least three independent experiments. Statistical significance was assessed by Student's t-test and P<0.05 was considered to indicate a statistically significant difference.

Results

Simvastatin promotes the osteogenic differentiation of BMSCs. Following incubation with simvastatin for 1 day, no significant difference in the ALP activity of BMSCs was observed between the control and simvastatin-treated groups. However, a 7 day incubation with simvastatin increased the ALP activity in BMSCs significantly compared with the control group, as shown in Fig. 1. BMSCs expressed osteocalcin and bone sialoprotein under normal conditions, however, their expression was increased significantly following incubation with simvastatin for 14 days (Fig. 2). After the BMSCs had been incubated with simvastatin for 21 days, their cellular morphology changed significantly from fusiform to polygonal or irregular shapes, and they also formed a nodular structure. In addition, von Kossa staining revealed brown calcium-positive staining (Fig. 3) following treatment with simvastatin.

Simvastatin reveals no affect on the low immunogenicity of BMSCs. To assess whether simvastatin-co-cultured BMSCs

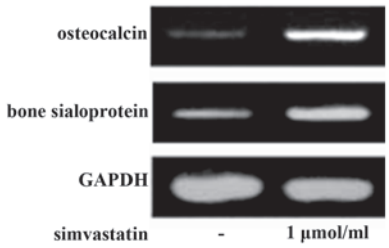

Figure 2. Treatment with simvastatin increases the mRNA expression levels of osteocalcin and bone sialoprotein. Following incubation with simvastatin for 14 days, the mRNA expression levels of osteocalcin and bone sialoprotein were determined by reverse transcription-polymerase chain reaction. Treatment with simvastatin significantly increased the mRNA expression levels of osteocalcin and bone sialoprotein in the bone marrow mesenchymal stem cells. GAPDH was used as an internal loading control.

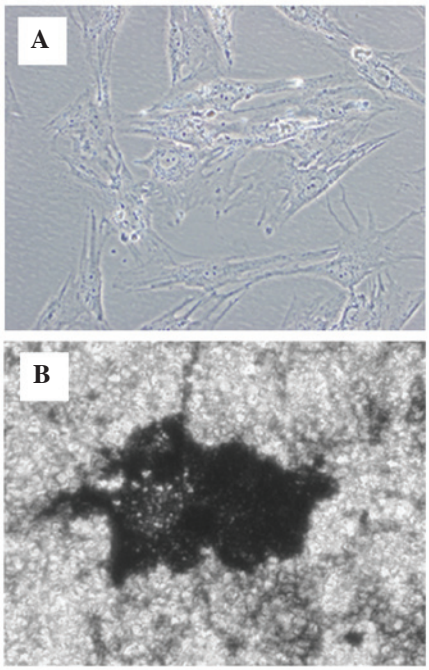

Figure 3. (A) Untreated BMSCs. (B) Following incubation with simvastatin for 21 days, von Kossa staining of the BMSCs revealed a brown calcium-positive reaction zone (magnification, x400). BMSCs, bone marrow mesenchymal stem cells.

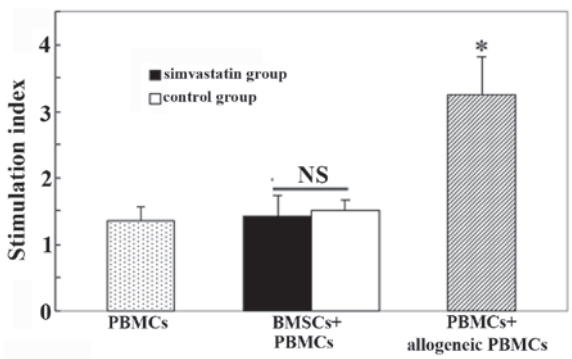

Figure 4. Treatment with simvastatin causes no induction of the proliferation of allogenic PBMCs. Following incubation with simvastatin for 7 days, the BMSCs caused no induction of the proliferation of allogeneic PBMCs (*P<0.05, vs. other groups). NS, no significant difference; BMSCs, bone marrow mesenchymal stem cells; PBMCs, peripheral blood mononuclear cells.

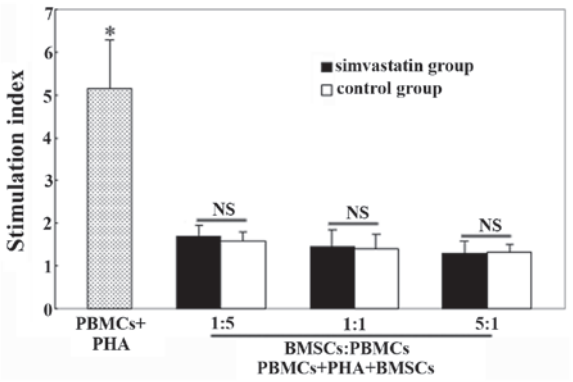

Figure 5. Simvastatin-treated BMSCs inhibited PHA-induced PBMCs proliferation to a similar extent as the untreated BMSCs (*P<0.05, vs. other groups). NS, no significant differences; BMSCs, bone marrow mesenchymal stem cells; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin.

induced the proliferation of allogeneic lymphocytes, the BMSCs were treated with simvastatin for 7 days, and subsequently incubated with an equal quantity of allogeneic PBMCs. Following 5 day incubation, analysis of lymphocyte proliferation revealed that simvastatin-treated BMSCs revealed no affect on the proliferation of allogeneic PBMCs, to an identical extent as the pure allogeneic PBMC group (Fig. 4), suggesting that simvastatin causes no affect on the low immunogenicity of BMSCs.

Simvastatin reveals no affect on the immunosuppressive effects of BMSCs. Treating PBMCs with 0.5 μg/ml PHA stimulated their proliferation. However, simvastatin-treated BMSCs and untreated BMSCs suppressed PHA-induced PBMC proliferation, independent of cell number (Fig. 5). Therefore, this suggested that simvastatin caused no affect on the immunosuppressive activity of BMSCs.

Discussion

BMSCs are easy to amplify *in vitro*. In addition, they have the capacity for self-renewal, multi-differentiation, and can specifically differentiate into bone, fat and cartilage cells. Therefore, BMSCs are good seed cells for use in tissue engineering (13), including in the field of trauma surgery. The ability of BMSCs to form bone tissue *in vivo* is relatively clear and so they have been used clinically to repair bone tissue in patients with tibia ulna defects (4), osteogenesis imperfecta (14) and mandibular defects (15). Additionally, previous studies revealed that BMSCs protected nerve cells following spinal cord injury and various factors that they secrete reduced local inflammation, and on the other hand, BMSCs can migrate to the damaged area and differentiate into specific cell types (16,17).

Previous studies suggested that simvastatin regulates bone morphogenetic protein-2 and vascular endothelial growth factor in osteoblasts, modulates bone metabolism and exhibits osteogenic potential; therefore, it has received increasing attention (18). The present study initially assessed the effects of simvastatin on BMSC differentiation. The data revealed that BMSCs incubated *in vitro* with 1.0 μmol/ml simvastatin exhibited significantly elevated ALP activity. In addition, von

Kossa staining revealed the presence of a nodular structure of calcium deposition. RT-PCR demonstrated that treatment with simvastatin for 14 days markedly increased the mRNA expression levels of osteocalcin and bone sialoprotein, suggesting that simvastatin potently induced osteogenic differentiation of BMSCs *in vitro*.

BMSCs have low immunogenicity and marked immune modulatory function, and they can inhibit proliferation, differentiation, chemotaxis, and the secretion of a variety of immune cells, including T lymphocytes, B lymphocytes, NK cells and antigen presenting cells (19). Therefore, BMSCs can be used for the treatment of graft-vs-host disease (20), systemic lupus erythematosus (21) and Sjögren syndrome (22). However, the effect of simvastatin on the immune function of BMSCs remains to be elucidated. In the present study, the treatment of BMSCs with simvastatin for 7 days, followed by the co-culture with an equal quantity of allogeneic PBMCs for a further 5 days revealed that BMSCs caused no affect on the proliferation of allogeneic PBMCs. This suggested that BMSCs had low immunogenicity and low antigen-presenting ability, and that simvastatin caused no affect on the immunogenicity of BMSCs. In addition, untreated BMSCs and simvastatin-treated BMSCs each suppressed the PHA-stimulated PBMC proliferation to an identical extent, suggesting that simvastatin-induced BMSCs retained their immunosuppressive properties.

The present study demonstrated that treatment with 1.0 μmol/ml simvastatin induced the significant bone differentiation of BMSCs without altering their immunomodulatory properties. Therefore, allogeneic simvastatin-induced BMSCs may be used for the treatment of patients suffering orthopedic trauma and bone non-union.

Acknowledgements

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