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Induction of alkaline phosphatase activity by L-ascorbic acid in human osteoblastic cells: a potential role for CK2 and Ikaros

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Abstract

Objective: To investigate the effect of L-ascorbic acid (AsA) on osteoblast differentiation, we examined the effects of AsA on in vitro osteoblastic differentiation markers such as collagen synthesis, alkaline phosphatase (ALP) activity, and receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) expression. The role of Ikaros and casein kinase 2 (CK2) in regulating osteoblast differentiation was also determined.

Methods: This study examined the expression of RANKL and OPG, collagen synthesis, and ALP activity in AsA-treated osteoblast-like cells (MG63) using reverse transcription-polymerase chain reaction and biochemical assays. In addition, Ikaros activity and CK2 expression were assessed by electrophoretic mobility shift assays and western blot assays, respectively.

Results: The results showed that AsA treatment slightly downregulated OPG mRNA expression, whereas the mRNA expression of RANKL and collagen was unaffected. AsA significantly increased ALP activity after 4 d, and this activation was inhibited by the CK2 inhibitors, 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole and 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazimidazole. Small interfering RNA-mediated depletion of CK2-α also decreased ALP activity in AsA-stimulated cells. Moreover, western blot analysis showed that AsA induced the activation of CK2. AsA dose-dependently decreased the DNA binding affinity of the transcription factor Ikaros, which is a bifunctional differentiation factor. Moreover, cells treated with AsA and CK2 inhibitor exhibited increased Ikaros activity compared with those treated with AsA alone.

Conclusion: These results suggest that AsA stimulates osteoblastic differentiation by enhancing ALP activity and suppressing Ikaros activity. Moreover, this process might be related to CK2 regulation.

Keywords: L-ascorbic acid; Osteoblastogenesis; Alkaline phosphatase; casein kinase-2; Ikaros

Introduction

Bone is a dynamic tissue that is continually broken down and re-formed in a turnover process known as remodeling, which occurs through interplay between bone-forming cells, called osteoblasts, and bone-resorbing cells, called osteoclasts [1]. A disturbance in the tight balance between the activities of osteoblasts and osteoclasts leads to excessive gain of bone mass (osteopetrosis) or loss of bone mass (osteoporosis). Osteoporosis occurs far more frequently and is a major burden on health care systems globally. Many patients with osteoporosis have already lost substantial amounts of bone. Therefore, a method to increase bone mass by stimulating new bone formation is needed [2]. Most bone matrix proteins are secreted and deposited by polarized mature osteoblasts that are aligned on the bone surface. Osteoblasts produce various bone matrix proteins such as type I collagen, which is the most abundant extracellular bone protein, and also take charge of the mineralization of the tissue [3]. In addition, the expression of a number of bone-related extracellular matrix proteins, the high enzyme activity of alkaline phosphatase (ALP), and the responses to

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osteotropic hormones and cytokines are the major characteristics of osteoblasts. Osteoblasts can regulate osteoclast activity through cell-to-cell contact whereby the osteoblast cell-surface receptor activator of nuclear factor-κB ligand (RANKL) binds to its cognate receptor, receptor activator of nuclear factor-κB (RANK). The interaction between RANKL and RANK is the key terminal factor in inducing differentiation of osteoclast precursor cells and activation of mature osteoclasts [4,5]. Osteoprotegerin (OPG) and RANKL, which are synthesized by stromal osteoblasts, have been identified as the two principal cytokines of osteoclast differentiation and activation [6,7]. OPG inhibits osteoclast differentiation by competing with the binding of RANKL to RANK [6]. Therefore, the RANKL/OPG/RANK system is an important regulator of bone formation.

L-ascorbic acid (AsA), also known as vitamin C, is an essential cofactor for the hydroxylation of proline and lysine residues in collagen, which is the most abundant protein in the body [8]. AsA is also an essential supplement for the differentiation of various types of cells in culture and is involved in myogenesis, chondrogenesis, and osteogenesis through formation of the collagen matrix [9]. Although the ability of AsA to induce osteoblastic differentiation has been related to collagen matrix formation in several osteogenic cells [10–12], the molecular mechanism of AsA action in osteoblastogenesis is not completely understood.

Ikaros, a hematopoietic cell-specific zinc finger DNA binding protein, is a key regulator of lymphocyte proliferative responses and development [13]. Inactivating mutations in Ikaros cause antigen-mediated lymphocyte hyperproliferation and the rapid development of leukemia and lymphoma. In the early stages of differentiation research on Ikaros, there was a growing focus on the bifunctional effects of Ikaros on the activation [14,15] or suppression [16,17] of the differentiation of hematolymphoid cell lineages [18].

Casein kinase-2 (CK2) is a serine/threonine kinase that has been implicated in multiple cellular functions, including growth control and proliferation [19–22]. Recently, CK2 was also implicated in cell survival or as a cell-protective mechanism in antiapoptotic responses to diverse stress inducers [23–25]. The enzyme exists as a tetramer consisting of two catalytic subunits (α1, α2) and two regulatory subunits (β) [26]. Elevated activities of CK2, of the subunits alone or of the heterotetrameric holoenzyme, might reflect the pleiotropy of this kinase, which is supported by the fact that more than 300 substrates have now been identified [27]. These substrates are involved in a wide variety of cellular functions, including the regulation of transcription, signal transduction processes, growth control, various steps in development, and the formation of cellular shape and architecture. However, the cellular functions of the protein kinase CK2 remain unclear.

Interactions between proteins are important for many biological functions. CK2 is a plasma membrane-associated enzyme with extracellular and intracellular binding partners and substrates [28]. Phosphorylation of these substrates or binding to plasma membrane-associated proteins seems to implicate CK2 as involved mainly in the regulation of cell proliferation. In addition, one study has suggested a regulatory role for CK2 in cell proliferation using antibodies directed against the α-subunit [29]. Thus, interaction of substrates with CK2 may prove to be important. Recently, it was reported that the DNA binding activity of Ikaros was regulated by its phosphorylation and that CK2 was responsible for Ikaros phosphorylation [28]. However, the roles of Ikaros and CK2 in osteoblastogenesis have not been elucidated.

This study examined the effects of AsA on in vitro osteoblastic differentiation markers such as collagen synthesis, ALP activity, and RANKL and OPG expression. The roles of Ikaros and CK2 in the regulation of the response to AsA were also determined.

Materials and methods

Materials

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium, penicillin, streptomycin, trypsin/ethylene-diaminetetra-acetic acid (EDTA), and fetal bovine serum were purchased from Gibco Co. (Grand Island, NY, USA). AsA was purchased from Sigma Chemical Co. The antibodies against CK2 and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

The human osteoblastic cell line, MG63, was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (100 IU/mL of penicillin and 100 μg/mL of streptomycin) and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Analysis of ALP activity

MG63 cells (2×10^4) were seeded on six-well plates and incubated with reagents for 2, 4, or 6 d. The cell layer was then washed with Dulbecco's phosphate buffered saline and dissolved in 0.25% Triton X-100. The cell lysate was then sonicated for 30 s on ice. The cellular protein concentration was determined after incubation in a protein assay reagent; bovine serum albumin containing 0.25% Triton X-100 was used as a standard. The ALP activity was measured using a spectrophotometric method with para-nitrophenyl-phosphate as the substrate. The optical density was measured at 405 nm using an enzyme-linked immunosorbent assay