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In vitro Activities of Polycalcium, a Mixture of Polycan and Calcium Lactate-Gluconate, on Osteoclasts and Osteoblasts

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The present study evaluated the beneficial effects of polycalcium (a mixture of Polycan and calcium lactate-gluconate 1:9 [*g*/*g*]) on osteoporosis using *in vitro* assays. Cell proliferation and alkaline phosphatase activities of osteoblasts (human primary osteoblasts) and osteoclast differentiation of RAW264.7 cells (murine osteoclast progenitor cells) treated with different concentrations of polycalcium for various periods were assessed. Osteoblast proliferation was stimulated and prevented RANKL-induced osteoclast differentiation of RAW264.7 cells. These results support the development of ideal anti-osteoporotic agents, such as polycalcium, that exhibit properties that accelerate bone formation and inhibit bone resorption.

Key words : Polycalcium, polycan, osteoclasts, osteoblasts

Introduction

Normal bone metabolism relies on a balance between bone formation and resorption regulated by osteoblasts and osteoclasts, respectively. When bone resorption is favored over formation, pathological bone destruction leads to osteoporosis [4,5]. The frequency of fractures significantly increases in patients with osteoporosis, and hip fractures pose a very serious problem because they often limit the patient's quality of life [25].

Numerous attempts have been made to develop new agents that treat or prevent bone diseases [15]. The current anti-resorptive agents are extensively used, but there still remains a demand for a highly efficacious resorptive inhibitor with an excellent safety and efficacy profile. Anabolic agents that stimulate bone formation are less known than anti-resorptive agents [6]. Continuous trials to develop these agents have been conducted with an advanced understanding of osteoblast differentiation and bone formation. Various approaches have searched for natural extracts that exert a therapeutic effect against bone loss.

Calcium (Ca) salts show anti-inflammatory activities [8,18] and have potentially favorable preventive and therapeutic effects on osteoporosis [7,20]. However, most people do not consume sufficient amounts of Ca, even though it is an essential element of living organisms and is highly recommended by nutrition experts [23,24].

Polycan (Glucan Corp., Korea), a commercial product of β-glucans that originated from *Aureobasidium pullulans* SM-2001, consists mostly of β-1,3/1,6-glucans and other organic materials such as amino acids, mono- or di-unsaturated fatty acids (linoleic and linolenic acids), and fibrous polysaccharides [16]. Recently, it has been reported that Polycan promotes fracture healing [14], exhibits anti-inflammatory activity [11,12], and has anti-osteoporotic properties, inhibiting bone loss and accelerating bone formation [17,19]. Several studies have shown that calcium phosphatase and vitamin D affect Ca resorption, but do not directly stimulate bone metabolism, especially bone formation.

The aim of this study was to confirm the beneficial effects of polycalcium (a mixture of Polycan and calcium lactate-gluconate 1:9 [*g*/*g*]) on osteoporosis, specifically cell proliferation and the alkaline phosphatase (ALP) activity of osteoblasts, using *in vitro* assays. Osteoclast differentiation of RAW264.7 cells was also evaluated.

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Materials and Methods

Reagents and cell culture

Dulbecco's Modified Eagle Medium (DMEM), minimal essential medium alpha (α-MEM), fetal bovine serum (FBS), penicillin/streptomycin, and trypsin-EDTA, which were used for cell cultures, were purchased from Gibco-BRL (Grand Island, NY, USA). Human primary osteoblasts (hOBs) and murine osteoclast progenitor RAW264.7 cells were a kind gift from Professor Daewon Jeong of Yeungnam University. For osteoblast analysis, ascorbic acid and β-glycerophosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA) and an ALP assay kit (AnaSpec, Inc., Fremont, CA, USA) was used. For osteoclast analysis, recombinant murine receptor activator of NF-κB ligand (RANKL) was purchased from BioVision (Mountain View, CA, USA).

Cell proliferation and ALP activity in human osteoblasts

The hOBs were grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2. The cells were seeded at a density of 1×10^3 per well in 96-well plates. After 24 hr, cells were transferred to fresh differentiation medium containing 0.05 mM ascorbic acid and 10 mM β-glycerophosphate every 3 days and were treated with different concentrations of polycalcium. Cell proliferation was determined using a commercially available kit (Cell Counting Kit-8, Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, hOBs (1×10^4 cells/well) were incubated, and 10 μl 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium monosodium salt (WST-8) was added to each well. After incubation for 1 hr, absorbance was measured at 450 nm using a microplate reader.

To measure ALP activity, hOBs were cultured in 24-well plates at a density of 1×10^4 cells/well with a differentiation medium and were treated with different concentrations of polycalcium. Cells were extracted with 0.05% Triton X-100 in the assay buffer and centrifuged at 2,500× g for 10 min at 4°C. Using the supernatant, ALP activity was determined by the colorimetric method using p-nitrophenyl phosphate (PNP) as a substrate at a wavelength of 405 nm and the protein content was determined by a bicinchoninic acid (BCA) protein assay kit. ALP activity was expressed as pg PNP/min/μg protein.

RANKL-induced osteoclast differentiation of RAW264.7 cells

RAW264.7 cells were grown in α-MEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2, seeded on a 96-well plate at a density of 2×10^3 cells/well, and treated with 100 ng/ml RANKL and with different concentrations of polycalcium. The medium was changed every 3 days and the number of undifferentiated RAW264.7 cells was evaluated using a commercially available kit (Cell Counting Kit-8, Dojindo, Kumamoto, Japan).

Statistical analyses

Multiple comparison tests for different dose groups were conducted. Variance homogeneity was examined using the Levene test. If no significant deviations from homogeneity were detected, the data were analyzed by one-way ANOVA followed by the least-significant differences (LSD) multi-comparison test to determine which pairs were significantly different. When there were significant deviations from homogeneity, a non-parametric comparison test (i.e., Kruskal-Wallis H test) was conducted. If significant differences were observed, the Mann-Whitney U test was performed to determine which specific pairs were significantly different. Statistical analyses were conducted using SPSS for Windows (Release 14K, SPSS Inc., USA).

Results and Discussion

Effects of polycalcium on cell proliferation and ALP activity in hOBs

To investigate the effect of polycalcium on cell growth at concentrations of 0, 10^{-5}, 10^{-3}, 10^{-1}, and 10 mg/ml for a treatment period of 3, 7, or 10 days, a cell proliferation assay was performed. The growth of hOBs gradually increased at 7 and 10 days, whereas 10 mg/ml polycalcium significantly ($p<0.05$) increased growth for a treatment period of 3, 7, and 10 days (Fig. 1).

We also examined the effect of polycalcium on the ALP activity of hOBs grown in differentiation medium at a concentration of 0, 10^{-5}, 10^{-3}, 10^{-1}, or 10 mg/ml for a treatment period of 7 or 14 days. Polycalcium increased ALP activity in a dose-dependent pattern. Significantly ($p<0.05$) increased ALP activity was detected for a treatment period of 7 or

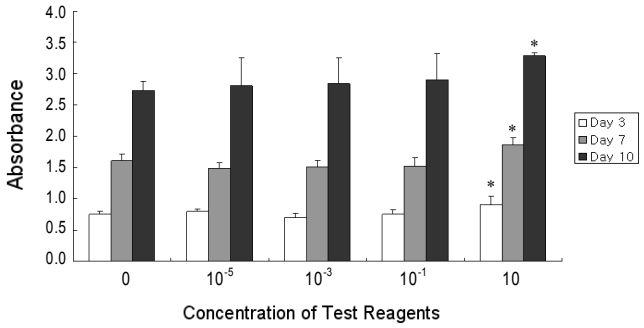

Fig. 1. Effects on osteoblast proliferation. A dose-dependent increase in the proliferation of human osteoblasts (hOB) after treatment with different concentrations of polycalcium (mg/ml) for 10 days is shown. All data are represented as the mean±S.D. of five independent experiments. *p<0.05 as compared to the control using the Mann-Whitney U test.

14 days with 10 mg/ml polycalcium. When ALP activity was corrected with protein concentrations, there were no significant changes (Fig. 2). These results suggest that polycalcium stimulates hOB proliferation dose-dependently, but does not promote hOB differentiation.

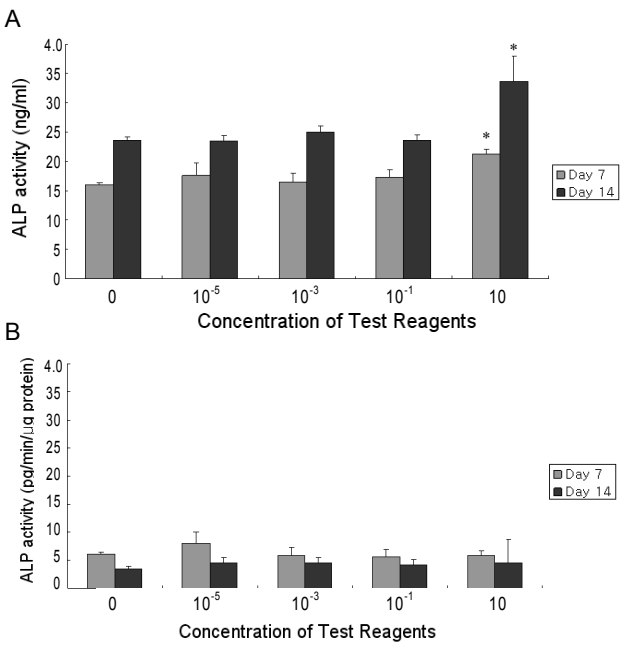

Fig. 2. Effects on alkaline phosphatase (ALP) activity. Polycalcium (mg/ml) increased ALP activity in a dose-dependent manner with significantly (p<0.05) high levels of activity after treatment of 10 mg/ml for 7 or 14 days (A). However, no significant changes in ALP activity were detected after correction for protein concentrations (B). All data are represented as the mean±S.D. of five independent experiments. *p<0.05 as compared to the control using the Mann-Whitney U test.

RANKL-induced osteoclast differentiation of RAW264.7 cells

Different concentrations of polycalcium (0, 10^{-5}, 10^{-3}, and 10^{-1} mg/ml) were added to RAW264.7 cells after stimulation with RANKL. As shown in Figure 3, no osteoclast differentiation of RAW264.7 cells in the control (untreated) group was observed. However, the number of undifferentiated RAW264.7 cells decreased after RANKL treatment, suggesting that RANKL stimulated osteoclast differentiation. When polycalcium was administered at 0, 10^{-5}, 10^{-3}, 10^{-1}, or 10 mg/ml for a treatment period of 1 or 4 days, osteoclast differentiation of RAW264.7 cells was inhibited dose-dependently (Fig. 3). In general, these results indicate that polycalcium directly inhibits RANKL-induced osteoclastogenesis.

Osteoblasts derived from mesenchymal cells synthesize and deposit bone matrix and increase bone mass [1]. The ontogenesis of bone-forming osteoblasts is a complex process and can essentially be divided into three steps: (1) commitment into osteoprogenitor cells and proliferation, (2) extracellular matrix development and maturation, and (3) matrix mineralization [1,2]. Therefore, the proliferation and activity of osteoclasts have important roles in bone formation. The efficacy of osteoblasts has been previously tested based on cell proliferation and activity [9].

Osteoclasts that originate from hematopoietic cells are key participants in bone remodeling because they are predominantly involved in bone resorption and can lead to an imbalance in bone remodeling. Osteoclastic bone resorption consists of multiple steps, including the differentiation of osteoclast precursors, fusion into multinuclear osteoclasts, and the activation of mature osteoclasts to resorb bone [10]. Mature, multinucleated osteoclast-like cells (OCLs) maintain the proper actin cytoskeletal organization, ruffled border, and acidic condition during bone resorption [22]. The efficacy of anti-osteoporotic agents, therefore, has been continually evaluated for the inhibition of osteoclastogenesis and its activity has been analyzed using the pit formation assay [9,21].

In the present study, we found that polycalcium stimulated the proliferation of hOB, and prevented RANKL-induced osteoclast differentiation of RAW264.7 cells in a dose-dependent manner. These results support the development of ideal anti-osteoporotic agents with properties that accelerate bone formation as well as inhibit bone resorption. However, polycalcium did not affect hOB ALP activity, which is an indication of osteoblast differentiation.

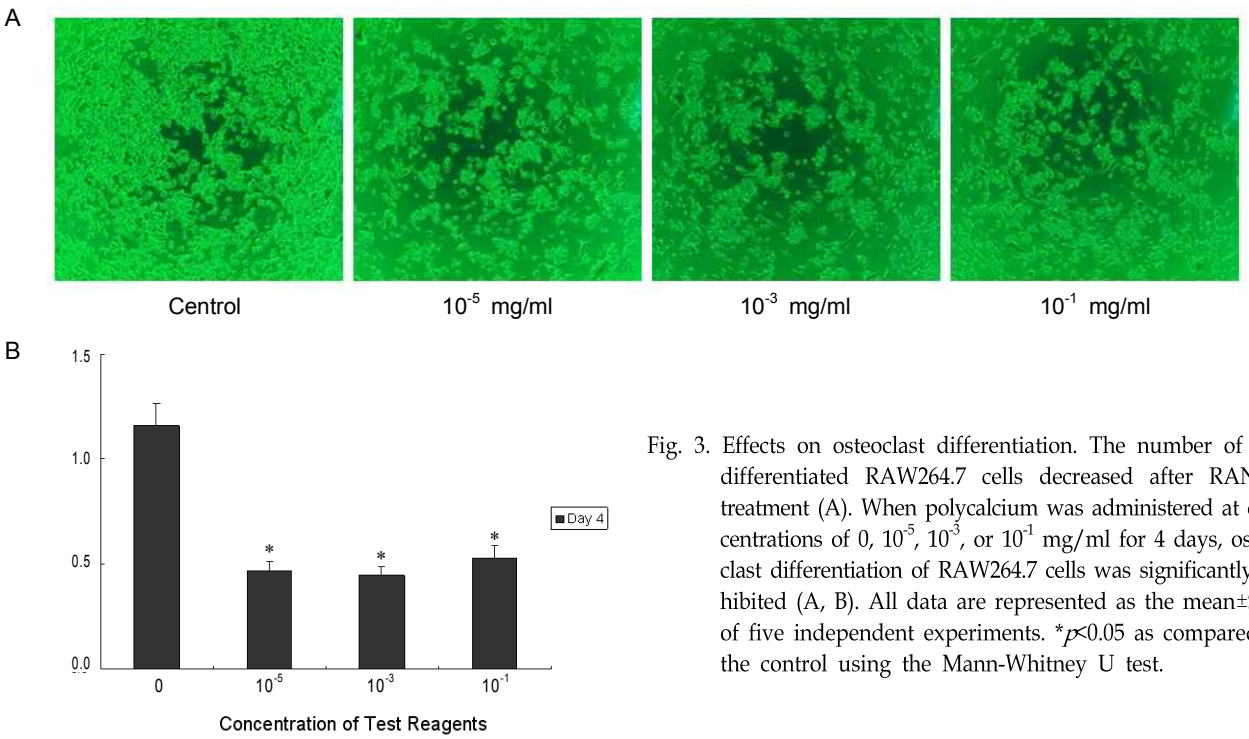

Fig. 3. Effects on osteoclast differentiation. The number of undifferentiated RAW264.7 cells decreased after RANKL treatment (A). When polycalcium was administered at concentrations of 0, 10^{-5}, 10^{-3}, or 10^{-1} mg/ml for 4 days, osteoclast differentiation of RAW264.7 cells was significantly inhibited (A, B). All data are represented as the mean±S.D. of five independent experiments. *p<0.05 as compared to the control using the Mann-Whitney U test.

Therefore, the data suggest that polycalcium affects only hOB proliferation, not hOB differentiation. Specific genes, such as ALP, collagen I, and osteocalcin, are expressed during osteogenic differentiation [3,13]. The expression of osteogenic genes during osteoblast differentiation should be further analyzed and the association of polycalcium and osteoblast proliferation should be confirmed.

In summary, we conclude that polycalcium is a potential candidate for anti-osteoporosis therapy. Further studies using *in vitro* and *in vivo* models are needed to elucidate the exact mechanism that confers the anti-osteoporotic effects of polycalcium.

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초록 : *In vitro*에서 polycalcium 복합조성물이 파골세포와 조골세포에 미치는 영향

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본 실험에서는 폴리칸(베타-글루칸)과 칼슘 락테이트 글루코네이트 1:9 (*g*/*g*) 복합 조성물인 Polycalcium의 시험관 내(*in vitro*) 골다공증에 대한 효과를 사람 유래 조골세포(human primary osteoblast)와 설치류 유래 파골전구세포(raw264.7 cell)를 이용하여 평가하였다. Polycalcium이 조골세포에 미치는 영향을 확인한 결과, 10 mg/ml 농도의 polycalcium 처리군에서 무처리 대조군에 비해 유의성 있는 조골세포의 수적 증가가 각각 배양 3, 7 및 10일 후에 확인되었으며, 또한 10 mg/ml 농도의 polycalcium 처리군에서 무처리 대조군에 비해 유의성 있는 ALP함량의 증가가 확인되었다. Polycalcium이 파골세포에 미치는 영향을 확인한 결과, 각각 10^{-5}, 10^{-3} 및 10^{-1} mg/ml polycalcium 처리군에서 무처리 대조군에 비해 유의성 있는 파골세포의 수적 감소가 배양 4일 후에 확인되었다. 이 같은 결과를 바탕으로, polycalcium이 조골세포의 증식 촉진 효과와 함께 파골세포 형성 억제 효과가 있는 것으로 확인되었다.