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Original Article

Osteogenic growth peptide enhances the proliferation of bone marrow mesenchymal stem cells from osteoprotegerin-deficient mice by CDK2/cyclin A

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To promote bone formation is one of the fundamental strategies in osteoporosis treatment and fractures repair. As one of the stimulators on bone formation, osteogenic growth peptide (OGP) increases both proliferation and differentiation of the osteoblasts *in vitro* and *in vivo*, in which osteoprotegerin (OPG) has been suggested being involved. In this study, we evaluated the effects of OGP on bone marrow mesenchymal stem cells (MSCs) from OPG-deficient mice *in vitro* by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, alkaline phosphatase (ALP) activity assay, real-time polymerase chain reaction, and western blot analysis. Results showed that OGP stimulated MSC proliferation and increased the expression of CDK2 and cyclin A in MSCs both at mRNA and protein levels. However, no differentiative effect of OGP was shown as ALP activity and the expression levels of Runx2 and Osterix were not increased significantly by OGP. Our study suggested that OGP may increase the bone formation in OPG-deficient mice by stimulating MSC proliferation rather than differentiation, and probably by triggering CDK2/cyclin A pathway.

Keywords osteogenic growth peptide; osteoprotegerin; mesenchymal stem cell; cell proliferation; CDK2/cyclin A

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Introduction

To promote bone formation is one of the fundamental strategies in osteoporosis treatment and fractures repair [1]. Osteogenic growth peptide (OGP), a tetradecapeptide initially isolated from regenerating bone marrow, is identical to the C-terminus of histone H4 [2]. OGP stimulates cell proliferation, alkaline phosphatase (ALP) activity, and matrix mineralization via an autoregulated feedback mechanism *in vitro* [2–4], increases trabecular bone mass [2], and modulates fracture callus structural and mechanical properties [5] by stimulation of bone formation *in vivo*. The active portion of OGP, the OGP (10–14) region, directly regulates progenitor rat mesenchymal stem cells (MSCs) differentiating into more osteoblasts and less adipocytes and might be relevant to the pathogenesis and the treatment of osteoporosis [6].

In our previous studies, we found that OGP could accelerate osteogenic cell proliferation [7] and fracture healing [8]. The osteoprotegerin (OPG)-deficient mouse is an excellent animal model for osteoporosis, which is useful for screening and evaluating medicines to treat osteoporosis [9]. OPG, released by osteoblasts [10], can bind to receptor activator for nuclear factor κB ligand (RANKL), acting as a decoy to prevent RANK and RANKL from coming in contact with each other. The balance of RANKL/OPG may be crucial in osteoporosis. The OPG-deficient mice exhibit an osteoporotic phenotype [11]. It has already been assumed that OGP (10–14) could increase OPG secretion and can restore the altered expression of OPG [12]. Recently, OGP was injected subcutaneously into OPG-deficient mice for 6 weeks. Its bone mineral density and serum ALP level were found to be elevated significantly [13]. So, we hypothesized that OGP could activate bone growth with other ways beside the OPG. Therefore, we used MSCs derived from the OPG-deficient mice to observe OGP's effects on its proliferation and differentiation. rhBMP2, a potent osteoinductive factor [14], which had been shown to induce osteogenic differentiation of mesenchymal cells [15], is available in clinical now for bone fusion [16], being used as the positive control.

Materials and Methods

Synthesis of OGP

The synthetic OGP was synthesized by an Fmoc solid-phase synthesis procedure in the Institute of Biochemistry

and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) [17]. Its amino acid sequence is Ala-Leu-Lys-Arg-Glu-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly with the purity of 99.2% measured by high-performance liquid chromatography and capillary electrophoresis [7].

Preparation and culture of bone marrow MSCs

Bone marrow MSCs were obtained from the OPG-deficient mice (male, 12 weeks old; Shanghai Research Center for Biomodel Organism, Shanghai, China). Femora and tibiae were removed under aseptic conditions. After removal of the surrounding muscle and connective tissue, the ends of bones were cut to expose the bone marrow cavity. The cavity was washed three times with Dulbecco's modified Eagle's medium-low glucose (Gibco, Gaithersburg, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, USA), streptomycin (100 μg/ml), and penicillin (100 U/ml). The bone marrow suspension was filtered through a fine-meshed sieve to remove bone particles and agitated by gentle pipetting to obtain a single cell suspension. Aliquots of the cell suspensions were counted with a hemocytometer.

Primary MSCs were plated into a 25-cm^2 flask, maintained at 37°C with 5% CO_2 in the above culture medium, and allowed to attach for 3 days before the removal of non-adherent cells [18]. When the culture reached the confluent stage, they were detached by 0.25% trypsin for 5 − 10 min at 37°C and then scraped from the surface of the culture plate. Adhered MSCs after first passage were used in all other experiments, and here defined as passage first (P_1) cells.

The P_1 cells were cultured in the above culture medium supplemented with 10^{-8} M dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml of ascorbic acid, which was termed an experimental culture medium. Cells were cultured in the experimental culture medium supplemented with 10^{-9} M OGP or 0.1 μg/ml of bone morphogenetic protein 2 (rhBMP2) or vehicle (4% fatty acid-free bovine serum albumin). The medium was replaced twice a week. The concentration of OGP (10^{-9} M) was determined by our series experiments that we had done before. We found that OGP was effective between 10^{-13} and 10^{-7} M and its peak effect usually appears between 10^{-9} and 10^{-11} M.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

The proliferative response was examined by a colorimetric assay system based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [19]. Briefly, 4 h before the end of culture, 5 mg/ml of MTT dissolved in minimum essential medium (Gibco) without phenol red was added to cultures in 96-cell plates. Plates were incubated for 4 h at 37°C in a CO_2 incubator. Media were then removed and 10% sodium dodecyl sulfate (SDS) was added. The plates were then incubated at 37°C for 2 h. Absorbance was read at 570 nm by an automicroplate reader (EL × 800; BioTek, Winooski, USA).

ALP assay

ALP activity was assessed by the time-dependent 4-nitrophenylphosphatehexahydrate (PNPP) method [20]. The medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS). Each well was added diethanolamine buffer to lyse the cells and PNPP as a substrate to react with ALP, and then shaken for 30 min at 37°C. The reaction was stopped by adding 0.2 M NaOH. The optical density was measured at 405 nm by an automicroplate reader (EL × 800; BioTek).

RNA purification and gene expression analysis by reverse transcriptase–PCR

MSCs (10^6 cells/dish) were cultured as described above. After 3 days of culture, total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, USA) following the recommendations of the manufacturer. Quantitation of RNA was performed by determining the absorbance at 260 and 280 nm. Total RNA was reverse transcribed to cDNA using the QuantiTect Rev Transcription kit (Qiagen, Hilden, Germany). The number of cDNA molecules in the reverse-transcribed samples was determined by real-time PCR analysis using a modified method with QuantiTect SYBR Green PCR kit (Qiagen) on the Mx3000P Real-Time PCR system (Stragene, La Jolla, USA) following the primers as shown in **Table 1**. The reaction mixture included 12.5 μl of master SYBR Green I, 0.25 μM of each 5′ and 3′ primers, and 2 μl of samples and/or H_2O to a final volume of 25 μl. A melting curve was obtained at the end of each run to discriminate specific from non-specific cDNA products. Content of cDNA was normalized by subtracting the cycle numbers of *GAPDH* from target gene ($\Delta C_t = C_t$ of target gene − C_t of *GAPDH*) and gene expression level was calculated using formula of $2^{-(\Delta C_t)}$ [21].

Western blot analysis

After 7 days of culture, the cells were washed twice with PBS and lysed using cell lysis buffer (Beyotime, Jinan, China) plus 1:100 volume of phenylmethanesulfonyl fluoride (100 mM). The cell lysates were centrifuged and the total protein content in the supernatants was measured using a BCA assay kit (Beyotime). The equivalent amount of protein was separated by 10% SDS–PAGE and transferred onto 0.45-μm PVDF membrane (Pierce Biotechnology, Rockford, USA). The membrane was first blocked with 5% non-fat milk in TBS with Tween 20 (TBST) for 1 h and then incubated overnight at 4°C with

Table 1 Primers used for real-time RT–PCR

Gene	Sequence (forward/reverse) (5′ → 3′)	Accession No.	Product size (bp)
GAPDH	AGCCTCGTCCCGTAGACA	XM_001473623.1	255
	CTCGCTCCTGGAAGATGG		
CDK2	CACAGGGCTTGCACGTCACT	NM_016756.4	253
	TGTCTCCTGGCCTGCATCAC		
Cyclin A	GTTCCTTACCCAGTACTTCC	NM_009828.2	234
	AGACAAGGCTTAAGACTCTC		
Runx2	TGTTCTCTGATCGCCTCAGTG	NM_001145920.1	146
	CCTGGGATCTGTAATCTGACTCT		
Osterix	CCCTTCTCAAGCACCAATGG	NM_130458.3	85
	AGGGTGGGTAGTCATTTGCATAG		

primary antibodies [rabbit anti-CDK2, 1:200; rabbit anti-cyclin A, 1:200; goat anti-Runx2, 1:200 (Santa Cruz, Santa Cruz, USA); rabbit anti-Osterix, 1:250 (Abcam, Cambridge, USA); mouse anti-GAPDH, 1:10,000 (Kangcheng, Shanghai China); and mouse anti-β-tubulin, 1:1000 (Beyotime)], respectively. After being washed three times with TBST, blots were incubated with horseradish peroxidase-labeled secondary antibody at a dilution of 1:5000 for 1 h at room temperature. Blots were visualized using BeyoECL Plus (Beyotime) and the protein bands were quantitatively analyzed by using an image analysis system (QuantityOne software; BioRad, Hercules, USA).

Statistical analysis

Data were analyzed using Stata 9.0 and shown as the mean ± standard error for description. A comparison of continuous variables was tested by ANOVA and the Kruskal–Wallis test. In addition, the Bonferroni-corrected *post hoc* test was conducted to adjust the observed significant level for multiple comparisons if the null hypothesis was rejected. Statistical significance level was set at 0.05.

Results

OGP stimulates MSC proliferation

As reported in the literature for other cellular experimental models, low doses of exogenous OGP seemed to enhance cell proliferation [2]. In comparison with MSC grown without OGP, the MSC proliferation rate stimulated with 10^{-9} M of OGP was increased by 15% ($P < 0.05$) on Day 3 and by 20% ($P < 0.05$) on Day 7 [**Fig. 1(A)**]. Although a significant increase was observed in the rhBMP2 group after 3 days of culture ($P < 0.05$), OGP had no significant variation in ALP activity vs. the non-treated control [**Fig. 1(B)**].

OGP regulates MSC proliferation via activating CDK2 and cyclin A

Quantitative reverse transcriptase–PCR (qRT–PCR) indicated that OGP up-regulated mRNA level of *CDK2* and *cyclin A* [**Fig. 2(A)**] in MSCs, whereas no effect on mRNA level of *Runx2* and *Osterix* [**Fig. 3(A)**]. Western blotting gave the same result as did by using RT–PCR analysis [**Fig. 2(B,C)** and **Fig. 3(B,C)**].

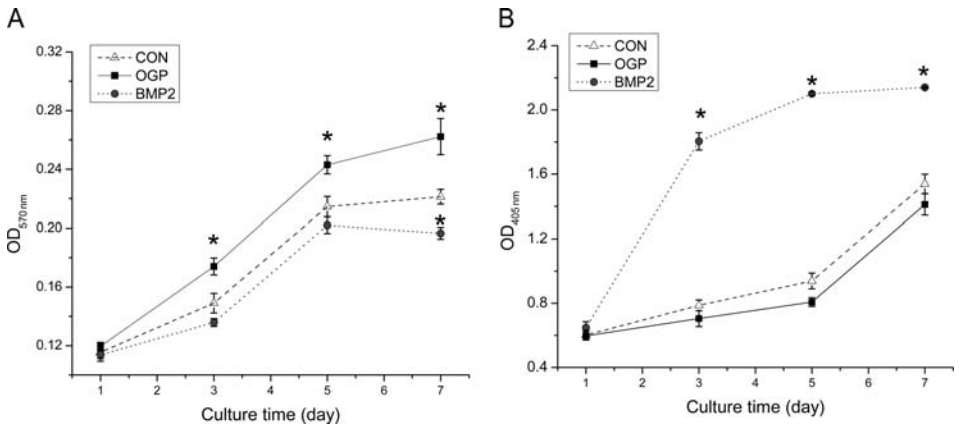

Figure 1 OGP's proliferation and differentiation effects on MSCs derived from OPG-deficient mice (A) MTT results of MSCs derived from OPG-deficient mice's tibial and femoral bones. Seven-day cultures. (B) ALP activity results of MSCs derived from OPG-deficient mice's tibial and femoral bones. Seven-day cultures. Values are presented as mean ± SE. *$P < 0.05$ vs. untreated control.

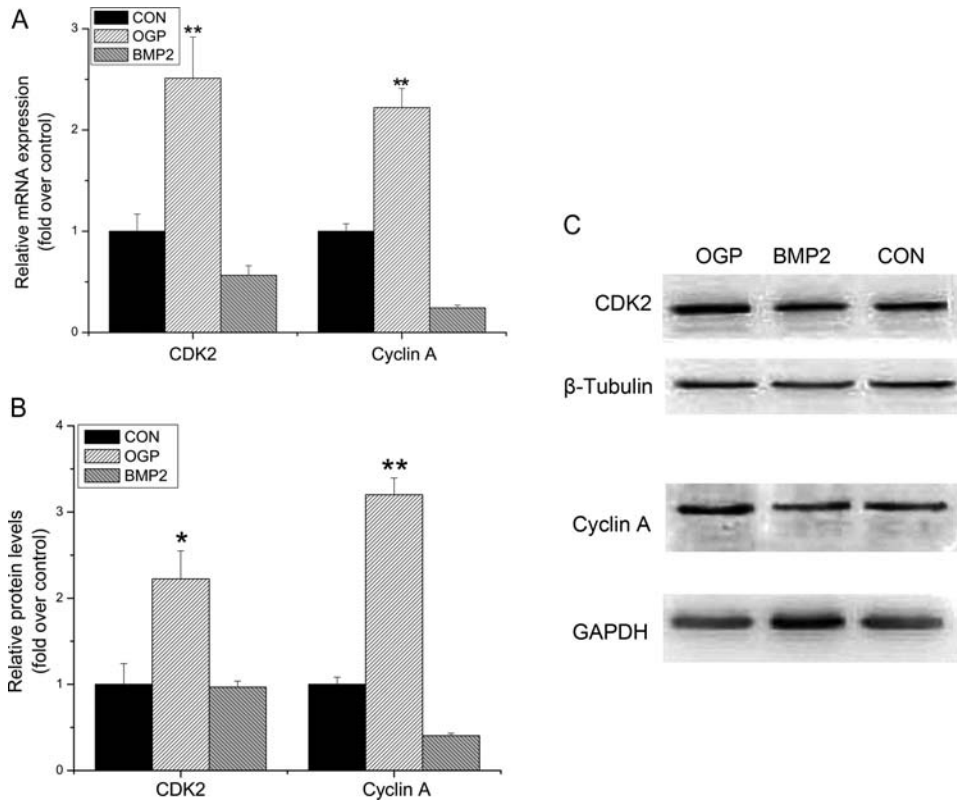

Figure 2 Expression of mRNA and protein levels of CDK2 and cyclin A in MSCs derived from OPG-deficient mice after treatment of OGP (A) Expression of mRNA levels of CDK2 and cyclin A in MSCs of OPG-deficient mice by qRT−PCR. The expression of indicated genes was normalized to expression of *GAPDH* and presented as fold change compared with untreated control. (B) Quantification of protein levels of CDK2 and cyclin A in MSCs of OPG-deficient mice by western blot normalized by β-tubulin or GAPDH. The graph represents as fold change compared with untreated control. (C) Western blotting analysis for CDK2 and cyclin A. β-Tubulin and GAPDH were used as loading controls. Values are presented as mean ± standard error. $*P < 0.05$ vs. untreated control, $**P < 0.01$ vs. untreated control. Experiments were performed in triplicate with essentially identical results.

Discussion

As we know so far, this experiment was the first time to evaluated OGP's effect on MSCs, which was derived from OPG-deficient mice. OGP increased trabecular bone density and stimulated fracture healing when administered to normal and osteoporotic experimental animals *in vivo* [22,23]. It also regulated the proliferation and osteogenic differentiation of rabbit MSCs *in vitro* [24]. In this experiment, OGP stimulated the proliferation of MSCs, and the expressions of CDK2 and cyclin A were increased at both mRNA and protein levels. Cyclin A-associated enzymes have been established as key promoters of progression through the S phase of the cell cycle [25]. CDK2 is a catalytic subunit of the cyclin-dependent protein kinase complex, whose activity is restricted to the G1/S phase, and essential for cell cycle G1/S phase transition. We presumed, for the first time, that up-regulation of CDK2 and cyclin A might be the reason of OGP's proliferation effects on MSCs derived from OPG-deficient mice.

We found that OGP had no effect on the differentiation of MSCs derived from OPG-deficient mice according to the results of ALP assay, mRNA, and protein expressions of Runx2 and Osterix. This was different with other literatures [6,12]. It might be due to several reasons. First, OPG was deficient in our animal model, so we presumed that the OPG might play some roles in MSC differentiation. Second, the OPG-deficient mice have increased osteoblast activity, which is secondary and compensatory for the increased bone resorption, and the serum ALP levels have already been elevated at a higher level [11]. Then the osteoblastic differentiation effect of OGP is hided. The reason is that OGP may need longer time (more than 7 days) to show its effect on MSC differentiation.

Chen and Chang [6] reported that Cbfa1 (also called Runx2) was a downstream molecule of OGP (10−14) action since OGP (10−14) up-regulated mRNA expression of *Cbfa1* in rMSCs. Interestingly, despite that Runx2 acts as Osterix upstream transcription factor [26,27], no expression changes of Runx2 and Osterix at mRNA and protein levels were found in our OGP group. These findings evoked a new question whether the OPG played a role in this pathway. Runx2-binding sites are present in all the genes expressed in an osteoblast and whose gene products

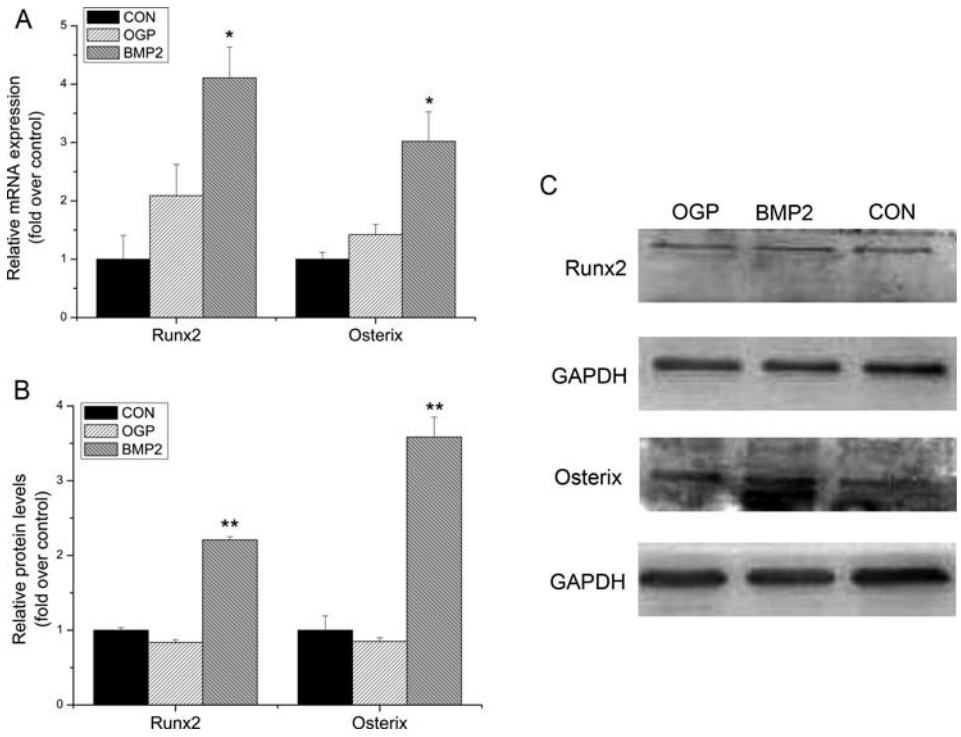

Figure 3 Expression of mRNA and protein levels of Runx2 and Osterix in MSCs derived from OPG-deficient mice after treatment of OGP (A) Expression of mRNA levels of Runx2 and Osterix in MSCs of OPG-deficient mice by qRT−PCR. The expression of indicated genes were normalized to expression of *GAPDH* and presented as fold change compared with untreated control. (B) Quantification of protein levels of Runx2 and Osterix in MSCs of OPG-deficient mice by western blot normalized by GAPDH. The graph represents as fold change compared with untreated control. (C) Western blotting analysis for Runx2 and Osterix. GAPDH was used as a loading control. Values are presented as mean ± standard error. $*P < 0.05$ vs. untreated control, $**P < 0.01$ vs. untreated control. Experiments were performed in triplicate with essentially identical results.

contribute to form a bone extracellular matrix [26,27]. So, we presumed that these genes, which were affected by the absence of OPG might interrupt the activation of the Runx2. Therefore, we hypothesized that the absence of OPG might cut down the effect of OGP on MSC differentiation. Further studies are needed.

In summary, OGP stimulates proliferation of MSCs derived from OPG-deficient mice by triggering CDK2−cyclinA pathway. In addition, OGP might interact with OPG and the absence of OPG might cut down the effect of OGP on MSC osteoblastic differentiation. Further work is required to measure the effect of OGP on wild-type animal MSCs *in vitro* to confirm the relationship between OGP and OPG.

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