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Ascorbic acid induces osteoblast differentiation of human suspension mononuclear cells

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Abstract

Background aims. Suspension mononuclear cells (MNCs) can be differentiated into osteoblasts with the induction of ascorbic acid and β-glycerophosphate. The aim of this study was to determine the ability of suspension MNCs to differentiate into osteoblasts using ascorbic acid only. *Methods.* Suspension MNCs were obtained by a combination of gradient centrifugation and culture selection. Suspension MNCs were subjected to differentiation assay by culturing them inside proliferation medium supplemented with 10 μg/mL, 30 μg/mL, 50 μg/mL, 60 μg/mL, 90 μg/mL and 500 μg/mL of ascorbic acid. Proliferation medium supplemented with 50 μg/mL ascorbic acid and 10 mmol/L β-glycerophosphate was used as a positive control for osteoblast induction, and proliferation medium without ascorbic acid was used as a negative control. Differentiation analysis was performed using alkaline phosphatase (ALP) assay, von Kossa staining and expression of osteoblast-related genes. *Results.* With all concentrations of ascorbic acid used, there was a significant increase ($P < 0.05$) in ALP-specific activity and mineralized nodule formation throughout the differentiation course compared with negative control. Ascorbic acid was also able to activate the expression of osteopontin (*SPP1*), osteonectin (*SPARC*) and runt-related transcription factor 2 (*RUNX2*) messenger RNA in positive control and ascorbic acid-induced MNCs (30 μg/mL and 90 μg/mL) but not in negative control. *Conclusions.* Ascorbic acid alone was sufficient to induce osteoblast differentiation from suspension MNCs; 30–90 μg/mL of ascorbic acid was found to be the optimal concentration. Ascorbic acid can be used as a nutritional supplement for cellular therapy of bone-related disease.

Key Words: *alkaline phosphatase, ascorbic acid, mineralization, osteoblasts, suspension mononuclear cell*

Introduction

Dynamic remodeling of bone in adults involves an exquisite interplay of developmental cues, signaling proteins, transcription factors and co-regulatory proteins that support differentiation of osteoprogenitor cells. *In vitro* osteogenic differentiation has been reported in response to various bioactive factors, such as osteogenin/bone morphogenetic protein 3 (1), bone morphogenetic protein 2 (2), osteogenic growth peptide (3), β-glycerophosphate (4) and the synthetic glucocorticoid dexamethasone (5). An understanding of the early morphogenetic signals necessary for regulating osteoblast gene expressions has provided intriguing insights into the important role of ascorbic acid for bone remodeling. The role of ascorbic acid in the expression of osteoblastic markers and for mineralization has been demonstrated in many osteoblast culture systems, including primary cultures of fetal rat calvarial cells (6), chick pre-osteoblasts (7), and human osteoblast-like cell lines (8). Ascorbic acid also serves as a potent inducer for mesenchymal stromal cells to differentiate into osteoblasts (9). Although many researchers have reported the importance of ascorbic acid for collagen matrix accumulation and expression of osteoblast phenotypes in various cell culture systems,

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the effects of ascorbic acid on differentiation potential of suspension mononuclear cells (MNCs) from human peripheral blood are unknown.

Ascorbic acid is an essential co-factor for the catalytic reaction of prolyl hydroxylase enzymes that involves synthesis of extracellular matrix. Ascorbic acid enhances development of collagen matrix at various stages, including gene transcription, messenger RNA (mRNA) stabilization, translation, hydroxylation and secretion (10). In addition to extracellular matrix synthesis, ascorbic acid modulates the differentiation of multi-potent progenitor cells into bone cells by activation of integrin signaling and promoting cell matrix interaction (11). The use of ascorbic acid as an inducer of osteoblast differentiation would be an advantage in cellular therapy especially in the treatment of bone-related diseases because ascorbic acid is a vitamin C (12) and possesses a protective role as an antioxidant and consumable supplement with no toxic effect when consumed within recommended dietary intake (13,14).

MNCs can be isolated from peripheral blood by density gradient centrifugation (15), and the cells can be cultured in well-defined routine tissue culture medium under standardized conditions for culture selection and elimination of mature MNCs (16). We previously reported that after culture selection, suspension MNCs were identified to express hematopoietic stem cell marker and retained the capacity to differentiate into osteoblasts on stimulation by standard osteoblast differentiation factors, which consist of proliferation medium supplemented with ascorbic acid and β-glycerophosphate (17,18). Our aim in the present study is to explore the possibility of using ascorbic acid only for osteoblast induction of human suspension MNCs. Analysis of differentiation involved biochemical assay of alkaline phosphatase (ALP) enzyme, von Kossa staining of osteoblasts and expression of osteoblast gene markers via molecular analysis.

Methods

Blood collection and isolation of MNCs

Blood of healthy humans 18–25 years old was drawn into ethylenediamine tetraacetic acid tubes (BD, Franklin Lakes, NJ, USA) following informed consent. Blood samples were diluted with Hank's Balanced Salt Solution (pH 7.; Sigma-Aldrich, St Louis, MO, USA) and layered on top of a Ficoll-Paque PLUS (GE Healthcare, Pittsburgh, PA, USA). After centrifugation at 400*g* for 25 min at room temperature, the high-density MNCs in the interface were collected, washed with phosphate-buffered saline (PBS), pH 7.4, and re-suspended in α-minimal essential medium supplemented with 10% (v/v) newborn calf serum and 2% (v/v) penicillin/streptomycin (Biowest, Nuaillé, France), subsequently referred to as proliferation medium. The cells were cultured at 1×10^5 cells/mL in proliferation medium and maintained at 5% CO_2 37°C for determination of proliferation of MNCs. After 7 days of culture selection, the non-adherent MNCs and adherent MNCs were distinguishable, and non-adherent MNCs, which were referred to as suspension MNCs, were collected and counted.

Differentiation of human suspension MNCs

Suspension MNCs were seeded at 1×10^5 cells/mL for osteoblast differentiation assay. MNCs were induced into osteoblasts by adding ascorbic acid (Sigma-Aldrich) at different concentrations, 10 μg/mL, 30 μg/mL, 50 μg/mL, 60 μg/mL, 90 μg/mL and 500 μg/mL, into the proliferation medium. As a positive control, proliferation medium with 50 μg/mL ascorbic acid and 10 mmol/L β-glycerophosphate (Sigma-Aldrich) was employed. Proliferation medium without ascorbic acid served as negative control. The cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. Differentiation analysis was performed on day 0, 1, 3, 7 and 14 on incubation with differentiation medium. Day 0 is defined as the day the supplement was added to the proliferation medium. Throughout the time course, the viability of MNCs cultured in proliferation medium and differentiation medium was determined by trypan blue exclusion assay.

Measurement of ALP activity profile

An assay for ALP activity was performed on 1×10^5 cells/mL cultured cells. The assay was carried out on day 0, 3, 5, 7, 10 and 14 of the osteoblast differentiation course. The cells were washed with PBS, and total protein concentration was determined by incubation in Bradford reagent (Sigma-Aldrich) containing 0.1% (v/v) Triton X-100 (Sigma-Aldrich) for 5 min at room temperature. The absorbance at 595 nm was measured against a reagent blank. For ALP assay, the cells were incubated in 0.1 mol/L sodium bicarbonate-sodium carbonate buffer (pH 10.0) containing 0.1% (v/v) Triton X-100, 2 mmol/L magnesium sulfate and 6 mmol/L p-nitrophenyl inorganic phosphate (Sigma-Aldrich) for 30 min at 37°C. The reaction was stopped by 1 mol/L sodium hydroxide (Sigma-Aldrich), and the absorbance was measured at 405 nm. Cellular ALP activity was determined by specific activity per number of cells.