SPANDIDOS PUBLICATIONS

Regulatory effect of exogenous regucalcin on cell function in osteoblastic MC3T3-E1 cells: Involvement of intracellular signaling factor

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Abstract. Bone loss is induced in regucalcin transgenic rats. The role of exogenous regucalcin in the regulation of osteoblastic cell function was investigated. Osteoblastic MC3T3-E1 cells with subconfluent monolayers were cultured for 24-72 h in medium containing regucalcin (10^{-10} or 10^{-9} M) without fetal bovine serum. The presence of regucalcin did not have a significant effect on cell number. Culture with regucalcin (10^{-9} M) for 24 h caused a significant decrease in protein and DNA contents in osteoblastic cells. The effect of regucalcin in decreasing cellular protein content was significantly inhibited in the presence of various kinase inhibitors including staurosporine (10^{-7} M), dibucaine (10^{-6} M), PD98059 (10^{-8} M), or wortmannin (10^{-8} M). Meanwhile, culture with regucalcin caused a significant decrease in cellular DNA content in the presence of various kinase inhibitors. The presence of regucalcin did not have a significant effect on protein and DNA contents in the cells cultured with cycloheximide (10^{-7} M), an inhibitor of protein synthesis, or 5,6-dichloro -1-ß-D-ribofuranosylbenzimidazole (10^{-6} M), an inhibitor of transcription activity; which each inhibitor caused a significant decrease in those contents. The effect of regucalcin in decreasing cellular protein content was seen in the presence of insulin-like growth factor-I (IGF-I; 10^{-9} or 10^{-8} M). Such an effect was not observed in cellular DNA content. The results of reverse transcription-polymerase chain reaction analysis with specific primers showed that the expression of Runx 2 (Cbfa 1) and alkaline phosphatase mRNAs in osteoblastic cells was significantly suppressed in the presence of regucalcin (10^{-10} or 10^{-9} M). Glyceraldhyde-3-phosphate dehydrogenase mRNA level was not significantly changed with culture of regucalcin (10^{-10} or 10^{-9} M). This study supports the view that exogenous regucalcin regulates the function of osteoblastic cells, and that the effect of protein is mediated through signaling factors.

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Introduction

Regucalcin was discovered in 1978 as a calcium-binding protein that does not contain EF-hand motif of Ca^{2+}-binding domain (1-3). The name regucalcin was proposed for this Ca^{2+}-binding protein, which may regulate Ca^{2+} and/or calmodulin effects on liver cell function (4,5). In recent years, regucalcin has been demonstrated to play a multifunctional role in maintaining cell homeostasis and function as the regulatory protein in intracellular signaling process in many cell types (6-8).

Rat and human regucalcin genes are localized on chromosame X (9,10). Regucalcin messenger ribonucleic acid (mRNA) and its protein are greatly present in liver and kidney cortex, and regucalcin is also found in other tissues (11,12). The expression of regucalcin mRNA is mediated through the Ca^{2+}-signaling mechanism (13,14). AP1, NFI-A1 and RGPR-p117 (novel protein) have been found to be transcriptional factors for the enhancement of regucalcin gene promoter activity (15-17).

Regucalcin plays a role in maintaining intracellular Ca^{2+} homeostasis, the inhibitory regulation of various Ca^{2+}-dependent protein kinases and tyrosine kinases, protein phosphatases, nitric oxide synthase, and the control of the enhancement of protein synthesis, nuclear deoxyribonucleic acid (DNA) and RNA synthesis in proliferative cells (6-8, 18-25). Recent study has demonstrated that regucalcin has suppressive effects on cell proliferation and apoptosis, which are mediated through many signaling factors, in cloned rat hepatoma H4-II-E cells and normal rat kidney proximal tubular epithelial NRK52E cells overexpressing regucalcin (26-28). Regucalcin has been proposed to play a role in maintaining cell homeostasis and function in many cell types (8).

We generated regucalcin transgenic (TG) rats to determine a regulatory role of endogenous regucalcin *in vivo* using a TG rat model (29). Bone loss and hyperlipidemia have been induced in regucalcin TG rats (30,31), supporting the view that regucalcin has an important role on pathophysiologic state.

The mechanism by which bone loss is induced in regucalcin TG rats has not been fully clarified. Regucalcin is expressed in rat bone marrow cells, and bone loss in regucalcin TG rats is partly involved in osteoclastic bone resorption (32,33). Regucalcin has been demonstrated to stimulate osteoclast-like cell formation in mouse marrow culture *in vitro*, and the protein stimulates bone resorption in rat femoral tissue *in vitro* (34). Also, regucalcin is found to have suppressive effects on cell differentiation and mineralization in osteoblastic MC3T3-E1 cells *in vitro* (35).

Furthermore, this study was undertaken to determine whether the effect of endogenous regucalcin on the function of osteoblastic cells *in vitro* is involved in intracellular signaling factors.

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. Staurosporine, PD98059, dibucaine, wartmannin, cycloheximide, 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole (DRB), and insulin-like growth factor-I (IGF-I) were obtained from Sigma Chemical Co. 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.

Isolation of regucalcin. Regucalcin is markedly expressed in the liver cytosol (11,12). Regucalcin was isolated from rat liver cytosol. The livers were perfused with Tris-HCl buffer (pH 7.4, containing 100 mM Tris, 120 mM NaCl, 4 mM KCl, cooled to 4˚C). The livers were removed, cut into small pieces, and suspended 1:4 (weight:volume) in Tris-HCl buffer (pH 7.4); the homogenate was spun at 5,500 x g in a refrigerated centrifuge for 10 min, and the supernatant was spun at 105,000 x g for 60 min. The resulting supernatant was heated at 60˚C for 10 min and recentrifuged at 38,000 x g for 20 min. Regucalcin in the supernatant was purified to electrophoretic homogeneity by gel filtration in Sephadex G-75 and G-50 followed by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose, as reported previously (1).

Cell culture. Osteoclastic 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 experiments, ~1x10^5 cells per dish were cultured for 3 days to obtain subconfluent monolayers in 35-mm plastic containing 2 ml α-MEM with 10% FBS. After the cells were rinsed with FBS, the medium was exchanged for medium without FBS containing either vehicle or regucalcin (10^{-10} or 10^{-9} M) in the presence or absence of various inhibitors, and the cells were cultured further for 24-72 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 resuspended 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 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. Protein concentration in the cell homogenate was determined by the method of Lowry *et al* (36) and expressed as the amount of protein (μg) per dish.

To measure DNA content in the cells, the cells were detached by using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}/free PBS and washed with PBS. The cells were shaken with 2.0 ml of ice-cold 0.1 N NaOH solution for 6 h after disruption (37). After alkali extraction, the samples were centrifuged at 10,000 x g for 5 min, and the supernatant was determined by the method of Ceriotti (38) and expressed as the amount of DNA (μg) per dish.

Determination of specific mRNA by reverse transcription-polymerase chain reaction (RT-PCR). Total RNAs were prepared as described previously (39). Osteoblastic MC3T3-E1 cells with subconfluency were cultured for 24 h in medium containing either vehicle or regucalcin (10^{-10} or 10^{-9} M). After culture, cells were washed three times with ice-cold PBS, and then cells were homogenized in 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.

RT-PCR was preformed with a Titam™ one tube RT-PCR kit (Roche Molecular Biochemicals) as recommended by the supplier. Primers for amplification of mouse Runx2 (type 1) cDNA were 5'-ATGCGTATTCCTGTAGA TCCGAG-3' (sense strand, positions 1016-1038 of cDNA sequence) and 5'-CATCATTCCCGGCCATGACGGTAAC-3' (antisense strand, positions 1451-1475) (40). The pair of oligonucleotide primers was designed to amplify a 459-bp sequence from the mRNA of mouse Runx2 (type 1). Primers for amplification of mouse alkaline phosphate cDMA were 5'-GATCGGGACTGGTACTCGGATAA-3' (sense strand, positions 729-751 of cDNA sequence) and 5'-CACATCA GTTCTGTTCTTCGGGTAC-3' (antisense strand, positions 860-884) (40). The pair of oligonucleotide primers was designed to amplify a 155-bp sequence from the mRNA of alkaline phosphatase. For semiquantitative 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'-GATTTGGCCGT ATCGGACGC-3' (sense strand) and 5'-CTCCTTGGAGG CCATGTAGG-3' (antisense strand). The pair of oligo-

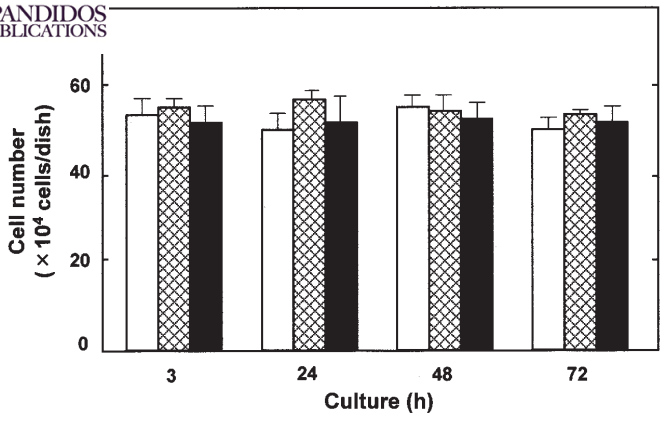

Figure 1. Effects of regucalcin on the number of osteoblastic MC3T3-E1 cells. Cells ($1x10^5$) were cultured for 72 h in medium containing 10% fetal bovine serum (FBS) to obtain subconfluent monolayers, and then in medium containing either vehicle or regucalcin (10^{-10} or 10^{-9} M) in the absence of 10% FBS. After medium change, cells were cultured for 3, 24, 48, or 72 h. At each time point, the number of cells was measured. Each value is the mean ± SEM of six experiments with separate cell culture. Data were not significant as compared with the control (none) value. White bars, control (none); double hatched bars, regucalcin (10^{-10} M); black bars, regucalcin (10^{-9} M).

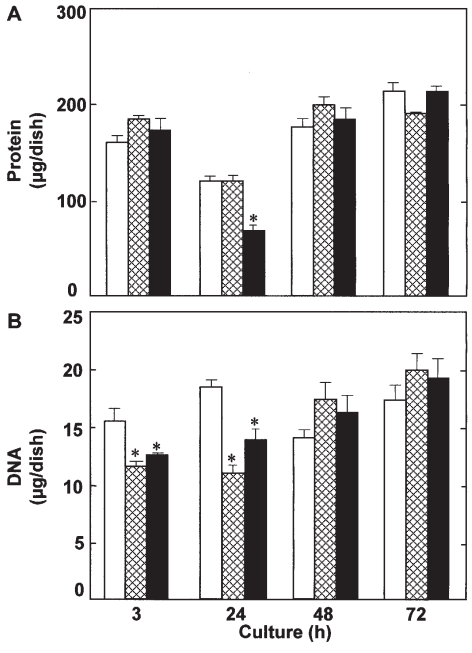

Figure 2. Effects of regucalcin on protein (A) and DNA (B) contents in osteoblastic MC3T3-E1 cells. Cells ($1x10^5$) were cultured for 72 h in medium containing 10% FBS. Cells with subconfluency were changed to medium containing either vehicle or regucalcin (10^{-10} or 10^{-9} M) in the absence of 10% FBS. At each time point, protein content in the cells was measured. Each value is the mean ± SEM of six experiments with separate cell culture. *P<0.01 compared with the control (none) value. White bars, control (none); double hatched bars, regucalcin (10^{-10} M); black bars, regucalcin (10^{-9} M).

nucleotide primers was designed to amplify a 977-bp sequence from the mRNA of rat G3PDH. RT-PCR was performed using reaction mixture (20 μl) containing 2 or 4 μg of total RNA, supplied RT-TCR buffer, Titam™ enzyme mix (AMV and Expand™ High Fidelity), 0.2 mM dNTP, 5 mM dithiothreitol,

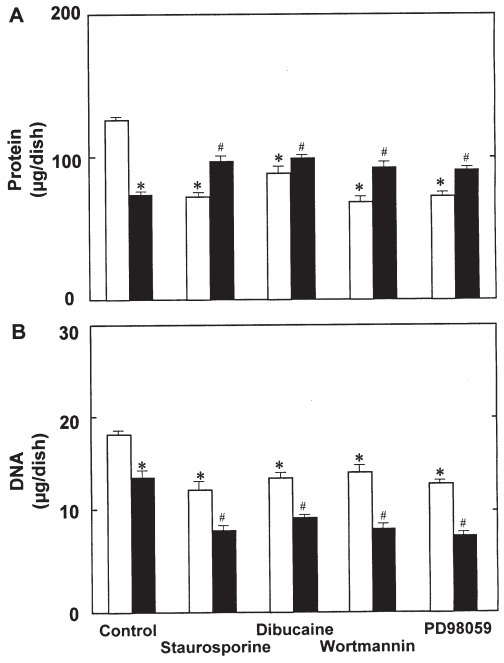

Figure 3. Effects of regucalcin on protein (A) and DNA (B) contents in osteoblastic MC3T3-E1 cells in the presence of signaling inhibitors. Cells ($1x10^5$) were cultured for 72 h in medium containing 10% FBS. Cells with subconfluency were cultured for 24 h in medium containing either vehicle or regucalcin (10^{-9} M) with or without staurosporine (10^{-7} M), dibucaine (10^{-6} M), worthmannin (10^{-8} M) or PD98059 (10^{-8} M) in the absence of 10% FBS. After culture, DNA content in the cell was measured. Each value is the mean ± SEM of six experiments with separate cell culture. *P<0.01 compared with the control (none) value. #P<0.01 compared with the value obtained from regucalcin alone. White bars, without regucalcin; black bars, with regucalcin.

5 U RNase inhibitor, and 0.3 μM primers. Samples were incubated at 50˚C for 30 min, and then amplified for 30 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 visualized by ethidium bromide staining. Image density was quantified using a FluoroImager SI (Amersham Pharmacia Biotech).

Statistical analysis. Data are expressed as the mean ± SEM. Statistical differences were analyzed using 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 groups.

Results

Effects of regucalcin addition in osteoblastic MC3T3-E1 cells. Osteoblastic MC3T3-E1 cells were cultured for 72 h in medium containing 10% FBS. The cells with subconfluency were cultured for 24-72 h in medium containing either vehicle or regucalcin (10^{-10} or 10^{-9} M) without FBS. The number of cells was not significantly changed in the presence of regucalcin (Fig. 1). The presence of regucalcin (10^{-9} M) caused a significant decrease in protein content in the cells cultured for 24 h (Fig. 2A). DNA content in the cells was significantly decreased with culture of regucalcin

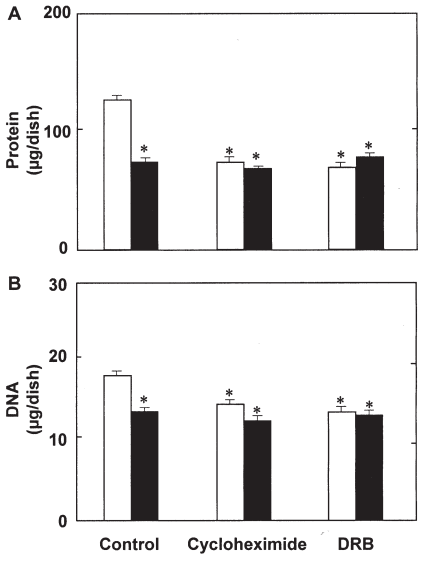

Figure 4. Effects of regucalcin on protein (A) and DNA (B) contents in osteoblastic MC3T3-E1 cells in the presence of cycloheximide or DRB. Cells ($1x10^5$) were cultured for 72 h in medium containing 10% FBS. Cells with subconfluency were cultured for 24 h in medium containing either vehicle or regucalcin (10^{-9} M) with or without cycloheximide (10^{-7} M) or DRB (10^{-6} M) in the absence of 10% FBS. After culture, protein content in the cells was measured. Each value is the mean ± SEM of six experiments with separate cell culture. *P<0.01 compared with the control (none) value. White bars, without regucalcin; black bars, with regucalcin.

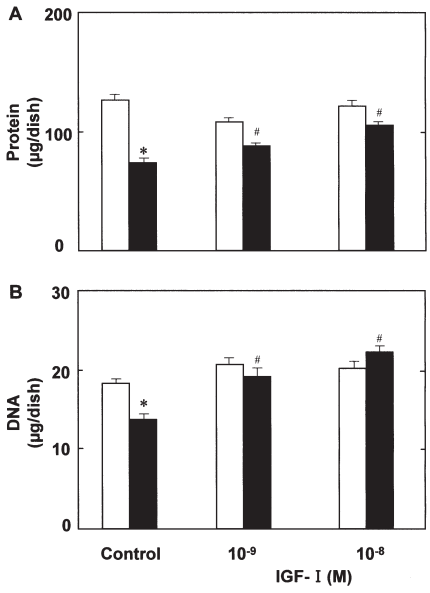

Figure 5. Effects of regucalcin on protein (A) and DNA (B) contents in osteoblastic MC3T3-E1 cells in the presence of IGF-I. Cells ($1x10^5$) were cultured for 72 h in medium containing 10% FBS. Cells with subconfluency were cultured for 24 h in medium containing either vehicle or regucalcin (10^{-9} M) with or without IGF-I (10^{-9} or 10^{-8} M) in the absence of 10% FBS. After culture, protein content in the cells was measured. Each value is the mean ± SEM of six experiments with separate cell culture. *P<0.01 compared with the control (none) value. White bars, without regucalcin; black bars, with regucalcin.

(10^{-10} or 10^{-9} M) for 3 or 24 h (Fig. 2B). Thus, culture with regucalcin (10^{-9} M) caused a significant decrease in protein and DNA contents in osteoblastic MC3T3-E1 cells, indicating that extracellular regucalcin has a regulatory effect on cell function.

Effect of regucalcin in osteoblastic MC3T3-E1 cells in the presence of various inhibitors. Osteoblastic MC3T3-E1 cells with subconfluency were cultured for 24 h in a medium containing either vehicle or regucalcin (10^{-9} M) in the presence or absence of staurosporine (10^{-7} M), an inhibitor of protein kinase C (41), dibucaine (10^{-6} M), an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase (42), wortmannin (10^{-8} M), an inhibitor of PI_3 kinase (43), or PD 98059 (10^{-8} M), an inhibitor of mitogen-activated protein kinase (44) with an effective concentration (Fig. 3). The presence of staurosporine, dibucaine, wortmannin, or PD98059 caused a significant decrease in protein and DNA contents in osteoblastic MC3T3-E1 cells. The effect of regucalcin in decreasing cellular protein content was significantly inhibited in the presence of staurosporine, dibucaine wortmannin, or PD98059 (Fig. 3A). The effect of regucalcin in decreasing cellular DNA content was also observed in the presence of staurosporine, dibucaine, wortmann, or PD98059 (Fig. 3B).

The effect of cycloheximide, an inhibitor of protein synthesis, or DRB, an inhibitor of transcription activity, on regucalcin action in osteoblastic MC3T3-E1 cells with subconfluency was examined (Fig. 4). Cells were cultured for 24 h in medium containing either vehicle or regucalcin (10^{-9} M) in the presence or absence of cycloheximide (10^{-7} M) or DRB (10^{-6} M). Cellular protein and DNA contents were significantly decreased with culture of cycloheximide or DRB. The presence of regucalcin did not have a significant effect on protein and DNA contents in cells cultured with cycloheximide or DRB.

Effects of regucalcin in osteoblastic MC3T3-E1 cells in the presence of IGF-I. Osteoblastic MC3T3-E1 cells with subconfluency were cultured for 24 h in medium containing either vehicle or regucalcin (10^{-9} M) in the presence or absence of IGF-I (10^{-9} or 10^{-8} M). Culture with IGF-I did not have a significant effect on cellular protein and DNA contents (Fig. 5). The effect of regucalcin in decreasing cellular protein content was also seen in the presence of IGF-I (10^{-9} or 10^{-8} M) (Fig. 5A). However, the effect of regucalcin in decreasing cellular DNA content was not observed in the presence of IGF-I (10^{-9} or 10^{-8} M) (Fig. 5B).

Effect of regucalcin on gene expression in osteoblastic MC3T3-E1 cells. Osteoblastic cells with subconfluency were cultured for 24 h in medium containing either vehicle or regucalcin (10^{-10} or 10^{-9} M) in the absence of FBS. The change in the expression of Runx2, alkaline phosphatase, or G3PDH mRNAs in osteoblastic cells was examined using RT-PCR analysis with specific primers (Fig. 6). The signal of bands for Runx2 or alkaline phosphatase mRNA expression was significantly reduced in cells cultured in the presence of regucalcin (10^{-10} or 10^{-9} M). The levels of G3PDH mRNA were not significantly changed in the presence of regucalcin (Fig. 6C).

Discussion

Regucalcin is expressed in rat bone marrow cells and femoral tissue (30,32). Bone loss is induced in regucalcin TG rats

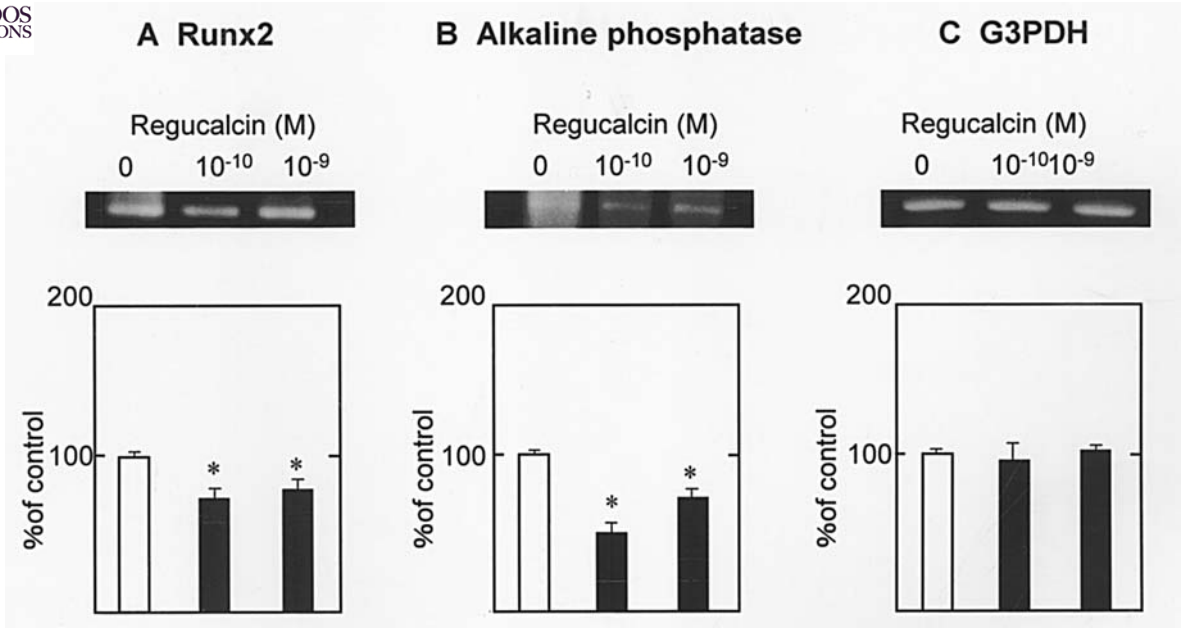

Figure 6. Effects of regucalcin on the expression of Runx2 (A), alkaline phosphtase (B) or G3PDH (C) mRNAs in osteoblastic MC3T3-E1 cells. Osteoblastic cells ($1x10^5$ cells) were cultured for 24 h in medium containing 10% FBS. Cells with subconfluency were changed to a medium without FBS in the presence or absence of regucalcin (10^{-10} or 10^{-9} M), and the cells were cultured for 24 h. Total RNA (2 μg) extracted from the cells was analyzed by RT-PCR using specific primers. One of five experiments with separate samples. The densitometric data for each mRNA level in the cells cultured for 24 h in the presence of regucalcin were indicated as % of control (mean ± SEM for five experiments). *P<0.01 compared with the control value.

(30). The mechanism by which bone loss is induced in regucalcin TG rats has not been fully clarified. Bone loss in regucalcin TG rats is partly involved in osteoblastic bone resorption (33,34). More recently, regucalcin has been shown to have suppressive effects on cell differentiation and mineralization in osteoblastic MC3T3-E1 cells that participate in bone formation and mineralization *in vitro* (35). Furthermore, this study has demonstrated that exogenous regucalcin has suppressive effects on protein and DNA contents in osteoblastic MC3T3-E1 cells *in vitro*.

The effect of regucalcin in decreasing protein and DNA contents in osteoblastic cells was observed at 24 h of culture with regucalcin addition. These decreases were restored at 48 and 72 h. Regucalcin did not have a significant effect on cell number in osteoblastic cells with culture for 72 h. It is speculated that exogenous regucalcin binds to the plasma membranes, and that the protein transmits signal(s) into osteoblastic cells. Iodinated regucalcin has been shown to bind to the plasma membranes isolated from rat liver *in vitro* (45). Specific binding sites for regucalcin in osteoblastic cells remain to be elucidated. Regucalcin may be released during longer culture times, and the protein signal may disappear in osteoblastic cells. This is in connection with the restoration of cellular protein and DNA contents reduced with culture of regucalcin.

The effect of regucalcin in decreasing protein contents in osteoblastic cells was inhibited in the presence of various kinase inhibitors including protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase, PI3 kinase, and MAP kinase. This result suggests that the effect of regucalcin in decreasing cellular protein content is partly mediated through various protein kinases that are involved in intracellular signaling process. Meanwhile, culture with regucalcin caused a significant decrease in DNA content in osteoblastic cells in the presence of various kinase inhibitors. The effect of regucalcin in decreasing cellular DNA content may be involved in other signaling mechanisms, which differ from its action on cellular protein content. From these observations, it is assumed that the effect of exogenous regucalcin is mediated through several signaling pathways in osteoblastic cells.

The effects of regucalcin on protein and DNA contents in osteoblastic cells were examined in the presence of IGF-I, which can stimulate cell proliferation (46). The effect of regucalcin in decreasing cellular protein content was also seen in the presence of IGF-I. However, the regucalcin-induced decrease in cellular DNA content was not observed in the presence of IGF-I. Presumably, the effect of regucalcin is transmitted independent of the intracellular signaling process of IGF-I action.

Culture with regucalcin suppressed the expression of Runx2, a transcription factor (47), and alkaline phosphatase, a key enzyme of mineralization (48), mRNAs in osteoblastic cells. This result supports the view that endogenous regucalcin has suppressive effects on differentiation and mineralization in osteoblastic cells (35). In addition, the effect of regucalcin is transmitted to that transcription process in the nucleus of osteoblastic cells.

Culture with regucalcin may have inhibitory effects on protein and DNA syntheses, which are mediated through different intracellular signaling processes, in osteoblastic cells. At present, the signaling factors which are involved in regucalcin action are unknown. Various protein kinases may partly be related to the intracellular signaling of regucalcin action in osteoblastic cells. Moreover, other signaling factors may be involved in the action of regucalcin in osteoblastic cells.

In conclusion, it has been demonstrated that exogenous regucalcin regulates the function of osteoblastic cells *in vitro*, and that regucalcin action is mediated through intracellular signaling factors.

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