Genistein and zinc synergistically enhance gene expression and mineralization in osteoblastic MC3T3-E1 cells

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Abstract. Genistein, a phytoestrogen, and zinc, an essential trace element, have an anabolic effect on bone components. We investigated whether the combination of genistein and zinc has additive and synergistic effects in osteoblastic cells *in vitro*. Osteoblastic MC3T3-E1 cells with subconfluency were cultured for 48 h in medium containing either vehicle, genistein (10^{-6} or 10^{-5} M), zinc sulfate (10^{-5} or 10^{-4} M) or genistein (10^{-6} or 10^{-5} M) plus zinc sulfate (10^{-5} or 10^{-4} M). The protein content in the osteoblastic cells was significantly increased in the presence of genistein (10^{-5} M) or zinc (10^{-4} M). This increase was significantly enhanced with a culture of genistein (10^{-5} M) plus zinc (10^{-4} M). The expression of mRNAs of $\alpha1(I)$ collagen and osteocalcin, which are bone matrix protein, in osteoblastic cells was demonstrated using reverse transcription-polymerase chain reaction (RT-PCR) analysis with a specific primer. This expression was not significantly increased with genistein (10^{-6} or 10^{-5} M) or zinc (10^{-5} or 10^{-4} M). The combination of genistein (10^{-6} or 10^{-5} M) and zinc (10^{-5} or 10^{-4} M) caused a significant increase in $\alpha1(I)$ collagen and osteocalcin mRNA expression in osteoblastic cells. Alkaline phosphatase mRNA expression in osteoblastic cells was significantly enhanced with a culture of genistein (10^{-5} M) plus zinc (10^{-4} M). The effect of genistein (10^{-5} M) plus zinc (10^{-4} M) in enhancing the expression of $\alpha1(I)$ collagen, alkaline phosphatase, or osteocalcin mRNAs in osteoblastic cells was completely prevented with a culture of cycloheximide (10^{-7} M), an inhibitor of protein synthesis, or 5,6-dichloro-1-ß-D-ribofuranosylbenimidazole (DRB) (10^{-6} M), an inhibitor of transcriptional activity. Moreover, a culture with genistein (10^{-5} M) or zinc (10^{-4} M) for 14 or 21 days caused a significant increase in mineralization. This increase was markedly enhanced with a culture of the combination of genistein (10^{-5} M) and zinc (10^{-4} M). This study demonstrates that the combination of genistein and zinc can synergistically enhance gene expression and mineralization in osteoblastic cells.

Introduction

Bone mass decreases with increasing age. Decrease in bone mass induces osteoporosis, which is widely recognized as a major public health problem. The most dramatic expression of this disease is represented by fracture of the proximal femur (1-3). Decrease in bone mass is due to increased bone resorption and reduced bone formation. Pharmacological and nutritional factors may prevent bone loss with increasing age (4,5). Chemical compounds in food that act on bone metabolism, however, are poorly understood.

Genistein is a natural isoflavonoid phytoestrogen found in *Leguminosae* and has been demonstrated to have an anabolic effect on bone metabolism, suggesting a role in the prevention of osteoporosis (reviewed in ref. 6). Genistein has a stimulatory effect on bone formation and mineralization (7,8) and an inhibitory effect on osteoclastic bone resorption (9,10). The dietary intake of genistein prevented bone loss in ovariectomized rats which are model animals of osteoporosis (11). Genistein is a useful biofactor in the prevention of osteoporosis.

Zinc, an essential trace element, has been demonstrated to have a potent stimulatory effect on osteoclastic bone formation (12,13) and an inhibitory effect on osteoclastic bone resorption (14,15). Zinc can stimulate protein synthesis in osteoblastic cells *in vitro* by activating aminoacyl-tRNA synthetase (16). The oral administration of a zinc compound prevented bone loss in an animal model of osteoporosis (17).

Whether the combination of nutritional factors reveals an additive or synergistic effect on bone components has not been fully determined. This knowledge may be important in the prevention of bone loss with increasing age. Recently, it has been shown that the combination of genistein and zinc has a synergistic effect on bone components in the femoral tissues of rats (18-20). Moreover, it has been demonstrated that the preventive effect of dietary fermented soybean on bone loss is enhanced by supplementation of isoflavone and zinc in ovariectomized rats (21). The intake of dietary genistein and zinc-supplemented fermented soybean has a stimulatory effect on serum bone-formation markers and a suppressive effect on serum bone-resorption markers in aged men and women (22).

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The cellular mechanism by which the combination of genistein and zinc has an additive and/or synergistic effect on bone metabolism has not been clarified. This study was undertaken to determine the effect of genistein and zinc on gene expression and mineralization in osteoblastic cells *in vitro*.

Materials and methods

Chemicals. α-minimal essential medium (α-MEM) and penicillin-streptomycin (5000 U/ml penicillin; 5000 μg/ml streptomycin) were obtained from Gibco Laboratories. Fetal bovine serum (FBS) was obtained from Bioproducts Inc. Cycloheximide, and 5,6-dichloro-1-ß-D-ribofuranosyl-benzimidazole (DRB), were obtain from Sigma Chemical Co. Zinc sulfate and other chemicals were of reagent grade and were obtained from Wako Pure Chemical Industries. All water used was glass distilled. Tissue culture plastic dishes were purchased from Falcon Plastics. Other materials used were commercial products of the highest grade available.

Cell culture. Osteoblastic MC3T3-E1 cells were cultured at 37˚C in a CO_2 incubator in plastic dishes containing α-MEM supplemented with 10% FBS. They were subcultured every 3 days using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS). For the experiments, 1.0×10^5 cells per dish were cultured for 3 days to obtain sub-confluent monolayers in 35-mm plastic dishes containing 2 ml α-MEM with 10% FBS. After the cells were rinsed with PBS, the medium was exchanged for medium without FBS containing either vehicle, genistein, zinc sulfate, or genistein plus zinc sulfate in the presence or absence of various inhibitors, and the cells were cultured for an additional 24 or 48 h. Cell viability was estimated by staining with trypan blue.

Cell counting. After trypsinization of the cells in each culture dish using a Ca^{2+}/Mg^{2+}-free PBS containing 0.2% trypsin and 0.02% EDTA for 2 min at 37˚C, cells were collected and wash-centrifuged in a PBS solution at 100 x g for 5 min. The cells were re-suspended in a 0.5 ml PBS solution, and an aliquot was stained with eosin. The cells were counted under a microscope using a Hemocytometer plate. For each dish, we took the average of two counts.

Analytical procedures. To determine the protein concentration in the osteoblastic cells, the cells were washed 3 times with PBS, scraped into 0.5 ml of ice-cold 0.25 M sucrose solution, and disrupted for 30 sec with an ultrasonic device. The protein concentration in the cell homogenate was determined by the method of Lowry *et al* (23) and expressed as the amount of protein (μg) per dish.

To assay alkaline phosphatase activity in the cells after the appropriate treatment periods, the cells were washed 3 times with PBS, scraped into 0.5 ml of ice-cold 0.25 M sucrose solution, and disrupted for 30 sec with an ultrasonic device. The supernatant, centrifuged at 600 x g for 5 min, was used to measure enzyme activity. The enzyme assay described below was carried out under optimal conditions. Alkaline phosphatase activity was determined by the method of Walter and Schutt (24). The enzyme activity was expressed as nmol of *p*-nitrophenol liberated/min/mg of protein.

Determination of specific mRNA by RT-PCR. Total RNAs were prepared as described previously (25). Osteoblastic MC3T3-E1 cells with subconfluency were cultured for 24 or 48 h in medium containing either vehicle genistein, zinc sulfate, or genistein plus zinc sulfate. After culturing, cells were washed 3 times with ice-cold PBS, and then the cells were homogenized in a buffer solution containing 4 M guanidinium thiocyanate, 24 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and isoamyl alcohol, and the phases were separated by centrifugation at 10,000 x g for 20 min at 4˚C. RNA located in the aqueous phase was precipitated with isoprepanol at -20˚C. RNA precipitates were pelleted by centrifugation, and the pellets were redissolved in diethyl-pyrocarbonate-treated water.

Reverse transcription-polymerase chain reaction (RT-PCR) was performed with a Titam™ one tube RT-PCR kit (Roche Molecular Biochemicals) as recommended by the supplier. Primers for amplification of mouse Runx2 cDNA were: 5'-GTATGAGAGTAGGTGTCCCG-3' (sense strand, positions 992-1011 of cDNA sequence) and 5'-ACATCCCC ATCCATCCACTC-3' (antisense strand, positions 1156-1175) (26). The pair of oligonucleotide primers was designed to amplify a 183-bp sequence from the mRNA of mouse Runx2. Primers for amplification of mouse α1(I) collagen cDNA were: 5'-TTCTCCTGGTAAAGATGGTGC-3' (sense strand, positions 2232-2252 of cDNA sequence) and 5'-GG ACCAGCATCACCTTTAACA-3' (antisense strand, positions 2466-2486) (27). The pair of oligonucleotide primers was designed to amplify a 254-bp sequence from the mRNA of α1(I) collagen. Primers for amplification of mouse alkaline phosphatase cDNA were: 5'-GGCTTCTTCTTGCTGGTGG A-3' (sense strand, positions 1158-1177 of cDNA sequence) and 5'-TGAGCGACACGGACAAGAAG-3' (antisense strand, positions 1387-1406) (28). The pair of oligo-nucleotide primers was designed to amplify a 244-bp sequence from the mRNA of alkaline phosphatase. Primers for amplication of mouse osteocalcin cDNA were: 5'-GGGG AAGGGACAACACATGA-3' (sense strand, positions 188-207 of cDNA sequence) and 5'-TCCTGGACATGGGGATTGA C-3' (antisense strand, positions 580-599) (29). The pair of oligonucleotide primers was designed to amplify a 412-bp sequence from the mRNA of osteocalcin. For semi-quantitative PCR, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control to evaluate total RNA input. Primers for amplification of G3PDH cDNA were: 5'-GAT TTGGCCGTATCGGACGC-3' (sense strand) and 5'-CTCC TTGGAGGCCATGTAGG-3' (antisense strand). The pair of oligonucleotide primers was designed to amplify a 977-bp sequence from the mRNA of rat G3PDH. RT-PCR was performed using a reaction mixture (25 μl) containing 2 or 4 μg of total RNAs, supplied RT-PCR buffer, Titam™ enzyme mix (AMV and Expand™ High Fidelity), 0.2 mM dNTP, 5 mM dithiothreitol, 5 U RNase inhibitor, and 0.3 mM primers. Samples were incubated at 50˚C for 30 min, and then amplified for 35 cycles under the following conditions: denaturation for 30 sec at 94˚C, annealing for 30 sec at 56˚C, and extension for 60 sec at 68˚C. The amplified products were separated by electrophoresis on a 1.5% agarose gel and were visualized by ethidium bromide staining. Image density was quantified with a FluoroImager SI (Amersham Pharmacia Biotech.).
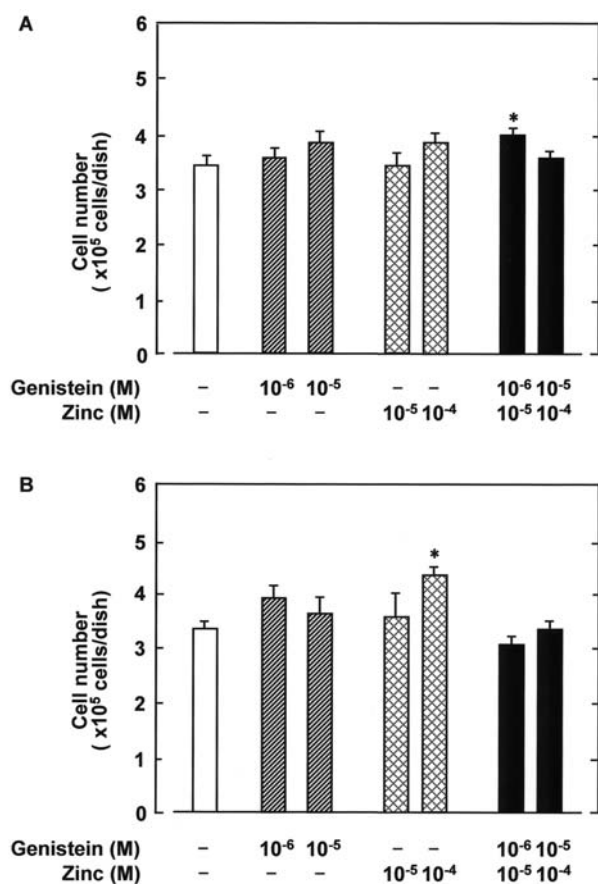
Figure 1. Effects of genistein and zinc on growth of osteoblastic MC3T3-E1 cells. (A) Osteoblastic cells (1x10⁵) were cultured for 72 h in medium containing either vehicle, genistein (10^{-6} or 10^{-5} M), zinc sulfate (10^{-5} or 10^{-4}) or genistein (10^{-6} or 10^{-5} M) plus zinc sulfate (10^{-5} or 10^{-4}) in the presence of 10% FBS. (B) Cells with subconfluency were cultured for 72 h in medium containing either vehicle, genistein (10^{-6} or 10^{-5} M), zinc sulfate (10^{-5} or 10^{-4}), or genistein (10^{-6} or 10^{-5} M) plus zinc sulfate (10^{-5} or 10^{-4}), without 10% FBS. After trypsinization of the cells in each culture dish, cells were collected and counted. Each value is the mean ± SEM of six cultures. *P<0.01 compared with the control (none) value.

Alizarin red staining. Osteoblastic MC3T3-E1 cells (1.0×10^5 cells) were cultured for 72 h in a α-MEM containing 10% FBS. Cells with subconfluency were changed to a Dullbecco's modified essential medium (DMEM) containing ascorbic acid (100 µg/ml) and 4 mM ß-glycerophosphate in the presence or absence of genistein, zinc sulfate, or genistein plus zinc sulfate with 10% FBS. After medium change, cells were cultured for 7, 14, or 21 days. The medium was changed every 3 days. At each time point, cells were rinsed with PBS, and fixed on ice with 70% ethanol for 15 min for alizarin red staining of calcium (30). The alizarin red solution (40 mM, pH 4.2) was filtered through Whatman paper and applied to the fixed wells for 30 min at room temperature. Nonspecific staining was removed by several washes in water.

Statistical analysis. Data are expressed as the mean ± SEM. Statistical differences were analyzed using a Student's t-test. P-values <0.05 were considered to indicate statistically significant differences. The ANOVA multiple comparison test was used to compare the treatment group.

Results

Effect of genistein and zinc on the proliferation of osteoblastic MC3T3-E1 cells. The effect of genistein and zinc sulfate on the proliferation of osteoblastic MC3T3-E1 cells reaching subconfluent monolayers was examined. Osteoblastic cells were cultured for 72 h in medium containing either vehicle, genistein (10^{-6} or 10^{-5} M), zinc sulfate (10^{-5} or 10^{-4} M), or genistein (10^{-6} or 10^{-5} M) plus zinc sulfate (10^{-5} or 10^{-4} M) with 10% FBS (Fig. 1A). The proliferation of osteoblastic cells was significantly increased in the presence of genistein (10^{-6} M) plus zinc sulfate (10^{-5} M). The stimulatory effect of genistein plus zinc sulfate on cell proliferation was not observed when the cells with sub-confluency were cultured for 72 h without 10% FBS (Fig. 1B). A culture with 10^{-4} M zinc sulfate caused a significant
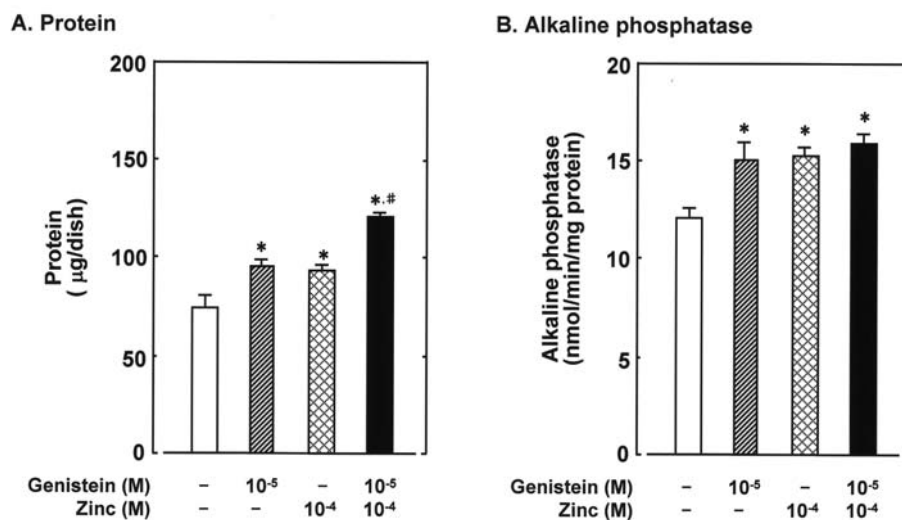

Figure 2. Effects of genistein and zinc on protein content and alkaline phosphatase activity in osteoblastic MC3T3-E1 cells. Osteoblastic cells (1x10⁵) were cultured for 72 h in a medium containing 10% FBS. After culturing, cells with subconfluency were cultured for 48 h in medium containing either vehicle, genistein (10^{-5} M), zinc sulfate (10^{-4}) or genistein (10^{-5} M) plus zinc sulfate (10^{-4}) without 10% FBS. Cells were washed with PBS and scraped to determine protein content and alkaline phosphatase activity. Each value is the mean ± SEM of six cultures. *P<0.01 compared with the control (none) value. #P<0.01 compared with the value obtained from genistein or zinc alone.
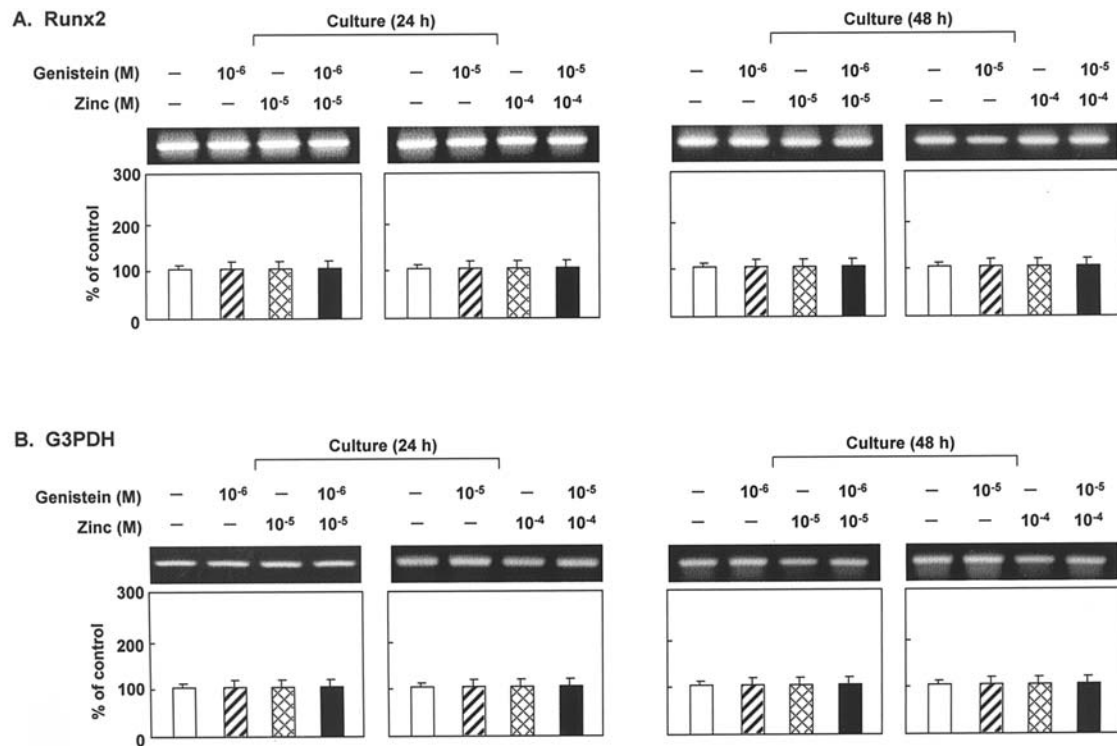
Figure 3. Effects of genistein and zinc on Runx2 or G3PDH mRNA expression in osteoblastic MC3T3-E1 cells using RT-PCR analysis. Osteoblastic cells (1×10^5) were cultured for 72 h in a medium containing 10% FBS. After culturing, cells with subconfluency were cultured for 24 or 48 h in medium containing either vehicle, genistein (10^{-6} or 10^{-5} M), zinc sulfate (10^{-5} or 10^{-4}), or genistein (10^{-6} or 10^{-5} M) plus zinc sulfate (10^{-5} or 10^{-4}), without 10% FBS. Total RNAs ($4 \mu g$) isolated from the cells were analyzed using RT-PCR with Runx2- or G3PDH-specific primer. The housekeeping gene G3PDH mRNA was used as an internal control by ethidium bromide staining. The figure shows one of five experiments with separate samples. The densitometric data for Runx2 or G3PDH mRNA expression represents % of control. These data were not significant as compared to that of control.
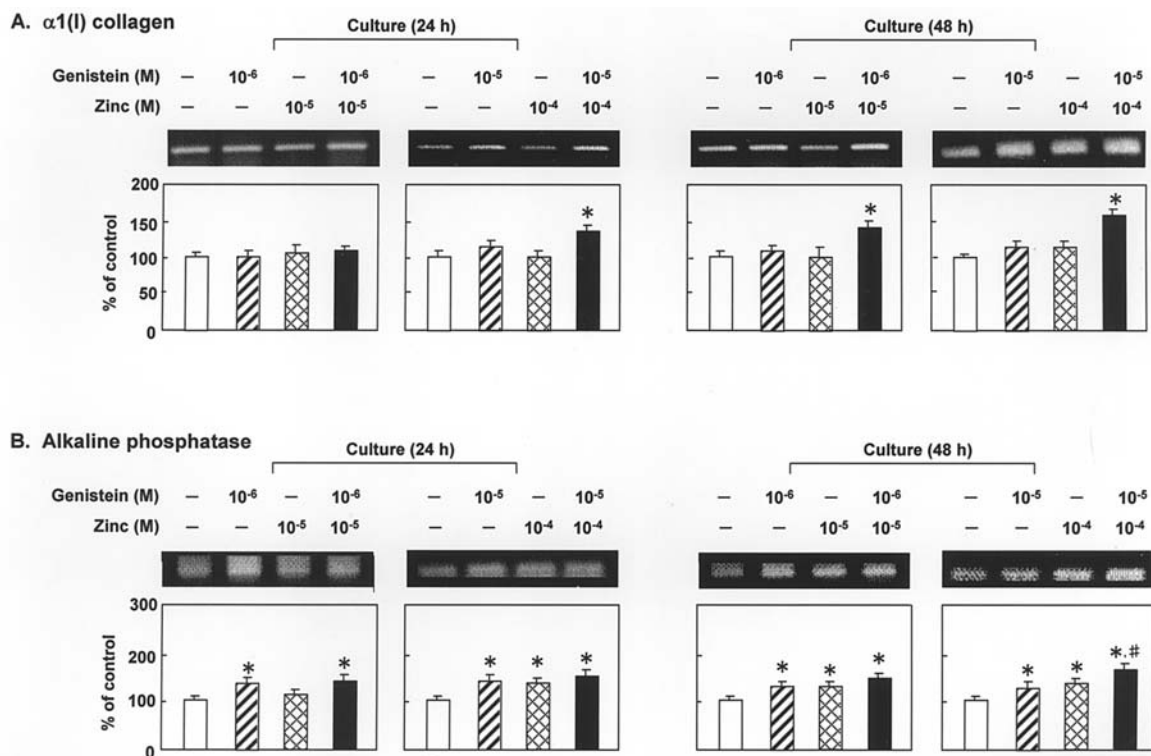

Figure 4. Effects of genistein and zinc on $\alpha1(I)$ collagen or alkaline phosphatase mRNA expression in osteoblastic MC3T3-E1 cells using RT-PCR analysis. Osteoblastic cells (1×10^5) were cultured for 72 h in a medium containing 10% FBS. After culturing, cells with subconfluency were cultured for 24 or 48 h in medium containing either vehicle, genistein (10^{-6} or 10^{-5} M), zinc sulfate (10^{-5} or 10^{-4}), or genistein (10^{-6} or 10^{-5} M) plus zinc sulfate (10^{-5} or 10^{-4}), without 10% FBS. Total RNAs ($4 \mu g$) isolated from the cells were analyzed using RT-PCR with $\alpha1(I)$ collagen- or alkaline phosphatase-specific primers. The figure shows one of five experiments with separate samples. The densitometric data for $\alpha1(I)$ collagen or alkaline phosphatase mRNA expression represents % of control. *$P < 0.01$ compared to the control (none) value. #$P < 0.01$ compared to the value obtained from genistein or zinc alone.
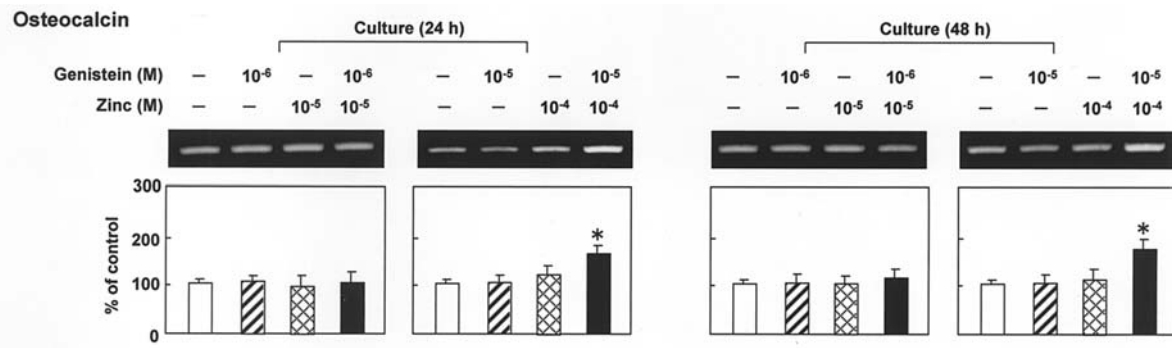
Figure 5. Effects of genistein and zinc on osteocalcin mRNA expression in osteoblastic MC3T3-E1 cells using RT-PCR analysis. Osteoblastic cells (1×10^5) were cultured for 72 h in a medium containing 10% FBS. After culturing, cells with subconfluency were cultured for 24 or 48 h in medium containing either vehicle, genistein (10^{-6} or 10^{-5} M), zinc sulfate (10^{-5} or 10^{-4}), or genistein (10^{-6} or 10^{-5} M) plus zinc sulfate (10^{-5} or 10^{-4}), without 10% FBS. Total RNAs (4 μg) isolated from the cells were analyzed using RT-PCR with osteocalcin-specific primer. The figure shows one of five experiments with separate samples. The densitometric data for osteocalcin mRNA expression represents % of control. *P<0.01 compared to the control (none) value.
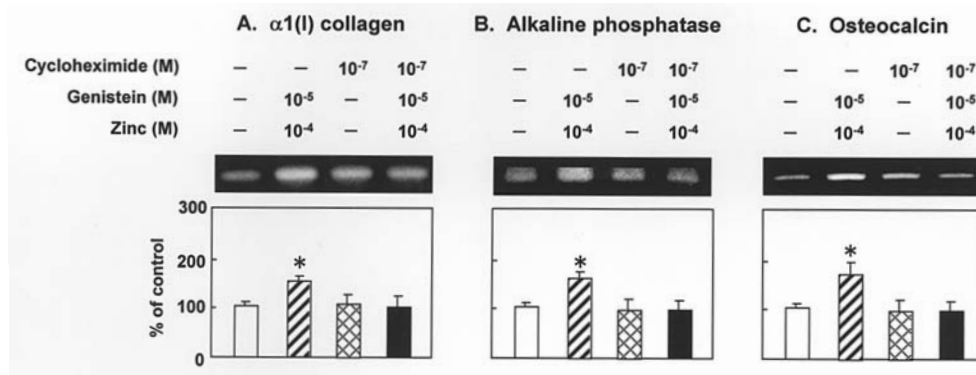

Figure 6. Effects of cycloheximide on the genistein- and zinc-induced increases in $\alpha1$(I) collagen, alkaline phosphatase, and osteocalcin mRNA expression in osteoblastic MC3T3-E1 cells using RT-PCR analysis. Osteoblastic cells (1×10^5) were cultured for 72 h in a medium containing 10% FBS. After culturing, cells with subconfluency were cultured for 48 h in medium containing either vehicle, genistein (10^{-5} M) plus zinc sulfate (10^{-4}), in the presence or absence of cycloheximide (10^{-7} M) without 10% FBS. Total RNAs (4 μg) isolated from the cells were analyzed using RT-PCR with $\alpha1$(I) collagen-, alkaline phosphatase-, or osteocalcin-specific primers. The figure shows one of five experiments with separate samples. The densitometric data for mRNA expression represents % of control. *P<0.01 compared to the control (none) value.

increase in cell number when cells with subconfluency were cultured for 72 h without FBS.

Effect of genistein and zinc on biochemical components in osteoblastic MC3T3-E1 cells. Osteoblastic cells were cultured for 72 h in medium containing 10% FBS. Cells with subconfluency were cultured for 48 h in medium containing either vehicle, genistein (10^{-5} M), zinc sulfate (10^{-4} M), or genistein (10^{-5} M) plus zinc sulfate (10^{-4} M). The change in protein content and alkaline phosphatase activity in osteoblastic cells was examined. The protein content in the cells was significantly increased with a culture of genistein (10^{-5} M) or zinc sulfate (10^{-4} M). These increases were significantly enhanced with a culture of the combination of genistein (10^{-5} M) and zinc sulfate (10^{-4} M) (Fig. 2A). Alkaline phosphatase activity in osteoblastic cells was significantly increased with a culture of genistein (10^{-5} M), zinc sulfate (10^{-4} M), or genistein (10^{-5} M) plus zinc sulfate (10^{-4} M) (Fig. 2B). The combination of genistein (10^{-5} M) and zinc sulfate (10^{-4} M) did not significantly enhance the effect of each factor on the enzyme activity.

Effect of genistein and zinc on gene expression in osteoblastic MC3T3-E1 cells. Osteoblastic cells with subconfluency were cultured for 24 or 48 h in medium containing either vehicle, genistein (10^{-6} or 10^{-5} M), zinc sulfate (10^{-5} or 10^{-4}), or genistein (10^{-6} or 10^{-5} M) plus zinc sulfate (10^{-5} or 10^{-4}) without 10% FBS. The expression of Runx2 or G3PDH mRNA in the cells was not significantly changed in the presence of genistein (10^{-6} or 10^{-5} M), zinc sulfate (10^{-5} or 10^{-4}) or genistein (10^{-6} or 10^{-5} M) plus zinc sulfate (10^{-5} or 10^{-4}) (Fig. 3A and B). $\alpha1$(I) collagen mRNA expression was significantly increased with a culture of genistein (10^{-6} M) plus zinc sulfate (10^{-5} M) or genistein (10^{-5} M) plus zinc sulfate (10^{-4} M) for 48 h, although its gene expression was not significantly changed with a culture of genistein (10^{-6} or 10^{-5} M) or zinc sulfate (10^{-5} or 10^{-4}) alone (Fig. 4A). Alkaline phosphatase mRNA expression was significantly increased with a culture for 48 h in the presence of genistein (10^{-6} or 10^{-5} M) or zinc sulfate (10^{-5} or 10^{-4}) (Fig. 4B). The effect of genistein (10^{-5} M) or zinc sulfate (10^{-4} M) in increasing alkaline phosphatase mRNA expression was significantly enhanced with the combination of genistein (10^{-5} M) and zinc
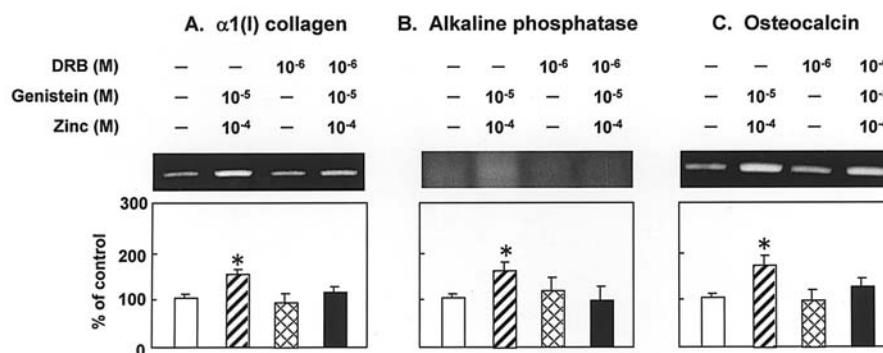
Figure 7. Effects of DRB on the genistein and zinc-induced increases in α1(I) collagen, alkaline phosphatase, and osteocalcin mRNA expression in osteoblastic MC3T3-E1 cells using RT-PCR analysis. Osteoblastic cells (1×10^5) were cultured for 72 h in a medium containing 10% FBS. After culture, cells with subconfluency were cultured for 48 h in medium containing either vehicle, genistein (10^{-5} M) plus zinc sulfate (10^{-4}), in the presence or absence of DRB (10^{-6} M) without 10% FBS. Total RNAs (4 μg) isolated from the cells were analyzed using RT-PCR with α1(I) collagen-, alkaline phosphatase-, or osteocalcin-specific primers. The figure shows one of five experiments with separate samples. The densitometric data for mRNA expression represents % of control. *P<0.01 compared to the control (none) value.
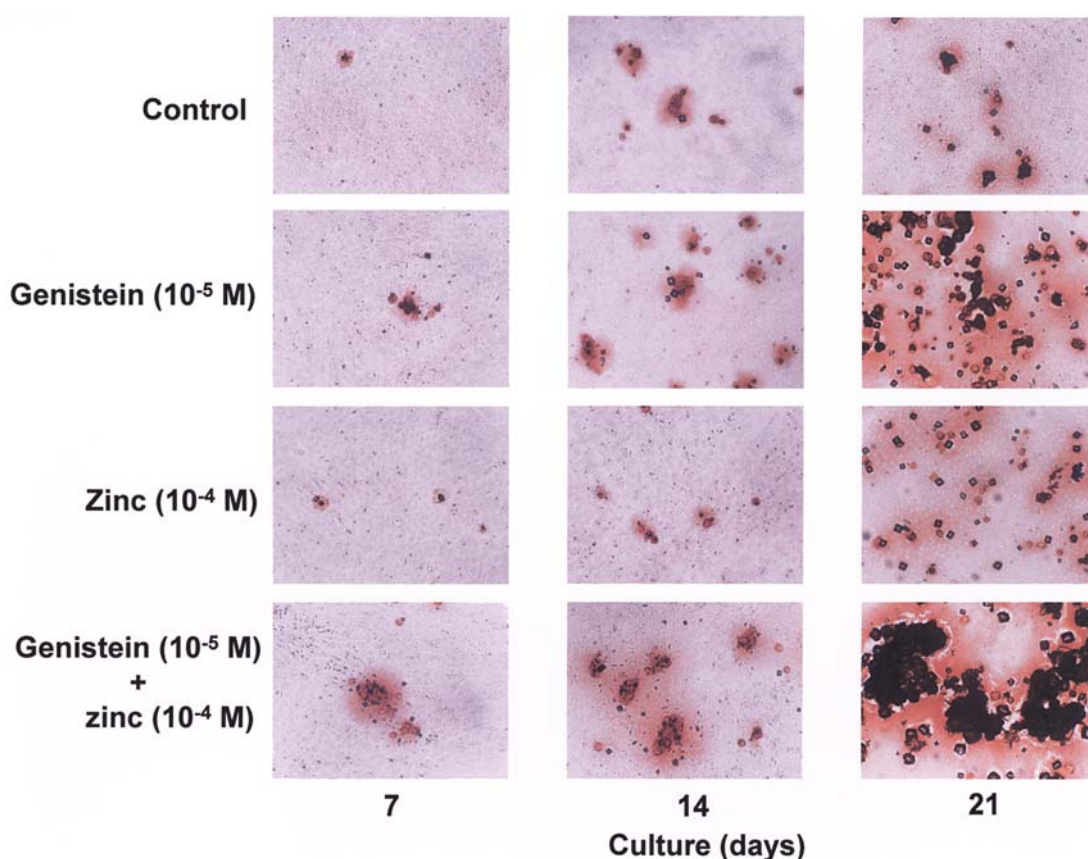

Figure 8. Effects of genistein and zinc sulfate on mineralization in osteoblastic MC3T3-E1 cells. Osteoblastic cells (1×10^5) were cultured for 72 h in medium containing 10% FBS. After culturing, cells with subconfluency were cultured for 7, 14, or 21 days in medium containing either vehicle, genistein (10^{-5} M), zinc sulfate (10^{-4} M), or genistein (10^{-5} M) plus zinc sulfate (10^{-4} M) with 10% FBS. Cells were washed with PBS and alizarin red stained. The figure shows one of five experiments with separate cultures.

sulfate (10^{-4} M) for 48 h (Fig. 4B). Osteocalcin mRNA expression in osteoblastic cells was significantly increased with a culture of genistein (10^{-5} M) plus zinc sulfate (10^{-4} M) for 24 or 48 h (Fig. 5). Genistein (10^{-5} M) or zinc sulfate (10^{-4} M) alone did not have a significant effect on osteocalcin mRNA expression in the cells.

The effect of cycloheximide, an inhibitor of protein synthesis, or DRB, an inhibitor of transcription activity, on genistein as well as the zinc sulfate-induced increase in gene expression in osteoblastic cells were examined. The effect of the combination of genistein and zinc sulfate in enhancing α1(I) collagen, alkaline phosphatase, or osteocalcin mRNA expression was completely prevented in the presence of cycloheximide (10^{-7} M) (Fig. 6) or DRB (10^{-6} M) (Fig. 7).

Effect of genistein and zinc on mineralization in osteoblastic MC3T3-E1 cells. Osteoblastic cells with subconfluency were cultured for 7, 14, or 21 days in medium containing either

vehicle, genistein (10^{-5} M), zinc sulfate (10^{-4} M), or genistein (10^{-5} M) plus zinc sulfate (10^{-4} M), and the mineralization was examined (Fig. 8). The results with alizarin red staining for calcium showed that the mineralization was significantly stimulated in the presence of genistein (10^{-5} M) or zinc sulfate (10^{-4} M). The enhancement of mineralization was observed from 14 days of culture. The effect was markedly enhanced with a 21-day culture in the presence of genistein or zinc sulfate. The combination of genistein and zinc sulfate caused a remarkable increase in mineralization as compared with that of genistein or zinc sulfate alone.

Discussion

The intake of the combination of dietary genistein and zinc prevents bone loss in ovariectomized rats which are model animals of osteoporosis (21). Genistein or zinc has been shown to activate aminoacyl-tRNA synthase, the rate-limiting enzyme of translational protein synthesis, and to stimulate protein synthesis in osteoblastic MC3T3-E1 cells (8,13,16). The cellular mechanism by which the combination of genistein and zinc has an anabolic effect on bone components in rats has not been determined. The present study demonstrates that the combination of genistein and zinc has a synergistic effect on gene expression and mineralization in osteoblastic MC3T3-E1 cells *in vitro*.

The combination of genistein and zinc was found to have an additive effect on protein content in osteoblastic cells *in vitro*. This effect may involve the activation of aminoacyl-tRNA synthase by genistein and zinc, since these factors can increases the enzyme activity in the cytosol of osteoblastic cells *in vitro* (8,15,16).

Alkaline phosphatase is an enzyme marker of osteoblasts, and the enzyme participates in bone mineralization (31). $\alpha1$ (I) collagen is a matrix protein that is related to bone formation and mineralization in osteoblast lineage cells (32). Osteocalcin is a bone matrix protein containing γ_2-carboxy-glutamin acid which is synthesized in osteoblasts, and is the protein involved in mineralization (32). The expression of alkaline phosphatase, $\alpha1$(I) collagen and osteocalcin mRNAs in osteoblastic cells was significantly enhanced with a culture of the combination of genistein and zinc as compared to the effect of each factor. This finding demonstrates that the combination of genistein and zinc has a synergistic effect on gene expression in osteoblastic cells.

The effect of the combination of genistein and zinc on gene expression in osteoblastic cells was completely prevented in the presence of cycloheximide, an inhibitor of protein synthesis, and DRB, an inhibitor of transcriptional activity. This result suggests that the combination of genistein and zinc stimulates the transcriptional process in osteoblastic cells. Genistein or zinc has a stimulatory effect on protein synthesis at the transcriptional process (8,13,16). Presumably, the combination of genistein and zinc stimulates both transcriptional and translational activities in osteoblastic cells. Each factor activates aminoacyl-tRNA synthetase at the transcriptional process in osteoblastic cells. It is speculated that genistein or zinc has an effect on the binding of the transcriptional factor to DNA in osteoblastic cells.

A culture with genistein or zinc was found to have a stimulatory effect on the mineralization in osteoblastic cells. The combination of genistein and zinc synergistically enhanced mineralization in osteoblastic cells. This finding suggests that the combination of genistein and zinc can effectively enhance bone mineralization, thereby increasing bone mass. The combination of genistein and zinc may be a useful tool in the prevention and therapy of osteoporosis.

In conclusion, it has been demonstrated that the combination of genistein and zinc has a synergistic effect on gene expression and mineralization in osteoblastic cells *in vitro*.

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