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Vitamin C-linker–conjugated tripeptide AHK stimulates BMP-2-induced osteogenic differentiation of mouse myoblast C2C12 cells

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ABSTRACT

Vitamin C-linker–conjugated Ala-His-Lys tripeptide (Vit C-AHK) is a derivative of Vitamin C–conjugated tripeptides, which were originally developed as a component of a product for collagen synthesis enhancement or human dermal fibroblast growth. Here, we investigated the effect of Vit C-AHK on bone morphogenetic protein (BMP)–2–induced osteoblast differentiation in a cell culture model. Vit C-AHK enhanced proliferation of C2C12 cells and induction of BMP-2–induced alkaline phosphatase, a typical marker of osteoblast differentiation. Vit C-AHK also stimulated the phosphorylation and translocation of Smad1/5/8 to the nucleus and phosphorylation of mitogen-activated protein kinases (MAPKs) including ERK1/2 and p38. In addition, Vit C-AHK enhanced the BMP-2–induced mRNA expression of osteoblast differentiation–related genes such as *ALP*, *BMP-2*, *Osteocalcin*, and *Runx2*. Our results suggest that Vit C-AHK exerts an enhancing effect on osteoblast proliferation and differentiation through activation of Smad1/5/8 and MAPK ERK1/2 and p38 signaling and without significant cytotoxicity. These results provide important data for the development of peptide-based bone-regenerative agents and treatment of bone-related disorders.

1. Introduction

Osteoblasts are the major cells arising from mesenchymal stem cells that affect bone density and produce osteoblast markers such as alkaline phosphatase (ALP) and bone matrix proteins such as osteocalcin (OCN) (Katagiri et al., 1994; Long, 2012). Induction of these osteogenic markers is modulated by the degree of balance between Smad1/5/8 and mitogen-activated protein kinase (MAPK) pathways (Suzukim et al., 2002; Wu et al., 2006; Eivers et al., 2008; Chae et al., 2002). Bone morphogenetic proteins (BMPs), part of the transforming growth factor-β superfamily, stimulate osteoblast differentiation and ossification (Phimphilai et al., 2006). Among the BMPs, BMP-2 is a major inducer of bone regeneration and osteoblastogenesis (Rosen, 2009; Wagner et al., 2010).

Many studies have investigated methods for restoring the balance between bone formation and resorption. Thus, anabolic agents that promote BMP-2–mediated osteoblast differentiation have been developed for the treatment of bone diseases (Rosen and Bilezikian, 2001; Garces and Garcia, 2006). However, existing chemotherapeutic agents for bone-related diseases require new effective applications because of limitations related to the administration method and cost (Garrett, 2007). In recent years, many companies have turned to existing drugs to reduce research and development (R & D) spending and have begun to adjust these drugs for other indications through the drug repositioning process (Longman, 2004; Stuart, 2004; Son et al., 2013). This model of drug development provides many pharmaceutical companies and investigators an effective method to reduce costs and risks such as pharmacokinetic/pharmacodynamic research and clinical trials.

Vitamin C (ascorbic acid) has a stimulating effect on osteoblast differentiation (Pustylnik et al., 2013; Hadzir et al., 2014a, 2014b). In addition, oligopeptides of fewer than 10 amino acids may act as messengers, stimulators, or neurotransmitters and are involved in various physiological processes such as proliferation, differentiation, and digestion (Loren et al., 1987). In particular, in skin regeneration, a tri-, tetra-, or hexa-peptide containing Gly-Lys-His, Gly-His-Lys, or Gly-Pro-Hyp (hydroxyproline) enhances the synthesis of collagen and glycosaminoglycan in the dermis. The Vitamin C-linker–conjugated Ala-His-Lys tripeptide (Vit C-AHK) is a derivative of a Vitamin C–

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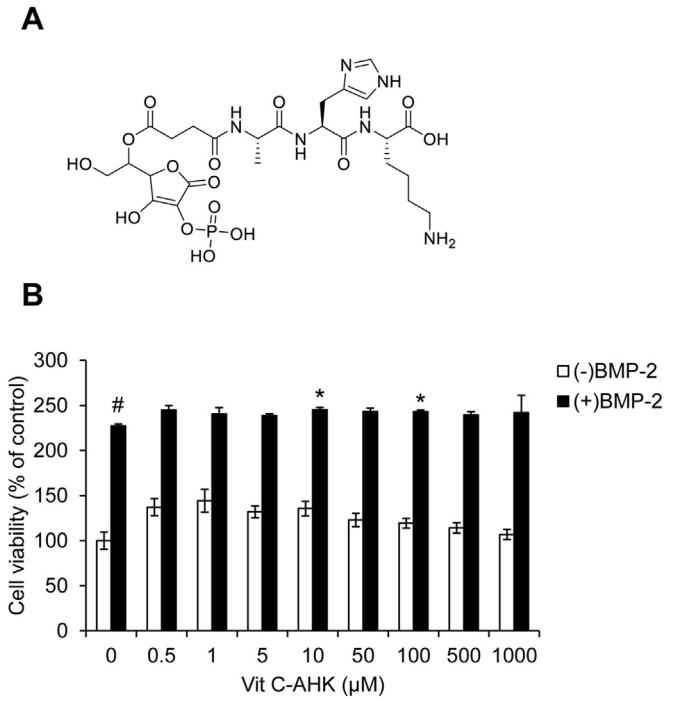

Fig. 1. The effect of Vit C-AHK on viability of C2C12 cells. (A) Chemical structure of Vit C-AHK. (B) C2C12 cells were treated with Vit C-AHK in the presence or absence of BMP-2 for 72 h. Cell viability was evaluated using the CCK-8 assay. Detailed experimental procedures are described in the Materials and Methods. Experiments were performed in triplicate.

conjugated tripeptide first designed as a component of products for improving collagen synthesis (Choi et al., 2009). This stimulating effect on the synthesis of collagen and proliferation of dermal fibroblasts has led to an in-depth investigation of Vit C-AHK, but no reports have evaluated associated bone homeostasis or osteogenic activity.

In this study, we investigated whether Vit C-AHK can stimulate the BMP-2–mediated signaling pathway and exert anabolic effects in osteoblast differentiation. Our results indicated that Vit C-AHK has stimulating effects on the expression of ALP and mRNA transcription of osteogenic genes through activation of Smad1/5/8 and MAPK signaling. These results demonstrate that Vit C-AHK has potent anabolic activity in BMP-2-induced osteoblast differentiation of bi-potential mesenchymal precursor C2C12 cells. In addition, the use of Vit C-AHK could be a therapeutic strategy for the development of drugs targeting bone-related disease treatment or osteogenesis.

2. Materials and methods

2.1. Materials

Vit C-AHK (500 mM stock solution in sterile distilled water) (Fig. 1A and Fig. S1) was synthesized by KY Park, a principal researcher of CHA Meditech Co., Ltd (Daejeon, Korea), and recombinant human BMP-2 was purchased from R & D Systems, Inc. (USA). Fetal bovine serum (FBS), Dulbecco's Modified Eagle's Medium (DMEM), and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) were purchased from Corning Inc. (USA). Antibodies against p-ERK1/2, ERK1/2, p-p38, and p38 were purchased from Cell Signaling Technology, Inc. (USA). Antibodies against p-Smad1/5/8, Smad1/5/8, horseradish peroxidase (HRP)-conjugated secondary antibodies, and HRP-conjugated actin were purchased from Santa Cruz Biotechnology, Inc. (USA). The ALP staining kit, MEK1/2 inhibitor PD98059, and p38 MAPK inhibitor SB202190 were purchased from Sigma Aldrich (USA).

2.2. Cell culture and differentiation

Mouse bi-potential mesenchymal precursor C2C12 (mouse myoblast cell line) cells and mouse fibroblast C3H/10T1/2 cells were purchased from ATCC (USA) and Korean Cell Line Bank (No.10226), respectively and maintained in DMEM containing 10% FBS and 1% antibiotics in a humidified atmosphere of 5% CO_2 at 37 °C. For osteoblast differentiation, C2C12 cells were seeded in a 96-well plate for 72 h and then treated with BMP-2 (50 ng/mL) alone or combined with Vit C-AHK in culture media containing 5% FBS. C2C12 cells were differentiated by replacing the medium with DMEM with 5% FBS and BMP-2 (50 ng/mL). The medium was changed every 3 days.

2.3. Cell viability assay

The C2C12 cells (4×10^3 cells/well) were seeded in a 96-well plate for 24 h. Cells were treated with Vit C-AHK for 72 h in culture media containing 5% FBS. Cell viability was assessed using the Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions. The absorbance was measured with a Multiscan™ FC microplate photometer (Thermo Fisher Scientific, Boston, MA, USA). Cell viability is presented as % of control (untreated cells). Experiments were performed in triplicate.

2.4. Alkaline phosphatase (ALP) staining and activity assay

The C2C12 cells (4×10^3 cells/well) were seeded in a 96-well plate and incubated for 24 h. Cells were treated with BMP-2 (50 ng/mL) alone or combined with Vit C-AHK in culture media containing 5% FBS for 72 h. After incubation, cells were fixed with 3.7% formaldehyde, rinsed with phosphate-buffered saline, and stained with the ALP staining kit (Sigma, USA). The ALP-positive cells were visualized by phase-contrast light microscopy (Olympus Optical, Japan), and ALP activity was evaluated using the 1-Step™ PNPP Substrate solution (Thermo Fisher Scientific, USA), with absorbance measured with a Multiscan™ FC microplate photometer (Thermo Fisher Scientific, Boston, MA, USA). Experiments were performed in triplicate.

2.5. Western blot analysis

C2C12 cells (4×10^4 cells/mL) were seeded in 6-well plates and incubated for 24 h. After treatment with BMP-2 (50 ng/mL) alone or combined with Vit C-AHK for 72 h, cytoplasmic and nuclear fractions of lysates were prepared as described previously (Lee et al., 2016). Lysates of cytoplasmic fractions and nuclear fractions were loaded on 8–15% polyacrylamide gels and transferred to nitrocellulose membranes. Specific primary antibodies were used to detect the expression of proteins. After incubation with HRP-conjugated secondary antibodies, the signals were visualized using Luminata™ Forte Western HRP Substrate (Merck Millipore, Darmstadt, Germany). The band intensities were measured to determine the relative protein expression using X-ray films and development solution (Fujifilm, Tokyo, Japan). Actin was the loading control. The detected bands were quantified based on the ImageJ software, and the relative ratio between each sample and untreated control was presented in the figures.

2.6. Quantitative real-time PCR (qRT-PCR) analysis

The C2C12 cells were treated for 72 h with BMP-2 (50 ng/mL) alone or combined with Vit C-AHK in culture media containing 5% FBS. Total RNA was isolated from C2C12 cells using the AccuPrep® RNA Extraction Kit (Bioneer Corp., Daejeon, Korea) and the cDNA synthesized from 1 μg of total RNA using oligo (dT) primers (Bioneer Corp., Daejeon, Korea) and the RocketScript™ Reverse Transcriptase Kit (Bioneer Corp., Daejeon, Korea). qRT-PCR was performed with the ExcelTaq 2 × Q-PCR Master Mix (SMOBiO, Hsinchu, Taiwan) and the