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Effect of whey protein on the proliferation and differentiation of osteoblasts

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ABSTRACT

Effects of whey protein on osteoblasts were evaluated. The whey protein was added to the culture medium at concentrations of 0.02 and 0.1 mg/mL. In vitro, whey protein stimulated the proliferation and differentiation of osteoblasts cultured in different concentrations of whey protein. The levels of osteocalcin and insulin-like growth factor-I in the culture medium also increased. Real-time reverse transcription-PCR results showed that the mRNA expression of osteoprotegerin (OPG) and receptor activator of nuclear factor-κ B ligand (RANKL) increased in the cells in a dose-dependent manner, and when the results were expressed as OPG/RANKL ratio, a significant increase could be seen in the 0.1 mg/mL whey protein group. These results showed that the active component in the whey protein plays an important role in bone formation and a potential therapeutic role in osteoporosis by activating osteoblasts.
Key words: whey protein, insulin-like growth factor-I, osteoblast, differentiation

INTRODUCTION

Whey protein is usually produced as a by-product of cheese or casein manufacturing. One liter of milk contains 5.5 g of whey protein, and the major proteins are α-lactalbumin (19%), β-lactoglobulin (48%), immunoglobulin (10%), and serum albumin (7%). Milk is a rich biological fluid that functions to provide nutrition at a time of very rapid skeletal growth and development in the neonate. Because of this, it contains growth regulators in addition to the simple substrates necessary for infant development (Lonnerdal and Iyer, 1995). Bone growth is controlled by many hormones and growth factors and bone is continually remodeled by the complex coupling of the actions of the bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts; Mundy, 1999). Whey protein was effective in increasing bone strength and the content of collagen-specific amino acids such as hydroxyproline in ovariectomized rats (Takada et al., 1996).

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The present study addresses the bone actions of whey protein using assessments of osteoblast development and activity in vitro. This study establishes whey protein as a potent novel anabolic factor in osteoblasts, and which also reduces bone resorption. These findings pose important questions regarding the role of whey protein in normal bone physiology during growth and in adulthood, and provide a novel mechanistic pathway as a target for drug development in the therapeutics of osteoporosis.

MATERIALS AND METHODS

Materials

α-Minimum essential medium (**α-MEM**) and fetal bovine serum (**FBS**) were purchased from Gibco (Grand Island, NY). Whey protein concentrate (Vitulas, Alberta, Canada) contained 80.05% protein, 6.2% lactose, 4.39% ash, and 0.9% fat (determined by AOAC, 2000; methods 930.29, 930.28, 930.30, and 932.06, respectively).

Osteoblast Culture

Rat osteoblast cells were derived from sequential collagenase digestion of 21-d fetal rat calvariae (Cornish et al., 1999). The cells were cultured in plastic dishes containing α-MEM plus 10% FBS in a CO_2 incubator (5% CO_2: 95% air) at 37°C and subcultured every 3 d at a dilution of 1:5 using 0.001% pronase E (Sigma, St. Louis, MO) plus 0.02% EDTA in Ca^{2+}-, Mg^{2+}-free PBS. The cells (5×10^4 per dish) were seeded in plastic dishes containing 2 mL of α-MEM supplemented with 10% FBS or in 96-well plates, each well containing 100 μL of medium, and cultured for 3 d until nearly confluent. Then, the cells were transferred to fresh serum-free medium containing 0.1% BSA and various concentrations of whey protein. The cells were further incubated for the indicated periods.

Cell Proliferation Assays

The methyl thiazolyl tetrazolium (**MTT**) assay was undertaken to assess cell proliferation following the method of Robinson et al. (1997). Methyl thiazolyl

Table 1. Primer design for GAPDH, osteoprotegerin (OPG), and receptor activator of nuclear factor-κ B ligand (RANKL)

Gene	Forward primer	Reverse primer
GAPDH	5′ CAGTCAGCCGCATCTTCTTTTG 3′	5′ TGGTTCACACCCATGACGAAC 3′
OPG	5′ CGAGTGATGAATGCGTGTA 3′	5′ TTCTGAAGTAGCAGGAGGC 3′
RANKL	5′ CCATCGGGTTCCCATAAAG 3′	5′ TGAAGCAAATGTTGGCGTA 3′

tetrazolium colorimetry is one of classic methods used to evaluate cell proliferation. In this method, MTT is reduced to amethyst crystal by butanedioic acid reductase, which is present in the mitochondria of living cells; the optical density (**OD**) value at 490 nm is directly proportional to the number of living cells, so it indirectly reflects the reproductive activity of osteoblasts. Briefly, the cells were cultured in 96-well plates; 15 μL of MTT and 150 μL of dimethyl sulfoxide were added to each well after removing the medium. The plates were shaken for 10 min, and were then read at 490 nm in a Bio-Rad 680 ELISA reader (Bio-Rad Laboratories, Hercules, CA). Results were presented as mean OD readings derived from triplicate wells.

Cell Differentiation Assays

The cells in a 24-well plate were washed with PBS and lysed in 0.5 mL of 10 m*M* Tris-HCl (pH = 7.5) containing 0.1% Triton X-100. The alkaline phosphatase (**ALP**) activity in the lysate was determined by using an ALP activity kit (Zhongshan Co., Beijing, China). Protein concentration was determined following the method of Lowry et al. (1951).

Osteocalcin and IGF-I Contents

After osteoblasts had been cultured in 35-mm dishes for 48 h of treatment with whey protein, the supernatant was used to determine the contents of osteocalcin and IGF-I following the method of Turan et al. (1997) and Gronbaek et al. (1995).

mRNA Expression of Osteoprotegerin and Receptor Activator of Nuclear Factor κB Ligand

Cells were seeded onto 6-well culture dishes (3×10^6 cells/well) and were cultured in α-MEM medium containing 0.1% BSA and various concentrations of whey protein (0.02 or 0.1 mg/mL). Then, RNA from the adherent cells was prepared using a commercially available kit for purification of nucleic acids (Nucleospin, Macherey-Nalgen, Düren, Germany). Samples were digested with a DNase. For reverse transcription, cDNA were synthesized from 2 μg of total RNA using reverse transcriptase (avian myeloblastosis virus) and oligo (dT) primers in a volume of 20 μL (First-Strand cDNA Synthesis Kit for RT-PCR, Roche Diagnostics, Indianapolis, IN). Polymerase chain reaction was performed with 1 μL of cDNA reaction mixture by using Taq polymerase (Q-BioTaqDNA polymerase, Q-Biogene, Dorset, UK) and primers (Table 1) for GAPDH, osteoprotegerin (**OPG**), and receptor activator of nuclear factor κB ligand (**RANKL**) in a volume of 50 μL. The PCR products were analyzed using 2% agarose gel electrophoresis.

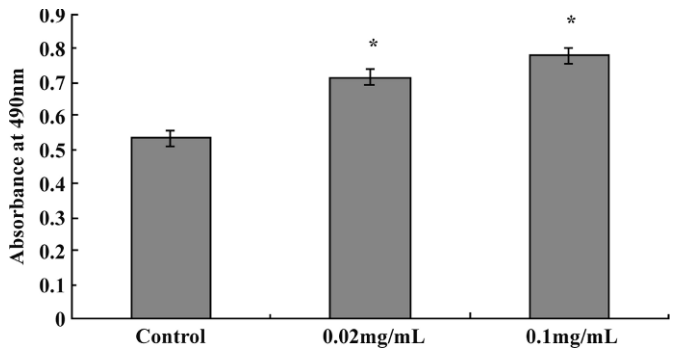

Figure 1. Effect of whey protein (0.02 or 0.1 mg/mL) on osteoblast growth. Results are expressed as the mean ± SE. $^*P < 0.05$: significant differences compared with control group.

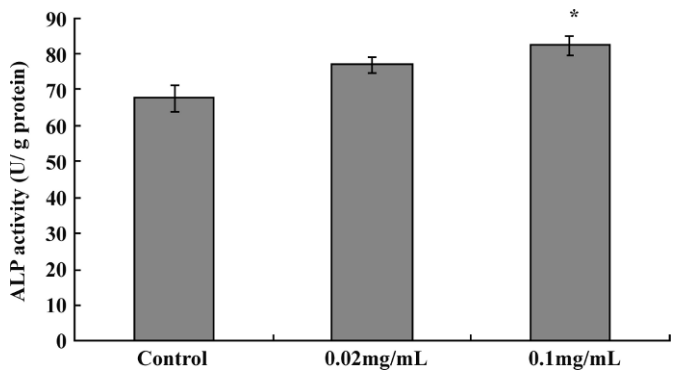

Figure 2. Effect of whey protein (0.02 or 0.1 mg/mL) on alkaline phosphatase (ALP) activity. Results are expressed as the mean ± SE. $^*P < 0.05$: significant differences compared with control group.

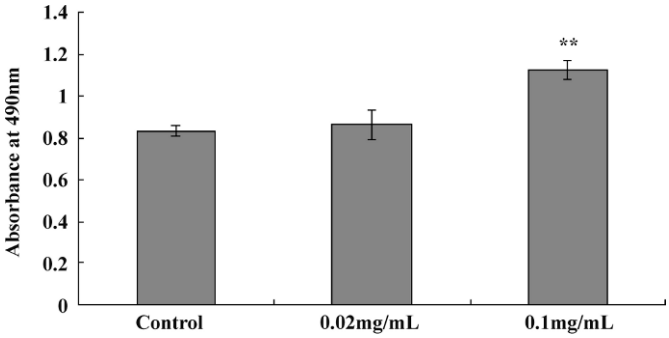

Figure 3. Effect of whey protein (0.02 or 0.1 mg/mL) on osteocalcin content. Results are expressed as the mean ± SE. $^{**}P < 0.01$: significant differences compared with control group.

Statistical Analysis

Data are expressed as means ± standard errors of 5 wells. Statistical analysis was performed using Student's unpaired *t*-test.

RESULTS

Whey Protein Stimulates Osteoblast Proliferation and Differentiation

In view of the effects of whey protein on osteoblasts, its effect on proliferation and differentiation of primary cultures of osteoblasts was assessed. Whey protein produced a dose-related increase of OD values and ALP activity in primary cultures of osteoblasts (Figure 1). At concentrations of 0.02 and 0.1 mg/mL, whey protein induced statistically significant increases of osteoblast proliferation. In the presence of the highest dose of whey protein (0.1 mg/mL), a significant increase of ALP activity was shown (Figure 2).

Osteocalcin and IGF-I Contents

Whey protein significantly stimulated the production of osteocalcin and IGF-I. In the presence of the lowest dose of whey protein (0.02 mg/L), no significant modulation of osteocalcin was shown. However, we observed that addition of whey protein at 0.1 mg/mL led to a dramatic increase in the content of osteocalcin (Figure 3). Whey protein stimulated the production of IGF-I at both concentrations of whey protein (Figure 4); the content of IGF-I reached a maximum at 0.1 mg/mL of whey protein.

mRNA Expression of RANKL and OPG

We performed RNA electrophoresis to evaluate the integrity of RNA. The OD_{260}/OD_{280} ratio of total RNA isolated from the cells of different groups were between 1.7 and 1.9, indicating that the purity of total RNA was acceptable. The RNA electrophoresis showed that there were 28S and 18S bands, with a ratio close to 2:1 (Figure 5A), indicating that the total RNA was intact.

Figure 5C and Figure 5D show mRNA expression of OPG and RANKL compared with the control group; the high-dose group always had the higher expression level of both OPG and RANKL. On the other hand, there was no significant difference in the regulation of the RANKL mRNA expression.

DISCUSSION

This study demonstrates that whey protein is a promoter of osteoblast growth in vitro under these conditions. In this study, we examined the effect of whey protein on the proliferation and differentiation of osteoblastic cells. Whey protein enhanced, in a dose-dependent manner, the proliferation and differentiation of cells; the characteristic osteoblast marker ALP increased after treatment with whey protein; and the MTT assay and ALP activity showed significant differences in the high-dose group (0.1 mg/mL of whey protein) compared with the control group. Moreover, whey protein also increased total osteocalcin and IGF-I contents of osteoblasts. At present, osteocalcin is the only known bone-specific protein produced by osteoblasts (Lian and Gundberg, 1988). This protein is reported to appear in a later stage of osteoblast differentiation (Strauss et al., 1990), presumably in mature osteoblasts, whereas ALP (Yoon et al., 1987) appears in less-differentiated osteoblasts. It is significant that whey protein induced osteocalcin protein synthesis in the high-dose group. Whether other bone growth stimulators have similar effects in inducing osteocalcin synthesis remains to be elucidated in the future. In conclusion, whey protein is a bone induction factor that induces differentiation of

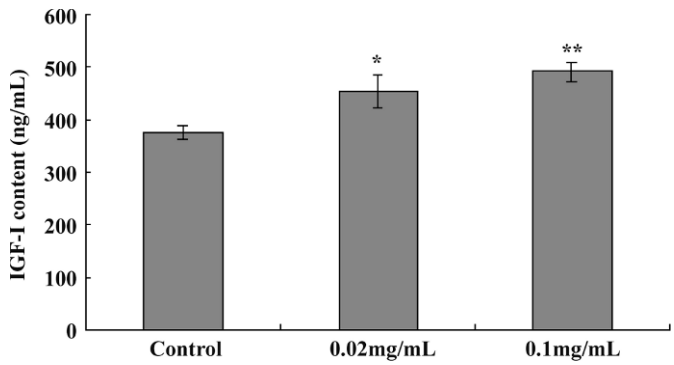

Figure 4. Effect of whey protein (0.02 or 0.1 mg/mL) on IGF-I content. Results are expressed as the mean ± SE. $^{*}P < 0.05$ and $^{**}P < 0.01$: significant differences compared with control group.

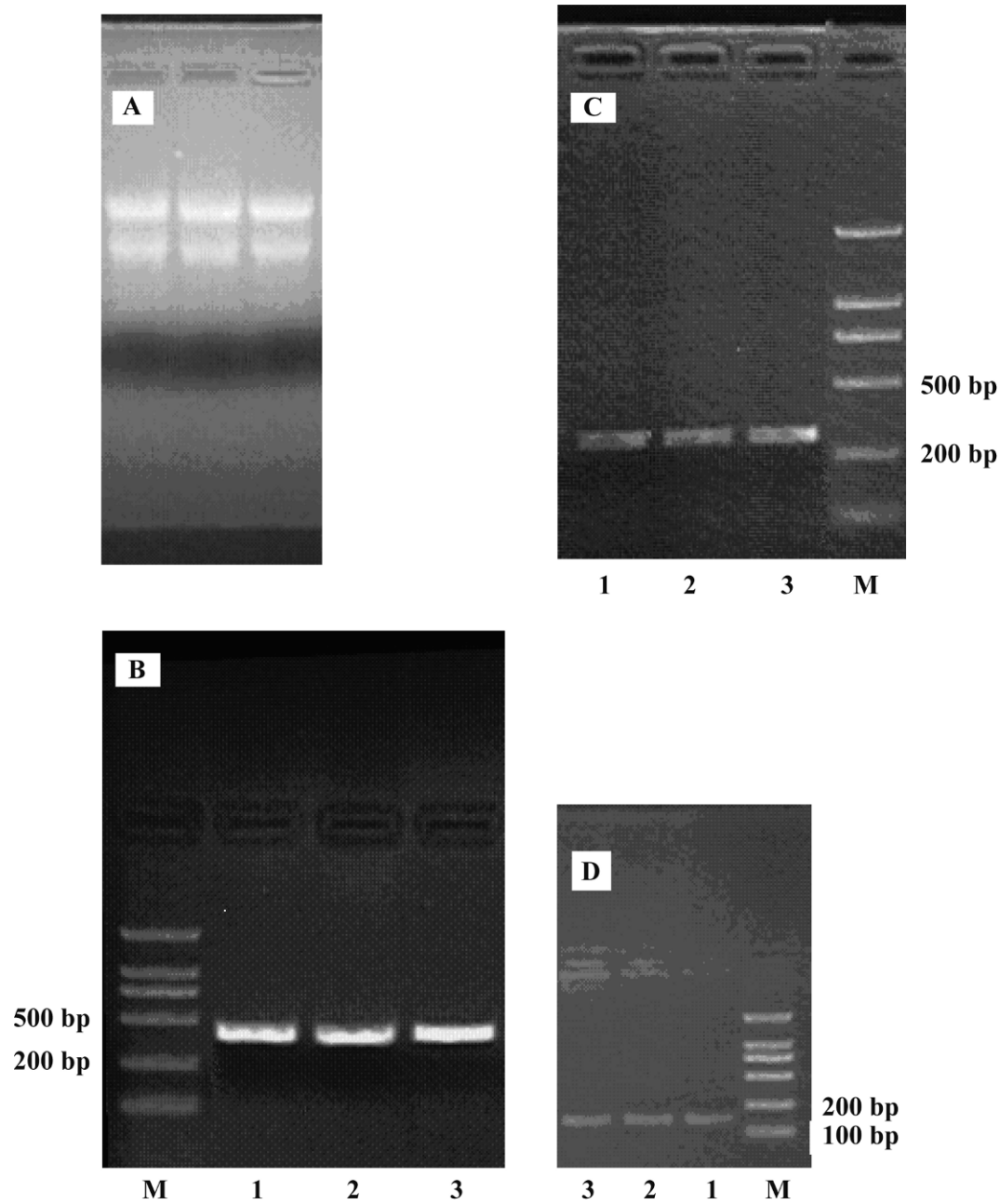

Figure 5. A) Total RNA electrophoresis; B) reverse transcription-PCR products of GAPDH in osteoblasts; C) reverse transcription-PCR of osteoprotegerin (OPG) in osteoblasts; D) reverse transcription-PCR products of receptor activator of nuclear factor-κ B ligand (RANKL) in osteoblasts. M = DNA marker; lane 1 = control group; lane 2 = low-dose group (0.02 mg/mL of whey protein); lane 3 = high-dose group (0.1 mg/mL of whey protein).

osteoblast progenitor cells into mature osteoblasts with the ability to synthesize osteocalcin.

We also detected the mRNA expression of some bone metabolism-related cytokines—OPG and RANKL. Our results showed that the high-dose group always had higher expression of OPG mRNA; OPG expression reached a peak in this group. However, when the results were expressed as OPG/RANKL ratio, there was a significant increase compared with the control group, because of the increase in OPG mRNA rather than a decrease in RANKL mRNA.

Taken together, these data demonstrate that whey protein is anabolic to bone, an effect that is consequent upon its potent proliferative and differentiated actions in osteoblasts. Whey protein may therefore have a physiological role in bone growth as well as a poten-

tial therapeutic role in osteoporosis or for local use in orthopedic practice to promote the healing of fractures or filling in of bone defects. We propose the possibility that the whey protein plays an important role in bone formation by activating osteoblasts.

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