

Combination of β -cryptoxanthin and zinc has potent effects on apoptotic cell death and suppression of bone resorption-related gene expression in osteoclastic cells

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Abstract. We investigated whether the effect of β -cryptoxanthin (CRP) on osteoclastic cells formed in the mouse marrow culture system *in vitro* is enhanced by culture with zinc. Bone marrow cells were isolated from mice. The macrophage colony-stimulating factor (M-CSF)-dependent bone marrow macrophages were cultured in the presence of M-CSF (10 ng/ml) and receptor activator of nuclear factor- κ B (NF- κ B) ligand (RANKL; 50 ng/ml) for 96 h. The osteoclastic cells formed were further cultured for 24 or 72 h in a medium containing either vehicle, CRP, zinc sulfate (zinc), or CRP plus zinc with or without M-CSF (10 ng/ml) and RANKL (50 ng/ml). The number of osteoclastic cells was significantly decreased after culture with the combination of CRP (10^{-7} M) and zinc (10^{-5} M) in the presence or absence of M-CSF and RANKL for 24 or 72 h as compared with the value for CRP or zinc alone. Agarose gel electrophoresis showed the presence of low-molecular weight deoxyribonucleic acid (DNA) fragments of adherent cells cultured with CRP (10^{-7} M) plus zinc (10^{-5} M) for 24 or 72 h in the presence of M-CSF and RANKL, indicating that the combination of the two chemicals induces apoptotic cell death. CRP plus zinc-induced decrease in osteoclastic cells was significantly inhibited in the presence of caspase-3 inhibitor (10^{-8} or 10^{-7} M). Culture with CRP (10^{-7} M) plus zinc (10^{-5} M) for 24 or 72 h caused a significant increase in caspase-3 mRNA expression in the presence or absence of M-CSF and RANKL as compared with the value for each chemical alone. CRP plus zinc-induced increase in caspase-3 mRNA expression was completely inhibited in the presence of cycloheximide (10^{-7} M), an inhibitor of protein synthesis, or 5,6-dichloro-1- β -D-ribo-

furanosylbenzimidazole (DBR; 10^{-6} M), an inhibitor of transcription activity. The mRNA expression of tartrate-resistant acid phosphatase (TRACP) and cathepsin K was significantly decreased after culture with CRP plus zinc in the presence or absence of M-CSF and RANKL for 72 h as compared with CRP or zinc alone. Nuclear factor of activated T cells c1 (NFATc1) mRNA expression was significantly decreased after culture with CRP plus zinc in the presence or absence of M-CSF and RANKL for 72 h as compared with each chemical alone, while NF- κ B mRNA expression was not significantly changed. This study demonstrated that the combination of CRP and zinc has potent suppressive effects on osteoclastic cells *in vitro*.

Introduction

Osteoporosis and its related fractures represent major public health problems that are expected to increase dramatically as the population ages. Bone loss with aging induces osteoporosis. Bone loss may be due to decreased bone formation and increased bone resorption. Food and nutritional factors may help to prevent bone loss with increasing age (1,2).

β -Cryptoxanthin (CRP), a kind of carotenoid, has unique anabolic effects on bone calcification *in vitro* (3,4); lutein, lycopene, β -carotene, and astaxanthin, which are carotenoids, do not have an effect on bone calcification in rat femoral tissue culture *in vitro*. CRP has direct stimulatory effects on osteoblastic bone formation (5,6) and inhibitory effects on osteoclastic bone resorption *in vitro* (7,8). Oral administration of CRP prevents bone loss in ovariectomized rats, an animal model of osteoporosis (9), suggesting that CRP has preventive effects on osteoporosis with aging.

Zinc is known to be an essential trace element for the growth of humans and other animals (10,11). Zinc deficiency results in a retardation of bone growth (12,13), suggesting that the element is required for the growth, development, and maintenance of healthy bone. Zinc has been shown to stimulate osteoblastic bone formation (14,15) and inhibit osteoclastic bone resorption (15-17). The pathophysiologic role of zinc in osteopenia and osteoporosis has also been shown. Bone zinc content is reduced with aging and skeletal unloading in rats (18,19). Osteoporosis patients have been shown to have lower levels of skeletal zinc than normal individuals (20). Women with osteoporosis excrete a great

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amount of zinc in urine (21). The oral administration of zinc compound has been shown to have preventive effects on bone loss in ovariectomized rats (22). Zinc may play a role in the prevention of osteoporosis with aging (19).

The anabolic effect of CRP on bone components in rat femoral tissues has been shown to enhance synergistically with zinc treatment *in vitro* (23) and *in vivo* (24). The combination of CRP and zinc may have a potent preventive effect on bone loss with aging. However, the cellular mechanism by which the combination of CRP and zinc has synergistic-anabolic effects on bone metabolism has not yet been clarified.

This study was undertaken to determine whether zinc modulates the suppressive effects of CRP on osteoclastic cells *in vitro*. We found that zinc enhances the stimulatory effects of CRP on apoptotic cell death and the suppressive effects of CRP on the gene expression of various molecules related to bone resorption in osteoclastic cells.

Materials and methods

Chemicals. α -minimal essential medium (α -MEM) and penicillin-streptomycin (5000 U/ml penicillin; 5000 μ g/ml streptomycin) were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS), cycloheximide, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), macrophage colony-stimulating factor (M-CSF, mouse), and receptor activator of NF- κ B ligand (RANKL, mouse) were obtained from Sigma (St. Louis, MO). β -cryptoxanthin (CRP) was obtained from Extrasynthase (Lyon-Nord, France). Caspase-3/ CPP 32 inhibitor W-1 (caspase-3 inhibitor), zinc sulfate, and other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan). All water used was glass distilled.

Animals. Female mice (ddY strain; 6 weeks old) were obtained from Japan SLC (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 1.1% calcium, 1.1% phosphorus and 0.012% zinc, and given distilled water. Mice were sacrificed by exsanguination.

Marrow culture and osteoclast differentiation. Bone marrow cells were isolated from mice for studies on primary osteoclast precursor, as reported elsewhere (25). Briefly, the bone ends of the femur were cut off, and the marrow cavity was flushed with 1 ml of α -MEM. The marrow cells were washed with α -MEM and cultured in the same medium containing 10% heat-inactivated fetal bovine serum M-CSF (10 ng/ml of medium) at 1.5×10^7 cells/ml in 24-well plates (0.5 ml/well) in a water-saturated atmosphere containing 5% CO₂ and 95% air at 37°C. After 2 days, adherent cells were used as the M-CSF-dependent bone marrow macrophage (M-BMM) after washing out the non-adherent cells, including lymphocytes. The cells were then cultured in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 4 days to generate mature osteoclasts. The purity of the osteoclastic cell preparation was usually in the range of 85-95% estimating tartrate-resistant acid phosphatase (TRACP) activity.

To determine the effect of CRP or zinc in the cell death and apoptosis of mature osteoclasts, they were incubated for 24 or 72 h in medium containing either vehicle (1% ethanol),

β -cryptoxanthin (10^{-8} or 10^{-7} M), or zinc sulfate (10^{-6} or 10^{-5} M) in the presence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml).

Enzyme histochemistry. After culture, cells adherent to 24-well plates were stained for TRACP, a marker enzyme of osteoclasts (26,27). Briefly, the cells were washed with Hanks' balanced salt solution and fixed with 10% neutralized formalin-phosphate (pH 7.2) for 10 min. After the culture dishes were dried, TRACP staining was applied according to the method of Burstone (26). The fixed cells were incubated for 12 min at room temperature (25°C) in acetate buffer (pH 5.0) containing naphthol AS-MX phosphate (Sigma) as a substrate, and red violet LB salt (Sigma) as a stain for the reaction product in the presence of 10 mM sodium tartrate. TRACP-positive multinucleated cells (MNCs) containing three or more nuclei were counted as osteoclast-like cells.

Analysis of DNA fragmentation. The osteoclastic cells formed were cultured for 24 or 72 h in a medium containing either vehicle, CRP (10^{-7} M), zinc (10^{-5} M), or CRP (10^{-7} M) plus zinc (10^{-5} M) with or without M-CSF (10 ng/ml) and RANKL (50 ng/ml). The culture supernatant was removed, and adherent cells were then lysed in 10 mM Tris-HCl, pH 2.4, 10 mM EDTA (neutralized), and 0.5% Triton X-100. Low-molecular weight DNA fragments were separated by electrophoresis in 1.5% agarose gel (28). The gels were visualized by ethidium bromide staining with an UV transilluminator (Funakoshi Co. Ltd., Tokyo, Japan). The DNA content in the cell lysate was determined by the method of Ceriotti (29).

Determination of specific mRNA by RT-PCR. Total RNAs were prepared as described previously (30). After the M-BMM were cultured in α -MEM containing 0.5% FBS for 24 h and were further cultured in the presence of M-CSF (10 ng/ml) and RANKL (25 ng/ml), the generated osteoclasts were cultured for 24 or 72 h in a medium containing either vehicle, CRP (10^{-7} M), zinc (10^{-5} M), or CRP (10^{-7} M) plus zinc (10^{-5} M). After culture, the cells were washed three times with ice-cold PBS, and were then 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 isopropanol at -20°C. RNA precipitates were pelleted by centrifugation, and the pellets were redissolved in diethylpyrocarbonate-treated water.

Reverse transcription-polymerase chain reaction (RT-PCR) was performed with a Titan™ One Tube RT-PCR kit (Roche Molecular Biochemicals) as recommended by the supplier. The primers for amplification of mouse caspase-3 cDNA were: 5'-GCTCTGGTACGGATGTGGACGCA-3' (sense strand, positions 254-276 of cDNA sequence) and 5'-CTC AATGCCACAGTCCAGCTCCG-3' (antisense strand, positions 560-582) (31). The pair of oligonucleotide primers was designed to amplify a 329-bp sequence from the mRNA of mouse caspase-3.

Primers for the amplification of mouse TRACP cDNA were: 5'-CAACGGCTACTTGCGGTTTC-3' (sense strand, positions 963-982 of cDNA sequence) and 5'-TGTGGGATC

SPANDIDOS PUBLICATIONS: 5'-TGTGG-3' (antisense strand, positions 1,281-1,300) pair of oligonucleotide primers was designed to amplify a 388-bp sequence from the mRNA of mouse TRACP.

Primers for the amplification of mouse cathepsin cDNA were: 5'-CAGCAGGATGTGGGTGTTCA-3' (sense strand, positions 47-66 of cDNA sequence) and 5'-ACACTGGCCTGGTTCTTGA-3' (antisense strand, positions 442-261) (33). The pair of oligonucleotide primers was designed to amplify a 415-bp sequence from the mRNA of mouse cathepsin K.

Primers for the amplification of mouse NFATc1 cDNA were: 5'-GGCGAAGCCCAAGTCTCTTT-3' (sense strand, positions 1,484-1,503 of cDNA sequence) and 5'-ACCACCA GCCACGAGATCAT-3' (antisense strand, positions 1805-1824) (34). The pair of oligonucleotide primers was designed to amplify a 341-bp sequence from the mRNA of mouse NFATc1. Primers for the amplification of mouse NF- κ B cDNA were: 5'-TCCACGAGGCAGCACATAGA-3' (sense strand, positions 3,028-3,047 of cDNA sequence) and 5'-CCA AAGGTGCTGGAGAATCG-3' (antisense strand, positions 3,291-3,310) (35).

The pair of oligonucleotide primers was designed to amplify a 283-bp sequence from the mRNA of mouse NF- κ B cDNA. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control to evaluate total RNA input.

Primers for the amplification of G3PDH cDNA were: 5'-GATTTGGCCGTATCGGACGC-3' (sense strand) and 5'-CTCCTTGGAGGCCATGTAGG-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 (20 μ l) containing 2 μ g of total RNAs, supplied RT-PCR buffer, TitanTM enzyme mix (AMV and ExpandTM 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).

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

Results

Effect on osteoclastic apoptosis. The effects of CRP or zinc on osteoclastic cells, induced in the presence or absence of M-CSF and RANKL in the mouse marrow culture were examined. Osteoclastic cells were cultured in a medium containing either vehicle (1% ethanol), CRP (10⁻⁸ or 10⁻⁷ M), zinc (10⁻⁶ or 10⁻⁵ M), CRP (10⁻⁸ M) plus zinc (10⁻⁶ M), or CRP (10⁻⁷ M) plus zinc (10⁻⁵ M) in the absence (Fig. 1) or presence

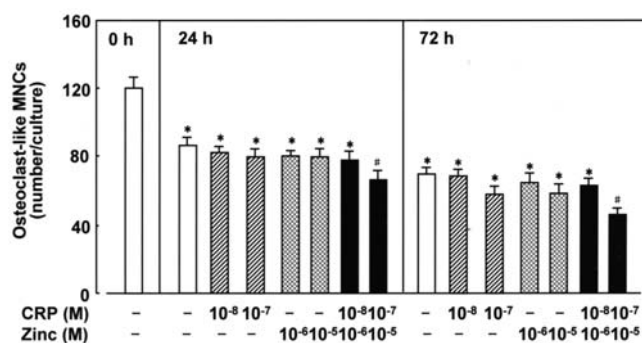


Figure 1. Effect of β -cryptoxanthin (CRP) and/or zinc on osteoclastic cell death in the absence of M-CSF and RANKL in mouse marrow culture. Mouse marrow cells were cultured as described in Materials and methods. The adherent cells were cultured for 96 h in a medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml). After medium change, the TRACP-positive MNCs formed were cultured for 24 or 72 h in a medium containing either vehicle (1% ethanol), CRP (10⁻⁸ or 10⁻⁷ M), zinc (10⁻⁶ or 10⁻⁵ M), or CRP (10⁻⁸ M) plus zinc (10⁻⁶ M), or CRP (10⁻⁷ M) plus zinc (10⁻⁵ M) in the absence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml). The cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean \pm SEM of six cultures. *P<0.01 compared with the control (none) value at zero time. #P<0.01 compared with the value for CRP or zinc alone.

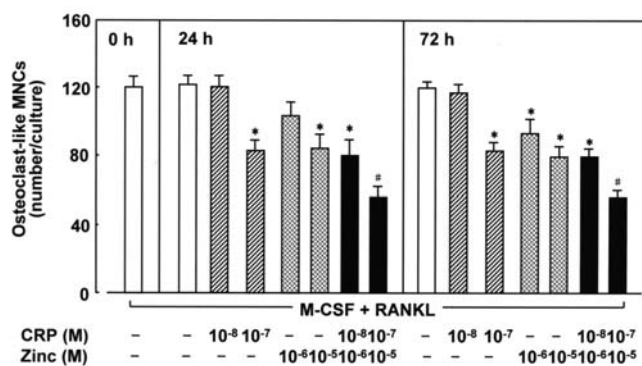


Figure 2. Effect of β -cryptoxanthin (CRP) and/or zinc on osteoclastic cell death in the presence of M-CSF and RANKL in mouse marrow culture. Osteoclasts were cultured as described in the legend for Fig. 1. The osteoclastic cells were cultured for 24 or 72 h in a medium containing either vehicle (1% ethanol), CRP (10⁻⁸ or 10⁻⁷ M), zinc (10⁻⁶ or 10⁻⁵ M), or CRP (10⁻⁸ M) plus zinc (10⁻⁶ M), or CRP (10⁻⁷ M) plus zinc (10⁻⁵ M) in the presence of M-CSF plus RANKL. The cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean \pm SEM of six cultures. *P<0.01 compared with the control (none) value at zero time. #P<0.01 compared with the control value at 24 or 72 h.

(Fig. 2) of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 24 or 72 h. Osteoclastic cells were significantly decreased after culture for 24 or 72 h in the absence of M-CSF and RANKL as compared with the value obtained at the zero time (Fig. 1). The number of osteoclastic cells was significantly decreased after culture with CRP (10⁻⁷ M) plus zinc (10⁻⁵ M) for 24 or 72 h as compared with the value obtained from CRP (10⁻⁷ M) or zinc (10⁻⁵ M) alone.

In the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 24 or 72 h, the osteoclastic cells formed were not significantly decreased as compared with the value obtained at the zero time (Fig. 2). The number of osteoclast-like cells

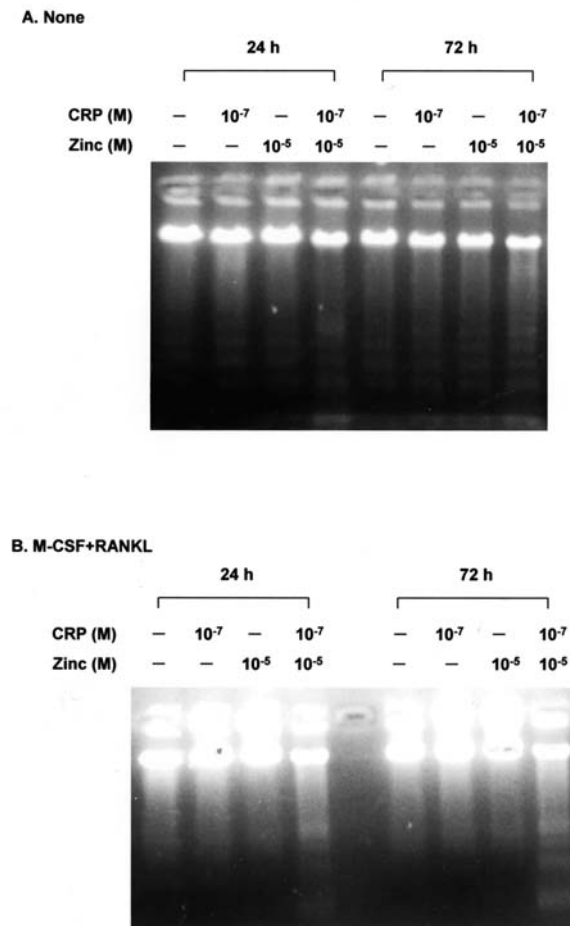


Figure 3. Effect of β -cryptoxanthin (CRP) and/or zinc on DNA fragmentation in osteoclastic cells formed in mouse marrow culture. Osteoclasts were cultured as described in the legend for Fig. 1. The osteoclastic cells were cultured for 24 or 72 h in a medium containing either vehicle (1% ethanol), CRP (10^{-7} M), zinc (10^{-6} M) or a combination, in the absence (A) or presence (B) of M-CSF (10 ng/ml) plus RANKL (50 ng/ml), and the lysate (containing 2 μ g DNA) of adherent cells was applied to agarose gel. The figure shows one of four experiments with separate samples.

was significantly decreased after culture with CRP (10^{-7} M), zinc (10^{-5} M), or CRP (10^{-8} M) plus zinc (10^{-6} M). Culture with CRP (10^{-7} M) plus zinc (10^{-5} M) for 24 or 72 h caused a significant decrease in osteoclast-like cells as compared with the value obtained from CRP (10^{-7} M) or zinc (10^{-5} M) alone in the presence of M-CSF and RANKL.

The effects of CRP or zinc on DNA fragmentation in osteoclastic cells formed with culture in the absence (Fig. 3A) or presence (Fig. 3B) of M-CSF and RANKL was examined. Osteoclastic cells were cultured for 24 or 72 h in a medium containing either vehicle (1% ethanol), CRP (10^{-7} M), zinc (10^{-5} M), or CRP (10^{-7} M) plus zinc (10^{-5} M) in the presence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml). The adherent cells were lysed, and then the lysate was separated by electrophoresis in agarose gel. Culture with CRP plus zinc caused DNA fragmentation in the presence of M-CSF and RANKL (Fig. 3B).

The effect of caspase-3 inhibitor on the CRP plus zinc-induced cell death was examined (Fig. 4). The adherent cells were cultured for 96 h in a medium containing M-CSF

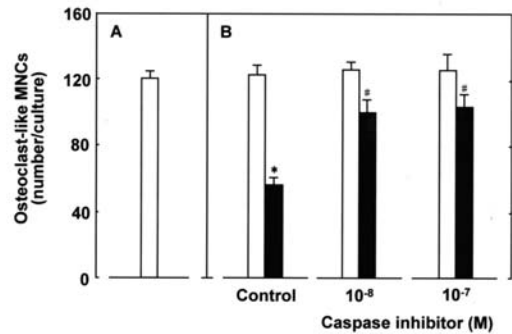


Figure 4. Effect of β -cryptoxanthin (CRP) plus zinc in the presence of caspase-3 inhibitor on osteoclastic cells formed in mouse marrow culture. Osteoclasts were cultured as described in the legend to Fig. 1. The adherent cells were cultured for 96 h in medium containing 0.5% FBS, M-CSF (10 ng/ml), and RANKL (50 ng/ml), and the number of osteoclastic cells was measured (A). After medium change, the osteoclast cells were cultured for 24 h in medium containing either vehicle (1% ethanol) or CRP (10^{-7} M) plus zinc (10^{-5} M) with or without caspase-3 inhibitor (10^{-8} or 10^{-7} M) in the presence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml) (B). The cells were then fixed and stained for TRACP, and the number of TRACP-positive MNCs was scored. Each value is the mean \pm SEM of six cultures. * $P < 0.01$ compared with the control (none) value with or without caspase-3 inhibitor. # $P < 0.01$ compared with the value for CRP plus zinc without caspase-3 inhibitor. (B) White bars, without CRP plus zinc; black bars, with CRP plus zinc.

(10 ng/ml) and RANKL (50 ng/ml) (Fig. 4A). The osteoclastic cells formed were cultured for 72 h in a medium containing either vehicle (1% ethanol) or CRP (10^{-7} M) plus zinc (10^{-5} M) with or without caspase-3 inhibitor (10^{-8} or 10^{-7} M) in the presence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) (Fig. 4B). The CRP plus zinc-induced decrease in osteoclastic cells was significantly inhibited after culture with caspase-3 inhibitor (10^{-8} or 10^{-7} M) as compared with the value obtained in the presence of CRP plus zinc without caspase-3 inhibitor (Fig. 4B).

Effect on caspase-3 mRNA expression in osteoclastic cells.

The effects of CRP or zinc on caspase-3 mRNA expression in osteoclastic cells were examined (Fig. 5). Osteoclastic cells were cultured in a medium containing either vehicle (1% ethanol), CRP (10^{-7} M), zinc (10^{-5} M), or CRP (10^{-7} M) plus zinc (10^{-5} M) in the presence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 24 or 72 h. Culture with CRP (10^{-7} M) or zinc (10^{-5} M) in the presence or absence of M-CSF and RANKL for 24 or 72 h caused a significant increase in caspase-3 mRNA expression in osteoclastic cells. Caspase-3 mRNA expression was synergistically enhanced after culture with the combination of CRP (10^{-7} M) and zinc (10^{-5} M) in the presence or absence of M-CSF and RANKL for 24 or 72 h as compared with that of each factor alone (Fig. 5A). G3PDH mRNA expression was not significantly changed in osteoclastic cells cultured in the presence of CRP (10^{-7} M), zinc (10^{-5} M), or CRP (10^{-7} M) plus zinc (10^{-5} M) with or without M-CSF and RANKL (Fig. 5B).

The effects of cycloheximide or DBR on the CRP plus zinc-induced increase in caspase-3 mRNA expression in osteoclastic cells were examined (Fig. 6). The CRP (10^{-7} M) plus zinc (10^{-5} M)-induced increase in caspase-3 mRNA

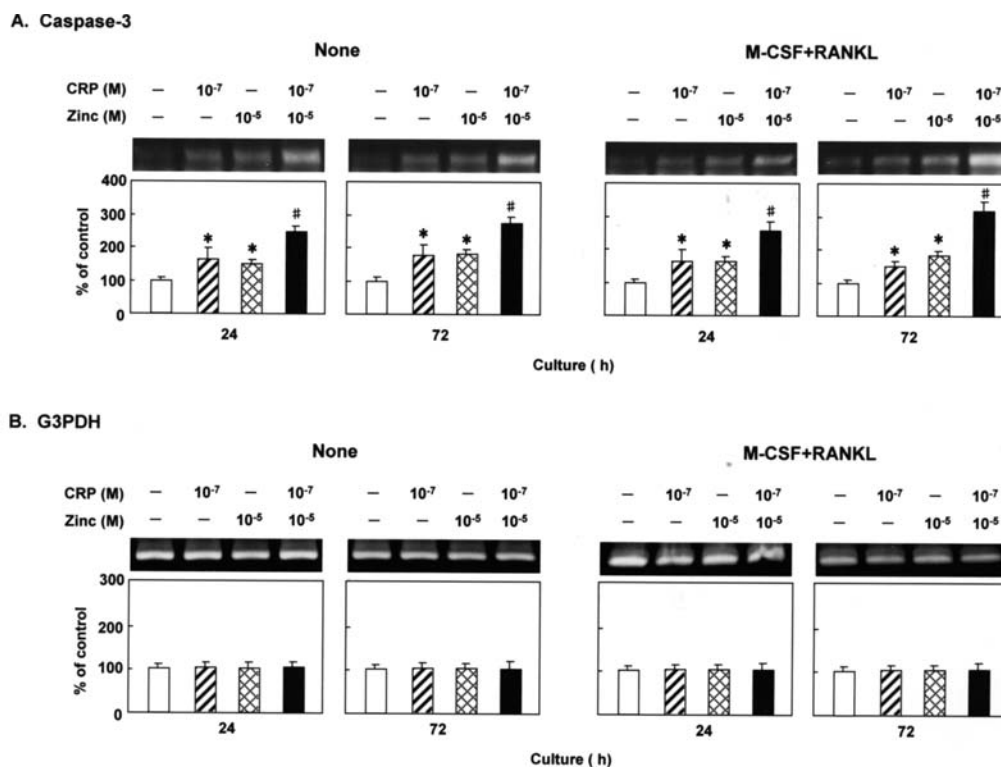


Figure 5. Effect of β -cryptoxanthin (CRP) and/or zinc on the mRNA expression of caspase-3 (A) and G3PDH (B) in osteoclastic cells formed in mouse marrow culture. Osteoclasts were cultured as described in the legend for Fig. 1. The osteoclastic cells were cultured for 24 or 72 h in medium containing either vehicle (1% ethanol), CRP (10^{-7} M), zinc (10^{-5} M), or CRP (10^{-7} M) plus zinc (10^{-5} M) in the presence or absence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml). Total RNAs (2 μ g) extracted from the adherent cells were analyzed by RT-PCR using specific primers. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA level were indicated as % of control (mean \pm SEM of five experiments). * $P < 0.01$ compared with the control (none) value.

expression was not seen in osteoclastic cells cultured with cycloheximide (10^{-7} M) (Fig. 6A), an inhibitor of protein synthesis, or DRB (10^{-6} M) (Fig. 6B), an inhibitor of transcriptional activity, in the presence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml). G3PDH mRNA expression was not significantly changed in this experiment (data not shown).

Effect on bone resorption-related gene expression in osteoclastic cells. The effects of CRP or zinc on the mRNA expression of TRACP or cathepsin K related to osteoclastic bone resorption were examined (Fig. 7). The expression of TRACP mRNA was significantly decreased in osteoclastic cells cultured with CRP (10^{-7} M) plus zinc (10^{-5} M) in the presence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 72 h (Fig. 7A). CRP (10^{-7} M) or zinc (10^{-5} M) alone did not have a significant effect on TRACP mRNA expression in osteoclastic cells with or without M-CSF and RANKL.

Cathepsin K mRNA expression in osteoclastic cells was significantly decreased after culture with zinc (10^{-5} M) in the presence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 72 h (Fig. 7B). Culture with CRP (10^{-7} M) did not cause a significant change in cathepsin K mRNA expression with or without M-CSF and RANKL. The combination of CRP (10^{-7} M) plus zinc (10^{-5} M) had a synergistic-suppressive effect on cathepsin K mRNA expression in osteoclastic cells cultured with M-CSF and RANKL for 24 or 72 h (Fig. 7B).

Effect on RANKL signaling-related gene expression in osteoclastic cells. The effects of CRP or zinc on the mRNA expression of NF- κ B or NFATc1, related to RANKL signaling, in osteoclastic cells, were examined (Fig. 8). NF- κ B mRNA expression in osteoclastic cells was not significantly changed after culture with CRP (10^{-7} M), zinc (10^{-5} M), or CRP (10^{-7} M) plus zinc (10^{-5} M) in the presence or absence of M-CSF (10 ng/ml) and RANKL (50 ng/ml) for 24 or 72 h (Fig. 8A).

NFATc1 mRNA expression in osteoclastic cells was significantly changed after culture with CRP (10^{-7} M) or zinc (10^{-5} M) in the absence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml) for 24 or 72 h (Fig. 8B). Culture with CRP (10^{-7} M) plus zinc (10^{-5} M) caused a significant decrease in NFATc1 mRNA expression in osteoclastic cells cultured without M-CSF and RANKL for 72 h. In the presence of M-CSF and RANKL, NFATc1 mRNA expression was not significantly changed in osteoclastic cells cultured with CRP (10^{-7} M) for 24 or 72 h, while it was significantly decreased after culture with zinc (10^{-5} M) for 72 h but not 24 h. Culture with CRP (10^{-7} M) plus zinc (10^{-5} M) caused a significant decrease in NFATc1 mRNA expression in osteoclastic cells cultured in the presence or absence of M-CSF and RANKL for 72 h. Such an effect was also seen in osteoclastic cells cultured in the presence of M-CSF and RANKL for 24 h.

G3PDH mRNA expression was not significantly changed in osteoclastic cells cultured in the presence of CRP (10^{-7} M), zinc (10^{-5} M), or CRP (10^{-7} M) plus zinc (10^{-5} M) with or without M-CSF and RANKL, as shown in Fig. 5B.

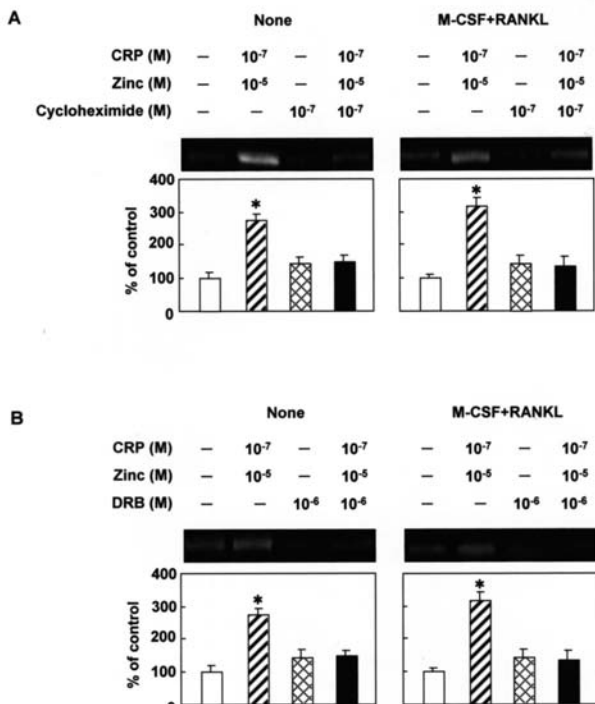


Figure 6. Effect of cycloheximide (A) or DRB (B) on the β -cryptoxanthin (CRP) and zinc-stimulated caspase-3 mRNA expression in osteoclastic cells formed in mouse marrow culture. Osteoclasts were cultured as described in the legend for Fig. 1. The osteoclastic cells were cultured for 72 h in medium containing either vehicle (1% ethanol), CRP (10^{-7} M), zinc (10^{-5} M), or CRP (10^{-7} M) plus zinc (10^{-5} M) with or without cycloheximide (10^{-7} M) or DRB (10^{-6} M) in the presence or absence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml). Total RNAs (2 μ g) extracted from the adherent cells were analyzed by RT-PCR using specific primers. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA levels were indicated as % of control (mean \pm SEM of five experiments). * $P < 0.01$ compared with the control (none) value.

Discussion

The anabolic effect of β -cryptoxanthin (CRP) on bone components in rat femoral tissues has been shown to enhance synergistically with zinc treatment *in vitro* (23) and *in vivo* (24). The cellular mechanism by which the combination of CRP and zinc has a synergistic-anabolic effect on bone metabolism has not been yet clarified. We found that the combination of CRP and zinc has potent stimulatory effects on cell death and suppressive effects on gene expression related to bone resorption in osteoclastic cells.

The number of osteoclastic cells was significantly decreased after culture with CRP plus zinc at the lower concentration in the presence or absence of M-CSF and RANKL as compared with the value of each chemical alone. DNA fragmentation in adherent osteoclastic cells was stimulated after culture with CRP plus zinc in the presence of M-CSF and RANKL for 24 or 72 h, suggesting that the combination of CRP and zinc has a potent stimulatory effect on apoptotic cells. CRP plus zinc-induced DNA fragmentation, however, was not markedly seen in adherent osteoclastic cells cultured in the absence of M-CSF and RANKL. The combination-induced DNA fragmentation may be dependent on M-CSF and RANKL.

The death of osteoclastic cells induced after culture with the combination of CRP and zinc was completely inhibited in the presence of caspase-3 inhibitor with M-CSF and RANKL. This result suggests that CRP plus zinc-induced apoptotic cell death is mediated through caspase-3 in osteoclastic cells. It cannot exclude the possibility, however, that other molecules are related to CRP plus zinc-induced apoptotic cell death.

The expression of caspase-3 mRNA in osteoclastic cells was significantly enhanced after culture with CRP plus zinc in the presence or absence of M-CSF and RANKL as compared with the value of each chemical alone, supporting the view that CRP plus zinc-induced apoptotic cell death is dependent on caspase-3. CRP plus zinc-induced increase in caspase-3 mRNA expression in osteoclastic cells cultured in the presence or absence of M-CSF and RANKL was completely inhibited after culture with cycloheximide, an inhibitor of protein synthesis, or DRB, an inhibitor of transcription activity. This result suggests that CRP plus zinc-enhanced caspase-3 mRNA expression in osteoclastic cells is related to newly synthesized protein synthesis.

The effect of the combination of CRP and zinc on the gene expression of molecules, related to bone resorption in osteoclastic cells, was examined in the presence or absence of M-CSF and RANKL. The expression of TRACP and cathepsin K mRNAs in osteoclastic cells was found to be significantly suppressed as compared with the value of each chemical alone. This suppressive effect was greater in the presence of M-CSF and RANKL. TRACP and cathepsin K are enzymes involved in the degradation of bone matrix components, and their enzyme activities are increased in RANKL-stimulated bone resorption (36,37). It is speculated that the combination of CRP and zinc has a potent-suppressive effect on bone resorption.

NF- κ B and NFATc1 are molecules related to RANKL signaling (35,37). Culture with the combination of CRP and zinc caused a significant suppression of NFATc1 mRNA expression in osteoclastic cells in the presence or absence of M-CSF and RANKL. The suppression effect was greatest in the presence of M-CSF and RANKL. NF- κ B mRNA expression was not significantly changed in the presence of CRP, zinc, or CRP plus zinc. NFATc1 is a transcriptional factor which enhances the gene expression of TRACP and cathepsin K in osteoclasts, and the binding of NFATc1 to promoter is involved in NF- κ B or AP-1 (37). It is speculated that the suppression of NFATc1 mRNA expression induced with the combination of CRP and zinc induces the decrease in NF- κ B protein level. This may partly contribute to the decrease in the TRACP or cathepsin K mRNA expression caused by their combination. This remains to be elucidated, however.

The combination of CRP and zinc that is present in food has been shown to have a potent anabolic effect *in vitro* (23) and *in vivo* (24), suggesting that the combination of food chemical factors plays an important role in the prevention of osteoporosis with increasing age. It would be useful to identify some of the foods that are high in CRP and zinc.

In conclusion, it has been demonstrated that zinc modulates the suppressive effects of CRP on osteoclastic cells *in vitro*. The combination of zinc and CRP was found to have potent effects on osteoclastic cell death *in vitro*.

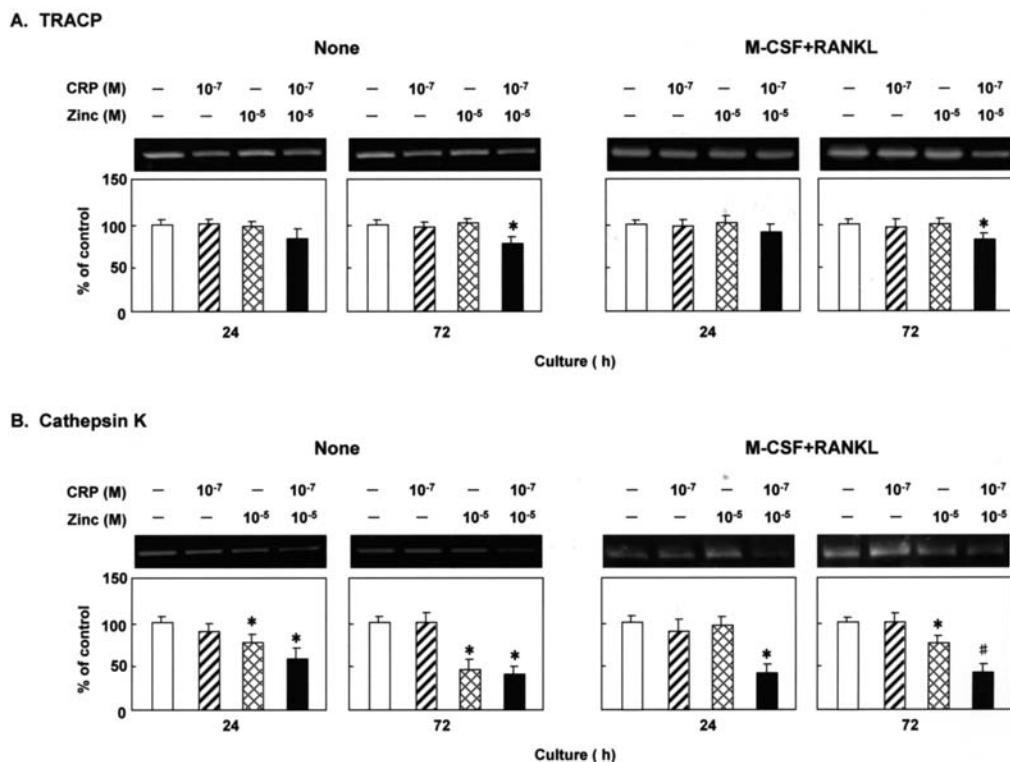


Figure 7. Effect of β -cryptoxanthin (CRP) and/or zinc on the mRNA expression of TRACP (A) or cathepsin K (B) in osteoclastic cells formed in mouse marrow culture. Osteoclasts were cultured as described in the legend for Fig. 1. The osteoclastic cells were cultured for 24 or 72 h in medium containing either vehicle (1% ethanol), CRP (10^{-7} M), zinc (10^{-5} M), or CRP (10^{-7} M) plus zinc (10^{-5} M) in the presence or absence of M-CSF (10 ng/ml) plus RANKL (50 ng/ml). Total RNAs ($2 \mu\text{g}$) extracted from the adherent cells were analyzed by RT-PCR using specific primers. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA level were indicated as % of control (mean \pm SEM of five experiments). * $P < 0.01$ compared with the control (none) value. # $P < 0.01$ compared with the value for CRP or zinc alone.

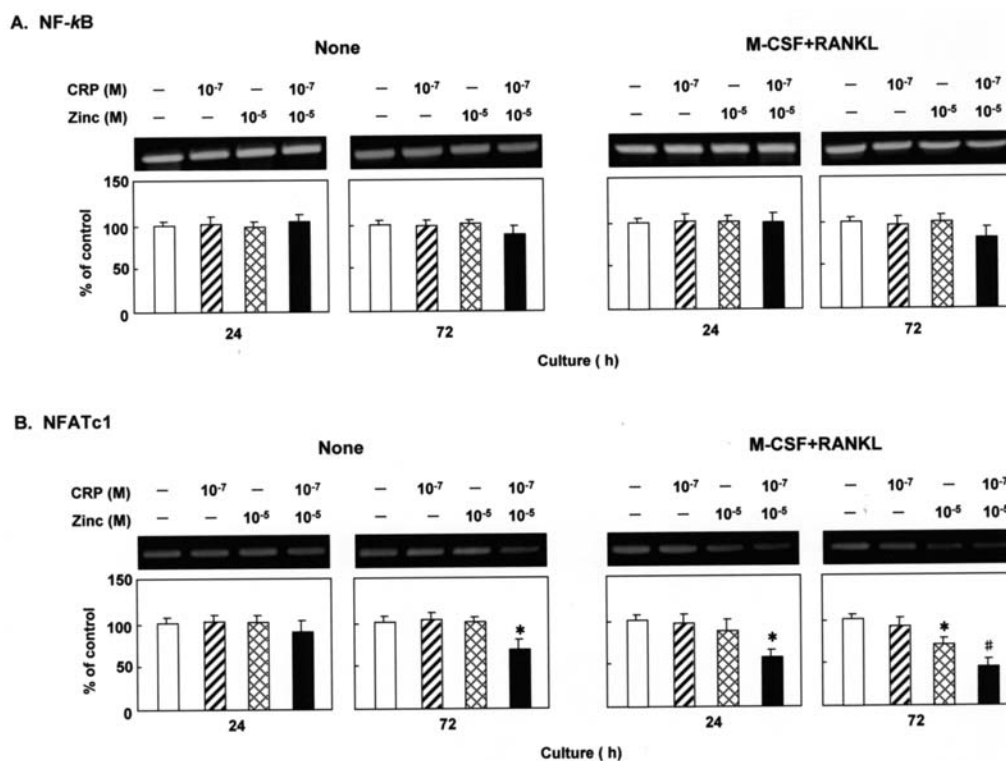


Figure 8. Effect of β -cryptoxanthin (CRP) and/or zinc on the mRNA expression of NF- κ B (A) or NFATc1 (B) in osteoclastic cells formed in mouse marrow culture. Osteoclasts were cultured as described in the legend for Fig. 1 and treated exactly as described in the legend to Fig. 7. Total RNAs ($2 \mu\text{g}$) extracted from the adherent cells were analyzed by RT-PCR using specific primers. The figure shows one of five experiments with separate samples. The densitometric data for each mRNA level were indicated as % of control (mean \pm SEM of five experiments). * $P < 0.01$ compared with the control (none) value. # $P < 0.01$ compared with the value for CRP or zinc alone.

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