Collagen-derived dipeptide prolyl-hydroxyproline promotes differentiation of MC3T3-E1 osteoblastic cells

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ARTICLE INFO

ABSTRACT

Prolyl-hydroxyproline (Pro-Hyp) is one of the major constituents of collagen-derived dipeptides. The objective of this study was to investigate the effects of Pro-Hyp on the proliferation and differentiation of MC3T3-E1 osteoblastic cells. Addition of Pro-Hyp did not affect MC3T3-E1 cell proliferation and matrix mineralization but alkaline phosphatase activity was significantly increased. Furthermore, cells treated with Pro-Hyp significantly upregulated gene expression of *Runx2, Osterix*, and *Col1α1*. These results indicate that Pro-Hyp promotes osteoblast differentiation. This study demonstrates for the first time that Pro-Hyp has a positive effect on osteoblast differentiation with upregulation of *Runx2, Osterix*, and *Collα1* gene expression.

1. Introduction

Collagen is a key extracellular matrix protein present mainly in skin and bone. Gelatin is a denatured form of collagen and collagen-derived peptides (CP) is formed by protease hydrolysis of this gelatin. Various di- or tripeptides are included in CP. Furthermore, it is thought that collagen in living tissues is degraded into collagen peptides by various enzymes secreted by osteoclasts or osteoblasts during the process of bone metabolism [1,2]. CP can be derived not only from food but also from collagen in connective tissues such as skin and bone.

Several food-derived collagen oligopeptides were identified in human blood after oral ingestion of CP [3,4]. Prolyl-hydroxyproline (Pro-Hyp) is the major constituent of CP, which remains within human blood after ingestion of CP [5–7]. Pro-Hyp or hydroxyproline-containing peptides are hard to be hydrolyzed *in vivo* and may play important functions in target tissues [8]. It has been reported that Pro-Hyp affects the growth of fibroblasts and regulates the differentiation of chondrocytes [9,10].

A previous study has shown that oral administration of CP increases bone mineral density in calcium-deficient rats [11].

Furthermore, some studies have indicated that crude CP has a beneficial effect on the proliferation or differentiation of osteoblastic cells [12–14]. Therefore, CP may also play an important role in bone remodeling. However, the role of specific sequences peptide including in CP on bone metabolism is currently unclear. In this study, we investigated the effect of Pro-Hyp on the proliferation and differentiation of MC3T3-E1 osteoblastic cells.

2. Materials and methods

2.1. Cell culture

Mouse calvarial, osteoblastic cell line, MC3T3-E1 cells were cultured in growth medium comprising alpha-modified Eagle's medium (α-MEM) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) and 100 U/mL penicillin. Cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂. For mineralization analysis, growth medium was replaced with osteoblast differentiation medium (ODM) comprising α-MEM supplemented with 10% FBS, 5 mmol/L β-glycerophosphate, 1 nmol/L dexamethasone, and 100 μg/mL ascorbic acid.

2.2. Cell proliferation

Cells were seeded in a 96-well plate at a density of 3×10^3 cells/well and cultured for 3 h in growth medium. Medium was then replaced with identical medium containing Pro-Hyp (0.01, 0.1,

and 1 mM, Bachem, Heidelberg, Germany). Cells were cultured with or without Pro-Hyp and cell proliferation was measured on day 2 of culture using the WST-1 assay (Cell Counting Kit; Dojindo Laboratories, Kumamoto, Japan). Plates were read using a microplate reader (Perkin Elmer, Inc., Waltham, MA, USA) at a wavelength of 450 nm.

2.3. Alkaline phosphatase (ALP) activity

Cells were cultured to confluence and growth medium was replaced with identical medium containing 0.1 or 1 mM Pro-Hyp. Cells were cultured for an additional 5 days. After incubation, cells were fixed with 20% formalin for 10 min and incubated in 0.05 mol/L 2-amino-2-methyl-1-propanol (AMP) buffer (pH 9.8), containing 10 mM naphtol AS-BI phosphate and 1 mM fastred violet LB salt for 30 min at 37 °C. The staining solution was aspirated and the cells were washed with deionized water [15]. The ALP stained areas were scanned using an image scanner and analyzed qualitatively using Image J software.

2.4. Mineralization assay

Cells cultured for 21 days in ODM and ODM + Pro-Hyp (0.1 and 1 mmol/L). ODM was changed every 2 days. After incubation, cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min. Cells were then washed twice with deionized water and 5% silver nitrate was added. Cells were exposed to ultraviolet (UV) light for 15 h. Finally, the cells were fixed with 5% sodium thiosulfate for 5 min [16]. Mineralized nodules were scanned using an image scanner and analyzed qualitatively using Image J software.

2.5. Quantitative real-time polymerase chain reaction (PCR)

MC3T3-E1 cells were seeded in growth medium in a six-well plate at a density of 9×10^4 cells/well. After culturing for 3 days, the medium was then replaced with identical medium containing 0.1 and 1 mM Pro-Hyp. The cells were cultured with or without Pro-Hyp for 3 h. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was converted with the PrimeScript™ reagent kit (Takara Bio Japan, Shiga, Japan). Quantitative real-time PCR was performed using the TaqMan gene expression assay (Applied Biosystems, Carlsbad, CA, USA). TaqMan probes were as follows: *Runx2* (Mm00501584_m1), *Osterix* (Mm04209856_m1), *Col1α1* (Mm00801666_g1). *GAPDH* (Mm03302249_g1) was used as the internal control for normalization of target gene expression.

2.6. Statistical analysis

Results are presented as means ± standard deviation (SD). Statistical analysis was performed using the Student's *t*-test. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Effect of Pro-Hyp on MC3T3-E1 cell proliferation

We evaluated whether Pro-Hyp treatment could enhance proliferation of MC3T3-E1 cells. MC3T3-E1 cells were cultured with 0.01–1 mM Pro-Hyp for 2 days and cell growth was measured using the WST-1 assay. The OD450 value for the control without Pro-Hyp was 0.79 ± 0.012. In the presence of 0.01, 0.1, and 1 mM of Pro-Hyp, the OD450 values were 0.79 ± 0.017, 0.081 ± 0.013,

and 0.79 ± 0.026, respectively. No significant differences were observed between the control and Pro-Hyp-treated cells (Fig. 1).

3.2. Effect of Pro-Hyp on ALP activity in MC3T3-E1 cells

We examined the effect of Pro-Hyp on ALP activity as a marker of osteoblast differentiation. MC3T3-E1 cells were cultured in the presence of 0, 0.1, or 1 mM Pro-Hyp for 5 days and cell differentiation was measured by ALP staining. The stained areas of the 0.1 and 1 mM Pro-Hyp-treated cells increased about 2.1- and 5.6-fold,
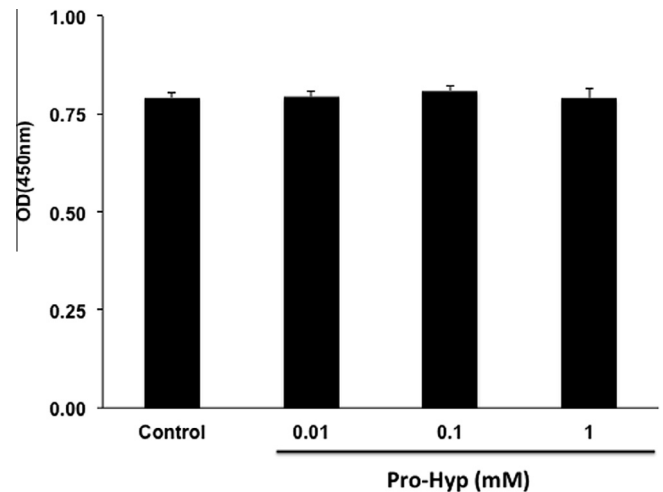

Fig. 1. Effect of Pro-Hyp on MC3T3-E1 cell proliferation. MC3T3-E1 cells were treated with Pro-Hyp at 0.01–1 mM for 2 days. MC3T3-E1 cell proliferation was measured using the WST-1 assay. Data are presented as means ± SD (n = 4).
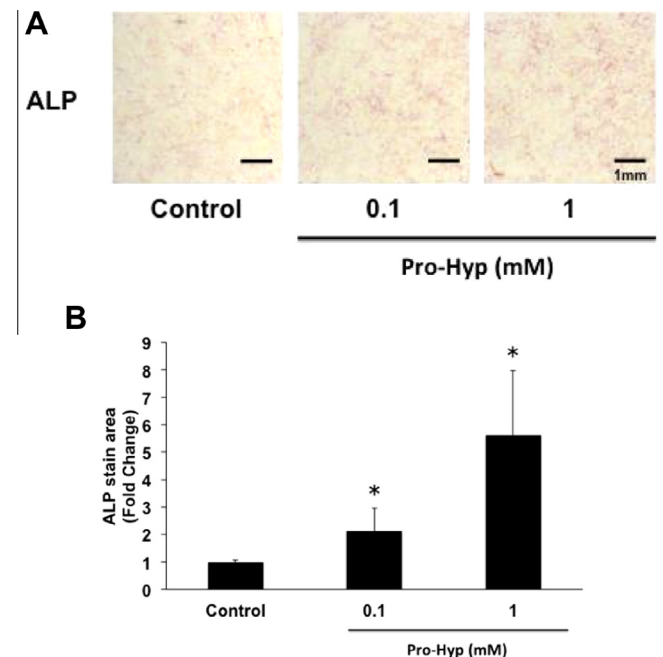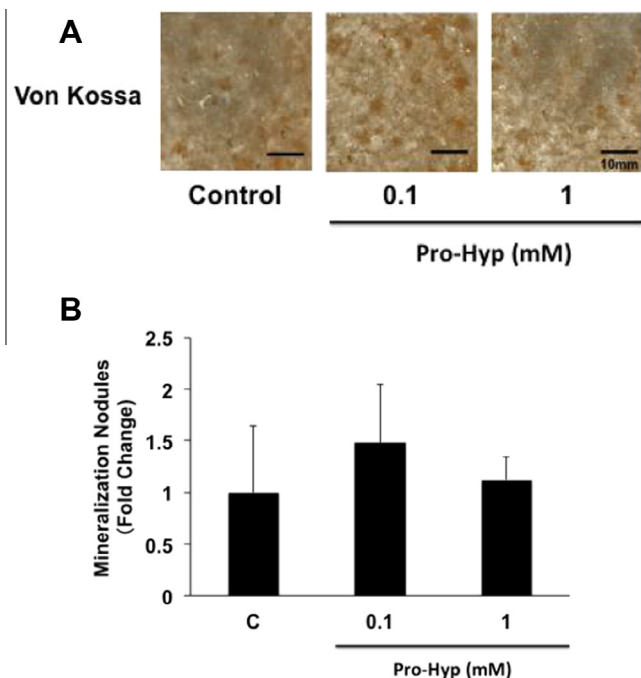

Fig. 2. Effect of Pro-Hyp on MC3T3-E1 alkaline phosphatase (ALP) activity. MC3T3-E1 cells were cultured for 5 days in osteoblast differentiation medium (ODM) with 0.1 or 1 mM of Pro-Hyp. ALP stained areas were scanned and analyzed qualitatively. (A) The scanned images show ALP staining in MC3T3-E1 cells in the presence or absence of Pro-Hyp (0.1 and 1 mM). The scale bar represents 1 mm. (B) ALP staining was evaluated using Image J software. Data are presented as means ± SD (n = 4), *$p < 0.05$.

Fig. 3. Effect of Pro-Hyp on mineralization of MC3T3-E1 cells. MC3T3-E1 cells were cultured for 21 days in osteoblast differentiation medium (ODM) with 0.1 or 1 mM of Pro-Hyp. The mineralized nodules were scanned and analyzed qualitatively. (A) The scanned images show Von Kossa staining in MC3T3-E1 cells in the presence or absence of Pro-Hyp (0.1 and 1 mM). The scale bar represent 10 mm. (B) Mineralized nodules were evaluated using Image J software. Data are presented as means ± SD (n = 3), *$p < 0.05$.

respectively, compared with the untreated ODM-only control cells ($p < 0.05$, Fig. 2).

3.3. Effect of Pro-Hyp on mineralization of MC3T3-E1 cells

To examine the effect of Pro-Hyp on osteoblast mineralization, MC3T3-E1 cells were cultured with ODM in the presence of 0, 0.1, and 1 mM Pro-Hyp for 21 days. Osteoblast mineralization was measured by Von Kossa staining. Mineralized nodules in the 0.1 and 1 mM Pro-Hyp-treated cells were increased about 1.37- and 1.07-fold compared with the untreated ODM-only control cells (Fig. 3).

3.4. Effect of Pro-Hyp on gene expression of Runx2, Osterix, and Col1α1 in MC3T3-E1 cells

To examine the effect of Pro-Hyp on osteoblastic gene expression in MC3T3-E1 cells, MC3T3-E1 cells were cultured with ODM in the presence of 0, 0.1, and 1 mM Pro-Hyp for 3 h and osteoblastic gene expression was determined by quantitative real-time PCR.

Runx2 and *Osterix* mRNA levels were increased in a dose-dependent manner in the presence of Pro-Hyp. Expression of *Runx2* increased about 1.43- and 2.21-fold in the 0.1 and 1 mM Pro-Hyp-treated groups, respectively, compared with the control ($p < 0.05$, Fig. 4A). The expression of *Osterix* increased about 1.41- and 2.88-fold in the 0.1 and 1 mM Pro-Hyp-treated groups, respectively, compared with the control ($p < 0.05$, Fig. 4B). *Col1α1* mRNA level was increased following treatment with high concentration of Pro-Hyp. The expression of *Col1α1* increased about 3.34-fold in the 1 mM Pro-Hyp-treated group compared with the control ($p < 0.05$, Fig. 4C).
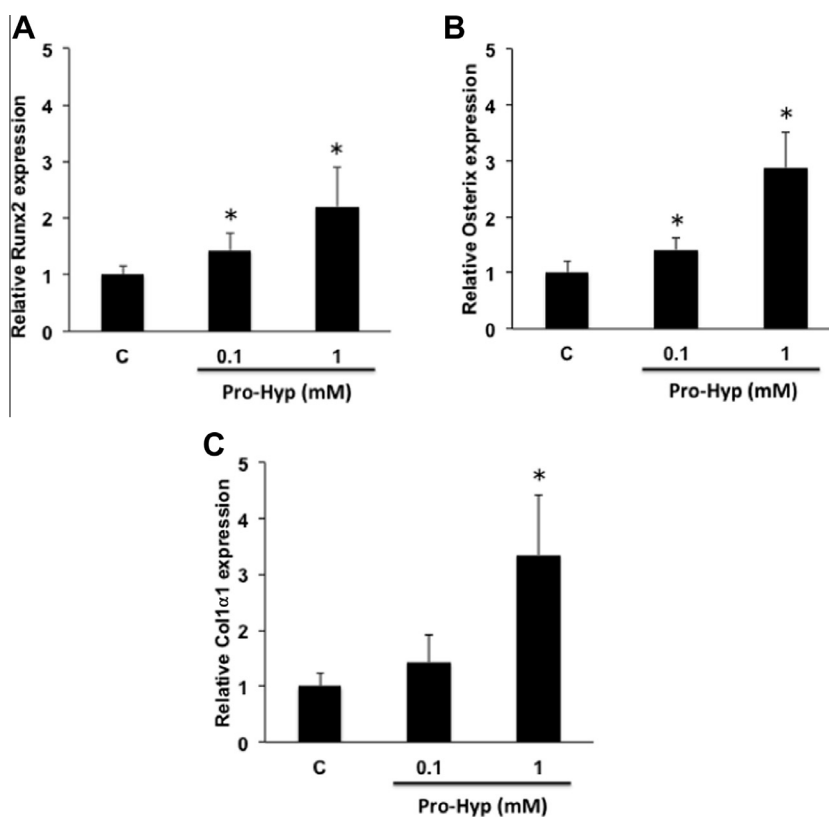
Fig. 4. Effect of Pro-Hyp on mRNA expression levels of *Runx2*, *Osterix* and *Col1α1* in MC3T3-E1 cells, analyzed by RT-PCR. After 3 days culture, medium was replaced with identical medium containing 0.1 or 1 mM of Pro-Hyp. Cells were cultured with or without Pro-Hyp for 3 h. RT-PCR analysis of the expression levels of *Runx2* (A), *Osterix* (B), and *Col1α1* (C) mRNA levels are shown. Results are expressed as relative values to *GAPDH*. Data are presented as means ± SD (n = 4), *$p < 0.05$.

4. Discussion

In this study, we investigated the effect of Pro-Hyp on the pro-liferation and differentiation of the MC3T3-E1 osteoblastic cell line. Pro-Hyp did not affect the proliferation of MC3T3-E1 cells. Previous reports have shown that crude CP stimulates the proliferation of osteoblast cells. In one previous report, crude CP was used that may include various collagen-derived di-, tri-, and oligo-peptides such as Pro-Hyp, Pro-Hyp-Gly, Hyp-Gly, or Ala-Hyp [12–14]. It is speculated that these collagen-derived peptides, except Pro-Hyp, have the ability to enhance osteoblast proliferation. Further studies are necessary to investigate the effects of other specific sequence peptides included in CP on the proliferation of osteoblasts.

During the osteoblast differentiation phase, osteoblasts synthe-size type 1 collagen, ALP, and various non-collagen proteins that are associated with matrix mineralization. ALP is a marker enzyme of early-stage osteoblast differentiation. Mineralized nodules appear as a marker of late-stage osteoblast differentiation [17–20]. We revealed that Pro-Hyp could significantly increase ALP activity in MC3T3-E1 cells compared with the control. This result suggests that Pro-Hyp enhanced osteoblast differentiation during the early stage. However, the formation of mineralized nod-ules in the MC3T3-E1 cells was not affected by the addition of Pro-Hyp. A previous study has shown that crude CP increases the collagen content in osteoblast cell cultures and promotes matrix mineralization [12]. Further studies are necessary to investigate the cooperative effect of Pro-Hyp and other di-, tri-, and oligo-peptides that are included in CPo promote matrix mineralization in MC3T3-E1 cells.

In addition, we examined the effects of Pro-Hyp on the regula-tion of osteoblast-associated genes. *Runx2* is a transcription factor that regulates the differentiation of osteoblasts and the expression of *Col1α1* and other extracellular matrix protein genes during bone formation [21]. Osterix is also an essential transcription factor for osteoblast differentiation [22]. We found Pro-Hyp significantly up-regulated *Runx2*, *Osterix* and *Col1α1* mRNA levels in MC3T3-E1 cells compared with the control. Furthermore, *Runx2* and *Osterix* mRNA levels were significantly increased with addition of lower concentrations of Pro-Hyp. These results indicate that Pro-Hyp can promote the differentiation of osteoblasts by upregulating gene expression of *Runx2* and *Osterix*. Previous reports have shown that treatment with crude CP increases the expression of the *Runx2* and the *Col1α1* genes in osteoblastic cells [13,14]. The regulation by Pro-Hyp in MC3T3-E1 cells would explain a mechanism of the protective effect of CP in bone metabolism.

In conclusion, this study demonstrates for the first time that Pro-Hyp, containing food-derived collagen hydrolysate and colla-gen metabolites in the blood, has a positive effect on osteoblast differentiation. These results indicate that bone formation is modulated by CP from oral ingestion (exogenous) or bone metabolites (endogenous). These findings may indicate the potential utility of Pro-Hyp as a food supplement or biomaterial in bone health.

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